THE IODOTHYRONINES:

MEASUREMENT IN SERUM AND APPLICATION OF THESE MEASUREMENTS TO THE DIAGNOSIS OF THYROID DISEASE.

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SUMMARY

The development of sensitive and specific techniques for the measurement of the iodothyronines has led to dramatic advances in the areas of thyroid hormone metabolism and action, and their regulation.

The current concepts of the regulation of thyroid hormone concentration and metabolic action by the hypothalamic-pituitary-thyroid gland axis are reviewed. Also reviewed in detail are those studies implicating the role of the peripheral target tissues in altering thyroid hormone concentration and metabolic action by regulating thyroxine (T_4) metabolism to the biologically active 3,3',5-triiodothyronine (T_3) and the biologically inactive 3,3',5'-triiodothyronine (rT_3).

A radioimmunoassay for rT_3 and the equilibrium dialysis-radioimmunoassay (E/D-RIA) technique for measuring the free thyroid hormones were developed in order to evaluate their usefulness in the diagnosis of thyroid disease, particularly where peripheral metabolism of T_4 was abnormal, as in non-thyroidal illness. The assay sensitivity and precision required were attained by empirical manipulation of the tracer and antibody concentrations.

The simplex technique was also used to establish the rT_3 assay. This led to the development of an assay with much lower binding than the empirical assay but similar sensitivity and precision. It was concluded that this technique provided an objective and practical approach to developing radioimmunoassays which relied less on develop-

mental experience than the conventional empirical techniques.

The empirically established serum-free thyroid hormone assays were compared to displacement curves generated using one and two binding site models of hormone binding to antibody. The models and the equations used to determine precision profiles were derived from the law of mass action. The generated precision profiles suggested higher imprecision and poorer sensitivity than was attained in practise. However, it was concluded that the theoretical prediction of precision profiles was a valuable aid to the rapid establishment of radioimmunoassays of a particular required sensitivity and precision.

Free thyroid hormone concentration was within the euthyroid reference range in nearly every subject with abnormal thyronine binding protein (TBP) concentration. A considerable number of these subjects had abnormal free thyroxine index (FTI) or free T_4 (fT_4) concentration as measured by the Corning Free T_4 assay. It was concluded that the Corning assay was prone to binding protein interference.

While all patients with thyroid disease studied had abnormal FTI and fT_4 (Corning), 37% had either fT_4 or fT_3 concentration (E/D-RIA) in the euthyroid reference range. The assay of rT_3 provided no additional information which might be helpful to the diagnosis of thyroid disease in these subjects.

A group of patients with moderate to severe non-thyroidal illness was characterized by the absence of symptoms

of hypothyroidism and low T_3 concentration but free T_3 was not depressed to the same extent suggesting an uncharacterized binding abnormality. The tissue supply of thyroid hormone was considered adequate in view of the normal fT_4 and TSH. Reverse T_3 was variable and provided no useful information in the diagnosis of thyroid disease in this group.

The cholecystographic agent, iopodate (Biloptin), caused a marked increase in total and free rT_3, reduction in total and free T_3, and stimulation of TSH secretion. The interference in T_4 metabolism caused by this agent necessitates the correct timing of thyroid function tests in patients undergoing cholecystography.

The stressful stimuli, acute strenuous exercise, caused an increase in both rT_3 and T_3. These results suggested that there were mechanisms operating in strenuous exercise which caused effects on thyroid pathophysiology different to those seen in other states of stress.

This is to certify that the work embodied in this thesis has not been previously submitted for the award of a degree in any other institution.

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The investigation of peripheral metabolism after ingestion of cholecystographic agents was performed in collaboration with Dr. Clive Beng, Department of Clinical Chemistry, The Queen Elizabeth Hospital, South Australia, and the study of thyroid pathophysiology during acute strenuous exercise in collaboration with Dr. Michael Hooper, Endocrine Unit, Royal Adelaide Hospital, South Australia.

CHAPTER 1

1.1 EARLY THYROID PHYSIOLOGY

In 1952, Recant and Riggs described a number of important observations made during a study of thyroid function in nephrotic patients:

The subjects had classical biochemical indications of hypothyroidism: low serum protein bound iodine (PBI), hypometabolism, and hypercholesterolemia.

There were no clear clinical symptoms of hypothyroidism.

Thyroid gland function was normal: no goitre was evident, ^{131}I-uptake was normal or elevated, ^{131}I-uptake was thyroxine (T_4) suppressible, and thyroid gland secretion was responsive to thyrotropin (TSH).

Pituitary function was normal: ^{131}I uptake by the thyroid gland was suppressible.

Marked proteinuria and albuminuria was present in all subjects.

Clearance of exogenous T_4 was normal, being twice the rate of clearance in a myxodemetous patient.

There was a tolerance to doses of exogenous T_4 higher than used for replacement therapy in hypothyroid subjects.

From these observations, two crucial conclusions were made:

1. The Supply of Hormone to the Tissues was Normal

It was postulated that circulating free and protein bound T_4 exist in dynamic equilibrium following the law of mass action. A reduction in circulating thyroid hormone binding protein due to proteinuria would lead to an increase in the proportion of free T_4. Despite reduced plasma PBI, free T_4 (fT_4) concentration would remain normal in the presence of normal thyroid gland function.

Assuming free T_4 to be the membrane permeable species, normal fT_4 concentration would ensure normal entry of hormone into the intracellular compartment, despite reduced circulating protein bound hormone.

2. The Subnormal Basal Metabolic Rate was not Associated with Hypothyroidism

In view of the normal supply of hormone to the tissue, it was concluded that the basal metabolic rate was reduced by other unknown mechanisms, and was not manifest in clinical symptoms.

These conclusions embody the two major themes of this thesis:

1. The Accurate Biochemical Assessment of Thyroid Status

The evolution of the free thyroid hormone hypothesis will be reviewed; the earlier indirect measurements of circulating free hormones will be described together with the recent direct equilibrium dialysis-radioimmunoassay and kinetic techniques; the establishment of equilibrium dialysis-radioimmunoassays for the free thyroid hormones is presented;

an evaluation of these assays in diagnosing abnormal thyroid function in thyroid and severe non-thyroidal disease is made; and a comparison with a commercial kit using the kinetic method carried out.

2. The Investigation of Peripheral Thyroid Hormone Metabolism, and its Relevance to the Diagnosis of Thyroid Disease in Severe Non-thyroidal Illness

Thyroid hormone metabolism is reviewed; the establishment and validation of a radioimmunoassay for the circulating iodothyronine, reverse triiodothyronine (rT_3), described; the diagnostic relevance of reverse triiodothyronine measurement is assessed; an investigation of the effect of the radiographic agent, iopodate, on peripheral thyroid hormone metabolism and thyroid function tests is made; and a model of thyroid hormone metabolism during stress is examined.

1.2 THE FREE THYROXINE HYPOTHESIS AND THE DIAGNOSIS OF THYROID DISEASE

THE NATURE OF CIRCULATING THYROXINE

Although T_4 was shown to be a small molecular weight hormone (Kendall, 1919), it displayed protein-like properties in the circulation. Circulating T_4 was non-ultrafiltrable, precipitable with trichloroacetic acid (Trevorrow, 1939) and nondialyzable (Riggs, Lavietes and Man, 1942). However, it was readily extractable with organic solvents (Taurog and Chaikoff, 1948) suggesting the hormone to be strongly, but non-covalently, bound to serum proteins;

it was subsequently shown to be specifically bound to albumin and an α 1, α 2-globulin (Gordon, Gross, O'Connor and Pitt-Rivers, 1952).

Just prior to this discovery, Scatchard (1949) had formulated a model of the interaction of small ions and serum proteins. Of particular importance was the concept of reversible binding of the ions to proteins, the bound and unbound species being in dynamic equilibrium and obeying the law of mass action.

The demonstration that ^{131}I-T_4 bound to serum proteins was readily exchangeable with added unlabelled T_4 *in vitro* (Albright, Larsen and Deiss, 1955) not only confirmed the non-covalent nature of the binding, but also established the reversible nature of T_4 binding to the serum thyroid hormone binding proteins. It was concluded that unbound or free T_4 was in dynamic equilibrium with T_4 bound to serum binding proteins.

CIRCULATING FREE THYROXINE

It was during this period that Recant and Riggs (1952) first pointed to the discrepancy between clinical thyroid status and PBI; they postulated that tissue supply of hormone in nephrosis was normal despite the low circulating total hormone concentration. Their conclusion assumed firstly that free T_4 existed in dynamic equilibrium with the T_4 bound and secondly, that only the free species was membrane permeable. Although there was no direct evidence at that time, subsequent studies provided convincing support for this hypothesis.

1. Correlation between Free Thyroxine Concentration and Clinical Thyroid Status

In 1957, Robbins and Rall calculated fT_4 concentration in serum using the law of mass action to formulate equations describing the binding of T_4 to albumin and thyroxine binding protein. Despite low thyroxine binding protein capacity and PBI in nephrosis, and elevated thyroxine binding protein capacity and PBI in pregnancy and during ingestion of estrogen, calculated free T_4 concentration was invariably normal. Free thyroxine concentration was elevated in thyrotoxicosis and low in hypothyroidism.

Christensen (1959) provided the first semiquantitative estimate of free T_4 by measuring the rate at which $^{131}I-T_4$ in a serum sample dialyzed through dialysis membrane; fT_4 was low in myxoedema, borderline low in nephrosis, normal in pregnancy, and elevated in thyrotoxicosis.

By using equilibrium dialysis to determine the percent dialyzable $^{131}I-T_4$, Sterling and Hegedus (1962) reported the first experimental quantitative estimations of fT_4 concentration. They confirmed the correlation between fT_4 concentration and clinical thyroid status in pregnancy, thyroidal and non-thyroidal disease.

Free thyroxine was subsequently shown to be normal in subjects with idiopathic deficiencies or elevations in thyroxine binding globulin (TBG) (Lee, Henry, Golub, 1964; Ingbar, Braverman, Dawber and Lee, 1965), further demonstrating the independent relationship between PBI and fT_4.

2. Free Thyroxine and Thyroxine Turnover

Although basal metabolic rate and the T_4 degradation rate were directly correlated to the PBI in myxoedema and hyperthyroidism, Sterling and Chodos (1956) reported a group of subjects with no evidence of endocrinological disease but who exhibited hypermetabolism and an increased T_4 degradation rate, despite normal PBI; in this small group of patients with severe non-thyroidal illness, PBI was not simply related to T_4 turnover.

The rate of organic iodine degradation was subsequently shown to be linearly related to the calculated fT_4 concentration in normal and thyrotoxic subjects (Robbins and Rall, 1957).

More convincing evidence for the relationship between fT_4 concentration and T_4 degradation rate was provided by the demonstration of normal degradation rate in conditions where TBG concentration was altered, but where fT_4 and basal metabolic rate had been previously shown to be normal; namely, in idiopathic alterations in TBG (Robbins and Nelson, 1958; Beierwaltes and Robbins, 1959; Ingbar, 1961); during pregnancy (Danowski, Johnson, Price, McKelvy, Stevenson and McCluskey, 1951); during ingestion of estrogen (Engbring and Engstrom, 1959; Ingbar and Freinkel, 1960); and during androgen administration (Federman, Robbins and Rall, 1958).

3. Free Thyroxine and Secretion of Thyrotropin

Reichlin and Utiger (1967) demonstrated an inverse relationship between fT_4 and TSH concentrations in those

hypothyroid subjects on L-thyroxine therapy and in whom the fT_4 concentration was below the normal range.

THE FREE THYROXINE HYPOTHESIS

These early observations were formulated into the free thyroxine hypothesis, containing the following postulates:

Circulating thyroxine existed as an unbound species in dynamic equilibrium with serum protein bound T_4.

Unbound T_4 was membrane soluble thereby establishing a rapid equilibrium between the extracellular and intra-cellular compartments.

Intracellular fT_4 concentration determined hormone action and thus clinical thyroid status.

Intracellular fT_4 concentration determined the rate of T_4 degradation by deiodination, oxidative deamination, and excretion as conjugate.

Pituitary thyrotrophes were sensitive to fT_4 concen-tration and maintained constant free hormone levels and tissue supply by regulating thyroid gland secretion through TSH.

There were several important consequences of these propositions:

In the event of alterations in circulating thyroid hormone binding protein, fT_4 concentration was auto-regulated thus maintaining normal free hormone concen-tration (Oppenheimer, 1968). For example, an increase in TBG concentration would cause a fall in circulating

bound and free T_4, and lowered intracellular fT_4 levels. The combination of resulting reduced T_4 degradation rate and constant thyroid gland secretion rate would lead to a compensating increase in circulating T_4 until fT_4 reached normal levels. At this point T_4 degradation rate would have returned to a level equivalent to thyroid gland production rate; T_4 flux through the system would be similar to its original rate and steady state conditions would prevail.

In primary thyroid disease, altered thyroid secretion would lead to changes in both hormone action and degradation.

It was quite possible, however, for alterations in T_4 clearance to take place without altering the euthyroid steady state. This was demonstrated by studies on the effect of phenobarbital on ^{131}I-T_4 degradation rate in intact and thyroidectomized rats (Bernstein, Artz, Hasen and Oppenheimer, 1968). Increased hepatic binding and degradation of T_4 was noted in phenobarbital treated rats; PBI remained constant in the phenobarbital treated intact rats but fell in the phenobarbital treated thyroidectomized rats on T_4 replacement. It was concluded that increased clearance of T_4 caused a fall in circulating T_4. TSH secretion was increased in response to the drop in fT_4 until thyroid gland production became sufficient to equal the clearance rate and maintain constant euthyroid fT_4 levels. A new steady state was attained in which T_4 flux through the system was increased, fT_4 was normal and the rats were eumetabolic.

In the thyroidectomized rats T_4 clearance rate remained in excess of the rate of exogenous T_4 administration leading to a continuous depletion of the circulating T_4 pool.

DIAGNOSIS OF THYROID DISEASE

This hypothesis suggested the measurement of fT_4 to be the most accurate determinant of thyroid status. Calculations using the law of mass action suggested fT_4 concentration to be about 60 pmol/L (Robbins and Rall, 1957), well below the sensitivity of existing chemical measurements of T_4. New techniques were developed in which the fraction of dialyzable ^{131}I-T_4 in serum under equilibrium dialysis conditions was used to estimate the fT_4 concentration from the PBI (Chapter 4).

This approach assumed that equilibrium between free and bound species *in vitro* was similar to that *in vivo*. There was no evidence to suggest that this was so and, in fact, it was known that intracellular T_4-binding components existed (Ingbar and Freinkel, 1960); if these had affinity for T_4 comparable to that of TBG, they would be important determinants of fT_4 concentration *in vivo*.

The equilibrium dialysis technique also assumed that fT_4 was in equilibrium with protein bound T_4 throughout the circulation. Oppenheimer (1968) pointed out that this was not necessarily so as the rate of hormone uptake by some tissues may exceed the rate of dissociation of T_4 from the binding proteins. Thus fT_4 concentration in some tissues may be lower than expected and might also be variable, depending

on the tissue demands at any particular time.

Despite these reservations, the measurement *in vitro*, if not an accurate estimate of fT_4 concentration, was considered to be proportional to the concentration *in vivo*.

FREE TRIIODOTHYRONINE

Free triiodothyronine (fT_3) analysis became possible with the development of an assay for circulating total T_3 (Nauman, Nauman and Werner, 1967). Although it was not known to what extent T_3 contributed to the clinical thyroid status, it had been shown to have greater metabolic potency than T_4 (Gross, Pitt-Rivers and Trotter, 1952). Subsequent demonstrations of extrathyroidal conversion of T_4 to T_3 (Schwartz, Surks and Oppenheimer, 1971), and T_3 specific nuclear receptors in rat liver and kidney (Oppenheimer, Koerner, Schwartz and Surks, 1972a) highlighted the importance of T_3 in the expression of thyroid status. However, the interpretation of both fT_4 and fT_3 concentration was not clear.

RECENT DEVELOPMENTS

Nuclear receptors for T_3 are now well characterized; they appear to be ubiquitous and there is a considerable body of evidence suggesting them to be the primary site of thyroid hormone action (Oppenheimer, Schwartz, Surks, Koerner and Dillman, 1976). The affinity of the nuclear receptors for T_3 is typically ten times that for T_4 suggesting that fT_3 reflects more the intracellular binding capacities and affinities whereas fT_4 reflects the circulating TBP capacities and affinities.

Although fT_3 measurement would appear to reflect directly the extent of thyroid hormone action, it should be remembered that T_4 is a peripheral source of T_3, accounting for up to half the daily T_3 production rate. The value of fT_4 measurement should not be underestimated.

The separate roles of the free thyroid hormones have been clarified by studies on the occupancy of nuclear receptors in rat pituitary, kidney, liver and heart muscle (Larsen, Bavli, Castonguay and Jove, 1980). Intracellular deiodination of T_4 to T_3, and the level of circulating T_3 appeared to contribute equally to the T_3 occupancy of pituitary receptors. In contrast, T_3 occupancy of receptors in kidney, liver and heart muscle could be accounted for by the circulating T_3 concentration alone. These results imply that the integrity of the pituitary-thyroid gland axis can only be evaluated by measuring both fT_3 and fT_4, whereas peripheral thyroid hormone action is effectively assessed by measuring fT_3.

A MODEL OF THE REGULATION OF THYROID HORMONE PRODUCTION, ACTION, AND METABOLISM

Figure 1.1 depicts a model of thyroid hormone physiology encompassing the points discussed and illustrating the free thyroid hormone hypothesis.

Equilibrium between the extracellular and intracellular free thyroid hormone pools is considered to be restored very quickly if a perturbation occurs in one of the pools; with the exception of the intrapituitary fT_3 pool, the intracellular and extracellular free hormone pools are

thus represented as being continuous for each hormone.

Three major compartments of thyroid hormones are considered: the intrathyroidal pool, the pituitary intracellular pool and the intracellular pool representing those cells containing nuclear T_3 receptors and/or metabolizing T_4 to T_3 and which are not pituitary cells, and the extracellular pool, of which the major component is the circulation. Free thyroid hormone is in equilibrium with protein bound hormone in each of these compartments. Although intracellular binding proteins (apart from nuclear T_3 receptors) have been identified in only a limited number of tissues (Oppenheimer, Schwartz and Surks, 1974), they are postulated to be ubiquitous for the purpose of this model. The extracellular binding proteins are those in the circulation, namely thyronine binding globulin (TBG), thyronine binding prealbumin (TBPA) and albumin.

In the intracellular compartments, free hormone is in equilibrium with hormone bound to the nuclear receptors. It is assumed that no modification to the T_3 or T_4 structure occurs whilst bound to the receptor. Although both receptor bound T_3 and receptor bound T_4 pools are shown, Silva, Dick and Larsen (1978) have estimated that receptor bound T_4 accounts for less than 10% of receptor bound iodothyronine in the pituitary.

The work of Larsen et al. (1980) would suggest that receptor occupancy by T_3 in the pituitary compartment is determined by the intracellular concentration of both free T_3 and free T_4. Circulating fT_4 contributes to nuclear T_3 receptor occupancy by virtue of intrapituitary deiodination

to T_3, which in turn joins the intracellular fT_3 pool. The pituitary intracellular fT_3 pool is represented as being separated from the circulating fT_3 pool while the intracellular fT_3 pools of other tissues are represented as being continuous with the circulating pool. This is done to bring out the conclusion of Larsen et al. (1980) that intrapituitary T_4 to T_3 deiodination contributes to T_3 nuclear receptor occupancy within the pituitary, while 5'-deiodination of T_4 in other tissues does not directly contribute to the T_3 nuclear receptor occupancy of the tissue within which the T_3 is generated. That is, the receptors in the pituitary are in some way influenced by locally generated T_3 but the T_3 generated in other tissues rapidly equilibrates with the circulation with the result that receptor occupancy is determined by the circulating fT_3 pool as a whole. It may be that circulating fT_3 concentration within the pituitary is higher than in the rest of the circulation due to very rapid pituitary T_3 production such that the pituitary intracellular fT_3 pool is in rapid exchange with, but not in equilibrium with, the circulation. The regulation of thyroid gland hormone biosynthesis and secretion is determined by the circulating TSH concentration which in turn is regulated by the level of iodothyronine nuclear receptor occupancy (Silva and Larsen, 1978).

The model depicts irreversible clearance of T_3 and T_4 from the free hormone pool in the intracellular compartments. This assumes that the hormones are not metabolized as protein bound units. Although the implication is that the clearance rate is determined by free hormone concentration,

several reports have shown a lack of correlation between free T_4 concentration and T_4 turnover rate (Bellabarba, Inada, Varsano-Aharon and Sterling, 1968; Schussler and Vance, 1968; Hennemann, Docter and Dolman, 1971; Lutz, Gregerman, Spaulding, Hornick and Dawkins, 1972; Premachandra, Gossain and Perlstein, 1976). These would suggest that thyroid hormone metabolism is regulated by other uncharacterized mechanisms, such as, for example, metabolizing enzyme concentrations and activities.

THE EFFECT OF ALTERING HORMONE FLUX

When the system is in steady state, secretion by the thyroid gland per day equals irreversible clearance of hormone per day. The quantity of hormone passing through the system per day is defined as the hormone flux and is equivalent to the clearance rate.

An increase in thyroid gland secretion rate will cause an increase in protein bound and free hormone with resulting increased clearance rate. A new steady state is reached when clearance matches secretion. The new steady state is characterized by increased hormone flux, raised free hormone concentration, elevated nuclear receptor occupancy and hypermetabolism. Reduction in secretion rate would cause converse changes.

In contrast an independent increase in the metabolism of thyroid hormone may occur without a significant alteration in hormone action. Thus increased clearance rate would lead in turn to reduced free hormone concentration, reduced pituitary T_3 receptor occupancy, increased TSH

secretion, and increased thyroid gland biosynthesis and secretion of hormone until secretion and clearance rates were equivalent. At this point free hormone concentration would have risen to its original level, TSH secretion would drop, and thyroid gland secretion rate would fall. Clearance rate again exceeds secretion rate and free hormone concentration falls. That is, a steady state would not be expected to ensue but instead there would be a continuous oscillation of clearance and secretion rates, free hormone concentration, T_3 receptor occupancy levels and TSH secretion rate.

Whether the period and amplitude of these oscillations would be large enough to accurately measure is not known. Unless clearance rate markedly exceeded secretion rate, it would seem unlikely that hypometabolism would result. Studies on the effect of phenobarbital on the turnover of T_4 in rats (Bernstein et al., 1968) confirm this conclusion.

The conclusion is that hormone turnover and hormone action are not necessarily interdependent. Concern that the free thyroxine hypothesis was in some way deficient because free hormone concentration did not reflect T_4 turnover (Oppenheimer, 1968) would appear to be unfounded in the light of recent studies on nuclear T_3 receptors. These latter investigations, in fact, highlight the relationship between free hormone concentration and hormone action.

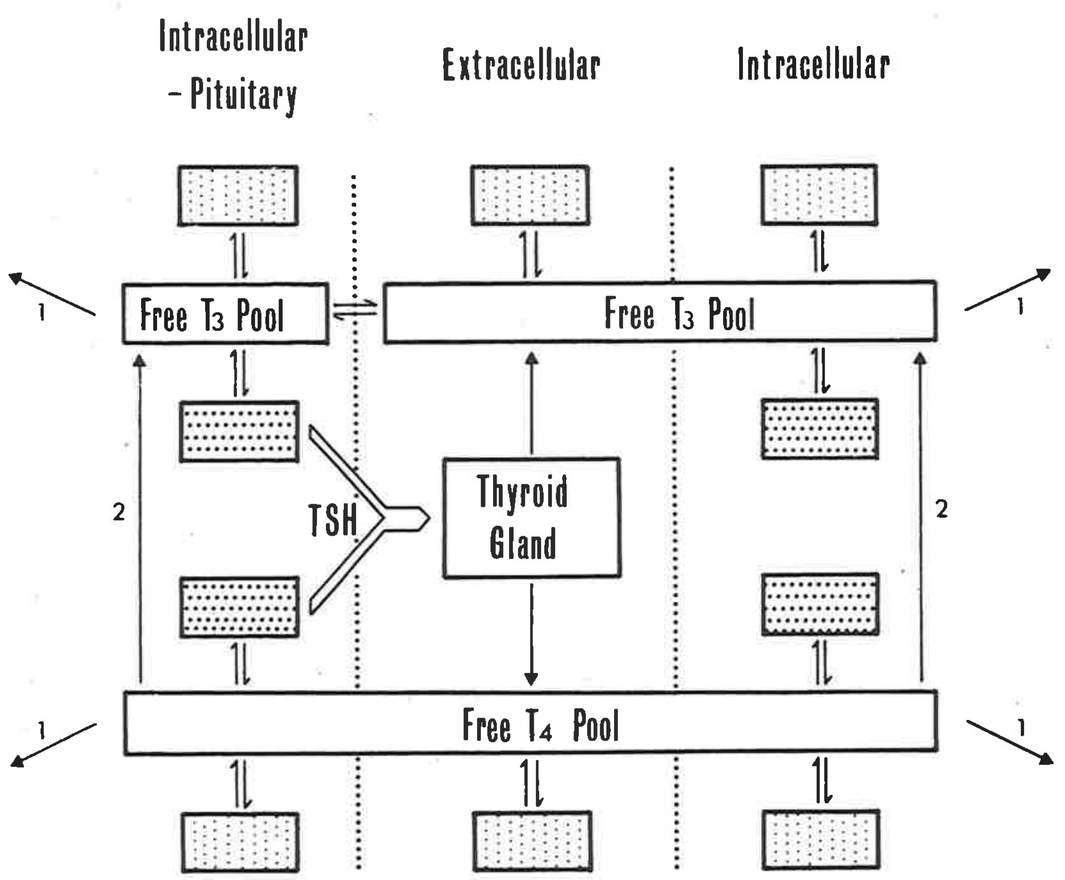
CLINICAL INTERPRETATION OF CIRCULATING FREE THYROID HORMONE CONCENTRATION

The measurement of free thyroid hormone concentration in the serum of patients with clinical hypothyroidism using

Figure 1.1 A model of the regulation of thyroid hormone production, action, and metabolism

1 Intracellular 5'-deiodination of T_4 to T_3, occurring principally in the liver, kidney and skeletal muscle (see Chapter 1 (1.3)).

2 Irreversible metabolism and excretion of T_3 and T_4 (metabolism of T_4 being by routes other than 5'-deiodination to T_3)


Intracellular
- Pituitary
Extracellular
Intracellular
Free T3 Pool
Free T3 Pool
TSH
Thyroid
Gland
Free T4 Pool
1
1
2
2
1
1

Binding Protein
Nuclear Receptors

the equilibrium dialysis-radioimmunoassay technique has revealed a considerable number of both fT_3 and fT_4 levels which fall in the respective reference ranges (Yeo, Lewis and Evered, 1977). Although these results challenge the free thyroid hormone hypothesis, it is not clear whether there are technical limitations in this method of analysis.

Data presented by Spector, Davis, Helderman, Bell and Utiger (1976) on thyroid function tests and clinical indices of thyroid status in patients with renal disease highlight the problems of interpreting free hormone data. The thyroid function tests gave data typical of patients with mild to severe non-thyroidal disease: normal total T_4, free T_4, TBG and TSH concentration, and low total T_3 and fT_3. The patients were judged clinically euthyroid and had normal basal metabolic rate; basal metabolic rate did not correlate with free T_4, free T_3, total T_4 or total T_3.

In the absence of hypothalamic or pituitary dysfunction these results could be taken to support the conclusion of Larsen et al. (1980) that TSH secretion is regulated by both T_3 and T_4. In contrast the lack of hypometabolism in the presence of diminished free T_3 would appear to argue against the proposal that thyroid hormone action in the peripheral tissues is determined principally by T_3. However, these discrepancies may be complicated by other effects of severe illness.

There is a considerable body of evidence supporting the hypothesis that free thyroid hormone concentration determines the degree of thyroid hormone action. Whilst

discrepancies between free hormone concentration, as determined by recent direct analysis *in vitro*, and clinical status appear to challenge this concept, these may be due to current technical limitations, uncertainty as to the relative importance of free T_3 and free T_4 concentration, and lack of information on the effect of illness on thyroid hormone physiology.

One aim of this thesis was to develop assays for the measurement of free thyroid hormone concentration and to investigate their usefulness in diagnosing abnormalities in thyroid status in both thyroidal and severe non-thyroidal disease.

1.3 DEIODINATIVE METABOLISM OF THE IODOTHYRONINES

AN HISTORICAL PERSPECTIVE

Prior to the 1950's, thyroxine was the only known secretory product and iodoamino acid component of the thyroid gland. In 1952, Gross and Pitt-Rivers identified ^{131}I-labelled 3,5,3'-triiodothyronine (T_3) in the plasma of thyrotoxic patients injected with ^{131}I-iodide, and reported the presence of T_3 in the thyroid tissue of animals. Using paper chromatography, synthesized T_3 was found to be identical to an unidentified ^{131}I-labelled compound previously demonstrated in the liver and feces of thyroidectomized rats and mice injected with ^{131}I-T_4 (Gross and Leblond, 1951).

Lower doses of T_3 than T_4 were effective in correcting the hypometabolism in myxoedema suggesting that T_3 not only had the same qualitative effects as T_4 but was also considerably more potent than T_3 (Gross, Pitt-Rivers and

Trotter, 1952; Lerman, 1953). The metabolic response to T_3 was also demonstrated to be faster than that to T_4 (Lerman, 1953; Selenkow and Asper, 1955). From these early observations the hypothesis was proposed that thyroid hormone action was effected by the metabolism of T_4 in the peripheral tissues to the metabolic stimulator, T_3. This hypothesis stimulated a decade of intensive investigation into the metabolism of T_4 in an effort to establish T_3 or other analogues as the effectors of T_4 action (Albright, Larson and Trust, 1954; Kalant, Lee and Sellers, 1955; Lassiter and Stanbury, 1958; Becker and Prudden, 1959).

It was during this period of interest in the biological activities of T_4 analogues, that 3,3',5'-triiodothyronine (rT_3) and 3,3'-diiodothyronine (3,3'-T_2) were identified in hydrolysates of thyroglobulin and in plasma (Roche, Michel, Wolf and Nunez, 1956a). They postulated that the thyroid gland synthesized and secreted four iodothyronines: T_4, T_3, rT_3 and 3,3'-T_2. While rT_3 displayed very low activity in the rat antigoitrogen and tadpole metamorphosis bioassays, 3,3'-T_2 had similar activity to T_4 (Roche, Michel, Truchot, Wolf and Michel, 1956b).

Labelled rT_3 was subsequently detected in the bile of dehepatectomized dogs injected with ^{131}I-T_4 (Flock, Bollman, Grindlay and Stobie, 1961) demonstrating that T_4 was extrathyroidally metabolized by deiodination of both the phenyl ring (5'-deiodination) and the tyrosyl ring (5-deiodination). The peripheral metabolism of T_4 to rT_3 was difficult to reconcile with the concept of obligatory peripheral deiodination to T_3 in order to effect thyroid hormone action,

particularly in view of the apparent biological inactivity of reverse T_3.

The iodothyronines, T_4, T_3, rT_3 and 3,3'-T_2 were also shown to be metabolized by the nondeiodinative routes of oxidative deamination and conjugation to glucuronic acid (Albert and Keating, 1952; Flock and Bollman, 1954; Roche and Michel, 1956; Pitt-Rivers and Tata, 1959; Roche, Michel, Nunez and Jacquemin, 1959).

Technical limitations did not allow a precise quantitative evaluation of these pathways of T_4 metabolism and what part they played in the initiation of thyroid hormone action. Although the accumulation of ^{131}I-T_3 in tissue after the administration of ^{131}I-T_4 was well known, there was no clear demonstration of the appearance of ^{131}I-T_3 in the plasma (Kalant et al., 1955; Lassiter and Stanbury, 1958). While this could be taken to support the concept of a local peripheral deiodination of T_4 to an effector at or near the site of hormone action, it was well documented that there was substantial thyroid gland secretion of T_3.

The relative contribution of the thyroidal and peripheral sources to the T_3 body pool, and the relative contribution of T_3 and T_4 to thyroid hormone action have become clearer with the recent development of specific and sensitive radioimmunoassays for the iodothyronines, and with the availability of high specific activity tracers. Braverman, Ingbar and Sterling (1970) unequivocally confirmed the peripheral deiodination of T_4 to T_3 by using a competitive protein binding assay technique to demonstrate the presence

of T_3 in the athyreotic patients maintained on Na-L-T_4. The additional evidence that euthyroid clinical thyroid status was maintained by administration of T_3 to rats on the T_4 blocking agent propylthiouracil (PTU) suggested that the biological activity of T_4 might be completely effected through its metabolite T_3 (Oppenheimer, Schwartz and Surks, 1972b).

Using radioimmunoassay, Chopra (1974) subsequently confirmed the presence of rT_3 in euthyroid human serum at the low concentrations predicted by Dunn and Stanbury (1958) from their observations of the very rapid clearance of ^{131}I-rT_3 from the circulation. The presence of only very low concentrations of rT_3 in the thyroid gland, and the demonstration of normal rT_3 levels in T_4-treated hypothyroid subjects, not only confirmed that rT_3 was a metabolite of T_4, but also suggested that T_4 was a major source of rT_3. Chopra further proposed that the increased rT_3 and decreased T_3 concentrations in cord serum were evidence of altered peripheral T_4 metabolism in the fetus. This was the first indication that T_4 metabolism to the active metabolite, T_3, might be regulated.

These early studies provided the background for the elaboration of thyroid hormone metabolism and led to an extensive revision of existing concepts of thyroid hormone action. This introduction outlines current knowledge of the peripheral deiodinative metabolism of the iodothyronines with particular reference to rT_3 physiology. Changes in circulating T_3 and rT_3 in a variety of pathological situations can best be interpreted with a knowledge of these metabolic pathways.

REVERSE TRIODOTHYRONINE AS A WEAK AGONIST OF THYROID HORMONE ACTION

The presence of rT_3 in the thyroid gland and circulation prompted a number of investigations to establish its biological activity. Roche et al. (1956b) found rT_3, in marked contrast to T_3, to have less than 5% the activity of T_4 in both the rat antigoitrogen and tadpole metamorphosis bioassays. The absence of any effect on the basal metabolic rate in rats confirmed the very low potency of rT_3 (Stasilli, Kroc and Meltzer, 1959).

In contrast, administration of massive doses revealed an antithyroxine effect of rT_3. Both basal metabolic rate and heart rate were significantly reduced in a Grave's disease patient on 120 mg/d of DL-rT_3 (Benua, Kumaoka, Leeper and Rawson, 1959); and basal metabolic rate was similarly reduced in T_4-replaced thyroidectomized rats and T_4-replaced myxoedemic subjects (Pittman, Tingley, Nickerson and Richardson Hill, Jr., 1960; Barker, 1960). On the other hand, Pittman et al. (1960) found no effect of rT_3 in either euthyroid or thyrotoxic subjects. The relevance of these findings was, however, diminished by the use of pharmacologic doses and by uncertainty about the purity of the preparations used.

A recent investigation *in vivo* supports the view that rT_3 has no detectable biological activity at physiological concentrations. Nicod, Burger, Strauch, Vagenakis and Braverman (1976a) have shown pituitary response to thyrotropin releasing hormone to be unaffected during administration of rT_3 doses sufficient to maintain mildly elevated

serum concentrations in euthyroid subjects.

Studies *in vitro* have revealed measurable but very low activity. Reverse T_3 had less than 1% the potency of T_3 in inhibiting TSH release from pituitary slices (Chopra, Carlson and Solomon, 1978), and had 0.1% the potency of T_3 in stimulating growth hormone production and glucose oxidation in cultured rat pituitary tumour cells (Papavasiliou, Martial, Latham and Baxter, 1977). In both systems very high concentrations of rT_3 had the same qualitative effects as T_3 and T_4, suggesting a weak agonistic action. Studies of binding to intracellular components substantiate the agonistic nature of rT_3. It has been shown to bind to an extracted cytosol receptor (Dillman, Surks and Oppenheimer, 1974); and to bind to solubilized thyroid hormone nuclear receptors from rat liver (Koerner, Surks and Oppenheimer, 1974), cultured rat pituitary tumour cells (Papavasiliou et al., 1977), rabbit fetal lung (Lindenberg, Brehier and Ballard, 1978), and from human lymphocytes (Lemarchand-Beraud, Holm, Bornand and Burger, 1978). The affinity of binding to the nuclear receptors was 0.1% that of T_3, supporting the conclusion of Roche et al. (1956b) that rT_3 has no significant biological activity.

The recent demonstration of high affinity binding of rT_3 to nuclear protein from pig liver seriously challenges the view that rT_3 has little biological activity (Smith, Robinson and Eastman, 1980). Binding affinity was greatest when no sulphydryl containing compounds were present in the binding assay, suggesting that nuclear binding of rT_3 may be significant in some patho-physiological situations associated

with reduced intracellular sulphydryl concentration.

REVERSE TRIIODOTHYRONINE PRODUCTION AND CLEARANCE

Although rT_3 had been detected in the circulation (Roche et al., 1956a), the lack of calorigenic activity cast doubt on its physiological importance. Nevertheless, recent quantitative estimates of iodothyronine turnover *in vivo* conclusively establish rT_3 to be a major metabolite of T_4. Peripheral deiodination to rT_3 accounts for the clearance of 40 - 45% of the approximately 100 nmoles of T_4 secreted per day, the remaining T_4 being either deiodinated to T_3 (25 - 35%) or metabolized by non-deiodinative pathways (20%) (Chopra, 1976; Gavin, Castle, McMahon, Martin, Hammond and Cavalieri, 1977; Eisenstein, Hagg, Vagenakis, Fang, Ransil, Burger, Balsam, Braverman and Ingbar, 1978; Lumholtz, Faber, Sorenson, Kirkegaard, Siersbaek-Nielsen and Friis, 1978a; Suda, Pittman, Shimizu and Chambers, 1978a).

The relative contribution of thyroidal secretion and peripheral T_4 deiodination to the triiodothyronine production rates has been difficult to assess accurately. Chopra (1976) measured the mass ratios, T_4/rT_3 and T_4/T_3, in extracts of digested normal human thyroid gland to be 75 and 9 respectively. Assuming the secretion of the triiodothyronines to be proportional to their abundance in the gland, he calculated that secretion accounts for less than 2.5% of the rT_3 production rate and for about one quarter the T_3 production rate. It is uncertain as to whether this method of estimating thyroid secretion of the triiodothyronines is accurate. Recent evidence suggests more rT_3 and T_3 are

secreted than their relative thyroid gland concentrations would suggest. Thus Laurberg (1978a,b) has shown the T_4/rT_3 and T_4/T_3 ratios in the canine thyroid vein to be much lower than the corresponding thyroidal tissue ratios. Similarly, the secretion ratios in humans during thyroid surgery were lower than expected (Westgren, Burger, Ingemansson, Melander, Tibblin and Wahlin, 1976; Hooper, Ratcliffe, Ratcliffe, Marshall, Young, Ngaei and Clark, 1978).

Although the exact contribution of thyroidal secretion to the daily production of the triiodothyronines remains uncertain, it is clear that there is increased secretion of the triiodothyronines relative to thyroxine when compared to their respective intrathyroidal concentrations. It now appears that this pattern of secretion may be due to both presecretory monodeiodination of T_4 and to preferential triiodothyronine secretion per se. Thus Laurberg (1978b) found the T_4/rT_3 and T_4/T_3 in venous effluent of the canine thyroid gland to increase during administration of the deiodinase blocker, PTU, until the effluent ratios were near the ratios in thyroid hydrolysates. It is of particular interest that thyroid tissue deiodinates T_4 to T_3 by a system similar to the deiodination systems found in liver and kidney (Green, 1978).

The triiodothyronines are rapidly cleared from the circulation, rT_3 (100 L/d) being cleared much more rapidly than T_3 (25 L/d) (Chopra, 1976; Gavin et al., 1977; Eisenstein et al., 1978; Lumholtz et al., 1978a; Suda et al., 1978a). Labelled 3,3'-T_2 and 3'-T_1 were detected during

clearance of injected $^{125}I-T_3$ and $3,3'-T_2$, $3',5'-T_2$ and $3'-T_1$ were identified in serum during clearance of $^{125}I-rT_3$ showing that the triiodothyronines undergo sequential monodeiodination (Sakurada, Rudolph, Fang, Vagenakis, Braverman and Ingbar, 1978; Figure 1.2). The glucuro-conjugates and sulfo-conjugates of these products were also demonstrated.

Using clearance studies, Gavin, Hammond, Castle and Cavalieri (1978) and Galeazzi and Burger (1980) have estimated that 60 - 80% of the clearance of the triiodothyronines is by way of deiodination to $3,3'-T_2$. In contrast, Geola, Chopra, Solomon and Maciel (1979) calculated that a substantially lower fraction of rT_3 produced per day is cleared to $3,3'-T_2$. They suggest that 60% of the rT_3 produced is cleared equally to $3,3'-T_2$ and $3',5'-T_2$ and the rest is metabolized by non-deiodinative routes.

PATHOPHYSIOLOGICAL DISTURBANCES IN THYROID HORMONE METABOLISM

1. Non-Thyroidal Illness

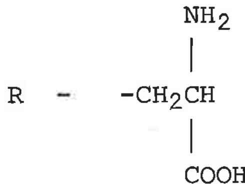
Some of the earliest studies on the physiology of rT_3 were in patients with mild to severe non-thyroidal illness and first drew attention to the importance of the peripheral deiodinative pathways of T_4 metabolism (Chopra, Chopra, Smith, Reza and Solomon, 1975a).

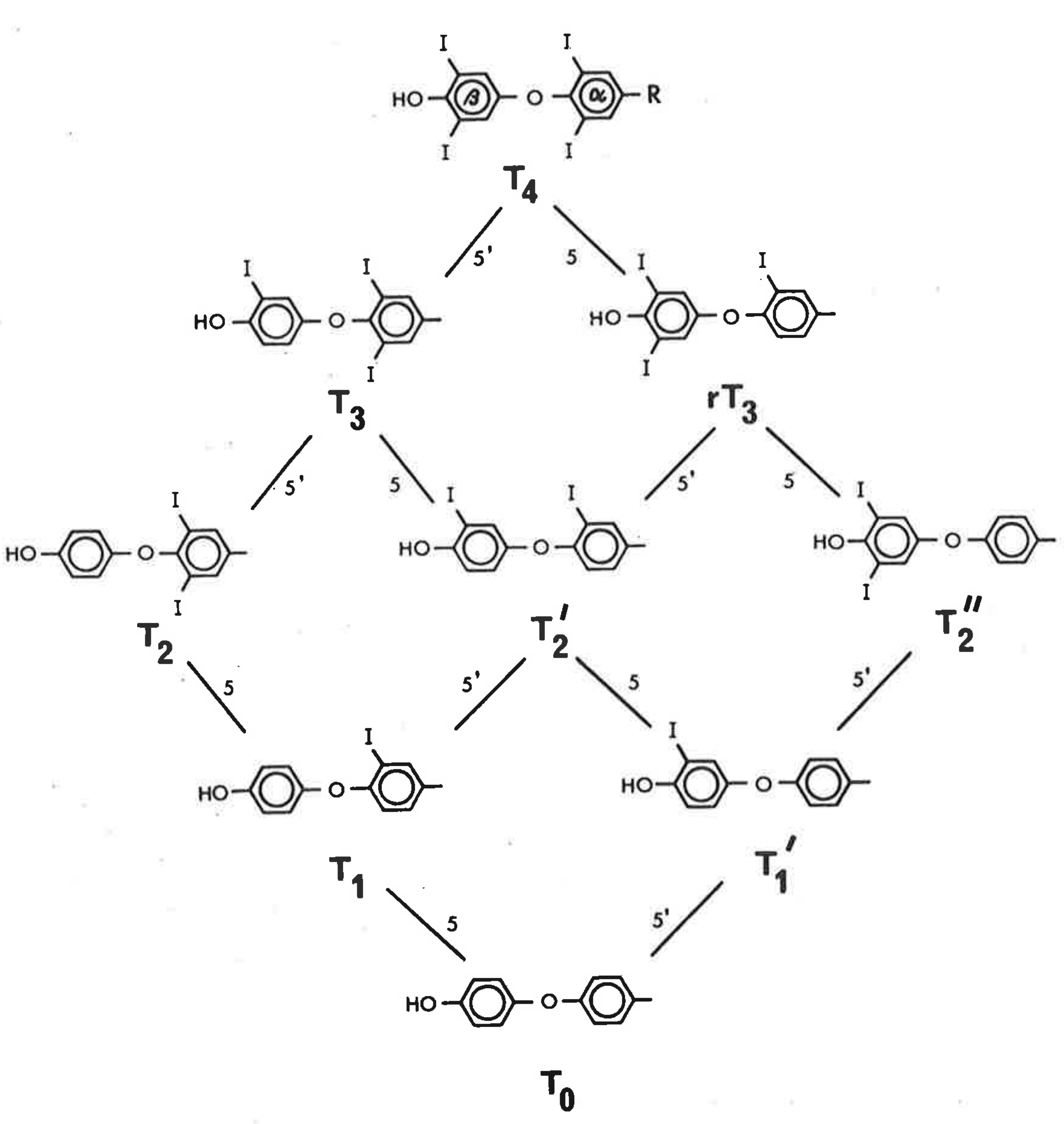
Although there are characteristic changes in thyroid function tests during mild to severe non-thyroidal illness which are suggestive of hypothyroidism, obvious clinical symptoms are most often absent. Typically, both total and free circulating T_3 concentrations are low, total

Figure 1.2 Sequential monodeiodination of thyroxine

T_2	3,5-diiodothyronine
T_2'	3,3'-diiodothyronine
T_2"	3',5'-diiodothyronine
T_1	3-monoiodothyronine
T_1'	3'-monoiodothyronine
T_0	thyronine
5'	5'-deiodination
5	5-deiodination

The complete sequential deiodination schemata is hypothetical. To date radioimmunoassays have been developed for all the iodothyronines depicted except 3-monoiodothyronine and the parent amino acid, thyronine. All those iodothyronines for which assays are available have been detected in serum.




HO
O
R
I
T4
5'
5
T3
rT3
T2
T2'
T2''
T1
T1'
T0

T_4 may be depressed, normal, or mildly elevated whilst fT_4 is usually normal or slightly elevated, and TSH concentration is either normal or mildly raised. *The association of depressed total T_3 and the absence of clinical signs of hypothyroidism during illness has been termed the sick euthyroid syndrome.* It is now clear that rT_3 concentration is often elevated during severe illness having been described during febrile illness (Chopra et al., 1975a; Talwar, Sawhney and Rastogi, 1977; Wartofsky, Burman, Dimond, Noel, Frantz and Earll, 1977), hepatic cirrhosis (Chopra et al., 1975a; Kodding, Janzen, Schmidt and Hesch, 1976), thermal injury (Becker, Wilmore, Johnson, Burman and Wartofsky, 1978), after myocardial infarction (Nilsson, Levin, Melander, Pettersson and Westgren, 1976), during a variety of acute and subacute diseases (Burger, Nicod, Suter, Vallotton, Vagenakis and Braverman, 1976; McLarty, Ratcliffe, Sammon, Ratcliffe, McColl, Meinhold and Wenzel, 1976), during protein calorie malnutrition (Chopra et al., 1975a), and during voluntary starvation (Vagenakis, Burger, Portnay, Rudolph, O'Brian, Azizi, Arky, Nicod, Ingbar and Braverman, 1975).

Considering the reciprocal changes in the concentration of the circulating triiodothyronines during illness, the predominantly peripheral origin of rT_3, and the peripheral tissue contribution to the daily production of T_3, Chopra et al. (1975a) postulated that the extrathyroidal generation of the triiodothyronines was regulated. They proposed the hypothesis that by regulating the peripheral production of T_3 the body could protect itself against the potent metabolic effects of this metabolite during the stress of, for example

severe illness or starvation.

While studies of circulating iodothyronine concentrations implicate altered peripheral metabolism of T_4 in certain situations, they provide little insight into the mechanism underlying the changes. Several recent studies on the clearance of the iodothyronines *in vivo* have helped clarify the mechanisms underlying the control of T_4 metabolism. The T_4 production rate is moderately decreased and T_3 production markedly depressed in both hepatic cirrhosis (Nomura, Pittman, Chambers, Buck and Shimizu, 1975; Chopra, 1976), and terminal renal failure patients on haemodialysis (Lim, Fang, Katz and Refetoff, 1977). Reduced peripheral metabolism of T_4 to T_3 accounted for the fall in T_3 production rate and, together with unaltered clearance of both T_3 and T_4, resulted in a net decrease in circulating T_3 and low to normal circulating T_4. In contrast, a marked diminution in rT_3 clearance and unaltered peripheral production resulted in substantial increases in serum rT_3 in hepatic cirrhosis (Chopra, 1976). Although these clearance studies explain the alterations seen in the triiodothyronines during acute and chronic diseases it should be kept in mind that altered peripheral deiodination may not be the only change in T_4 metabolism. Thus Lumholtz et al. (1978a) have recently reported a threefold increase in the percentage of T_4 cleared by non-deiodinative metabolism in a patient with heptatic cirrhosis on T_4 replacement for myxoedema.

2. Starvation

Similar changes in T_3 and rT_3 metabolism occurred in obese patients during prolonged starvation (Vagenakis, Portnay, O'Brian, Rudolph, Arky, Ingbar and Braverman, 1977; Eisenstein et al., 1978) and in euthyroid subjects on T_4 replacement after fifteen days fasting (Suda et al., 1978a). Again, reduced peripheral metabolism of T_4 accounted for the drop in T_3 production rate, whereas the peripheral production of rT_3 was unaltered and its clearance decreased. Suda et al. (1978a) found that the ratio of the rT_3 and T_4 production rates ($PRrT_3/PRT_4$) was increased whereas the total production rate of the triiodothyronines relative to T_4 production [$(PRrT_3 + PRT_3)/PRT_4$)] was unaltered. That is, there appears to be no increase in non-deiodinative metabolism of T_4 during fasting, but there is evidence to suggest that with a decrease in deiodinative metabolism of T_4 to T_3, metabolism by the T_4 to rT_3 pathway deiodinates that fraction of T_4 normally converted to T_3.

3. Diabetes

The reciprocal changes in the concentration of the circulating triiodothyronines observed in uncontrolled diabetes are similar to those which occur during fasting (Suda, Chambers, Thurston and Pittman, 1977). As in prolonged starvation, the rate of conversion of T_4 to T_3 and the rT_3 metabolic clearance rate were markedly reduced, while the clearance of T_3 and $PRrT_3/PRT_4$ were not significantly different to those values calculated for patients with controlled diabetes. Again, the major alteration

appears to be in 5'-deiodination rather than 5-deiodination. What is more, similar results were seen regardless of whether the uncontrolled diabetics were mildly ketoacidotic or not, suggesting that it is not acidosis *per se* but a diminished supply of fuel to the cells which induces the changes in T_4 metabolism.

4. Altered Nutritional Intake

More detailed analysis of the effects of hypocaloric intake on T_4 metabolism have revealed that neither T_3 or rT_3 concentrations were altered where carbohydrate intake was adequate (Spaulding, Chopra, Sherwin and Lyall, 1976). As the carbohydrate content of the hypocaloric diet was reduced, rT_3 became elevated and T_3 concentration fell; while there was a significant correlation between T_3 and glucose concentrations, no significant inverse relationship between rT_3 and glucose concentrations was evident. More prolonged studies have revealed a dissociation in the changes in T_3 and rT_3 concentration during reduced dietary intake. During a six week hypocaloric diet, obese subjects showed persistently decreased T_3 while rT_3 concentration rose transiently and returned to prediet levels by the fourth week (Visser, Lamberts, Wilson, Docter and Hennemann, 1978a).

Furthermore, during feeding on isocaloric and hypercaloric diets, rT_3 and T_4 concentrations were unaltered despite variations in carbohydrate composition. However, T_3 concentration was low where carbohydrate content was reduced, but where the carbohydrate content was adequate, T_3 rose with increasing carbohydrate caloric intake (Davidson and

Chopra, 1979).

5. Summary

In summary, fasting and severe illness partially inhibit the 5'-deiodinative processes of T_3 production and rT_3 clearance, but only slightly, if at all, alter the 5-deiodinative pathways of rT_3 production and T_3 clearance. These observations clearly support the existence of two peripheral deiodinating activities, one removing the 5'-iodine, the other removing the 5-iodine. More significantly, the data strongly suggest that these processes are not random, but are regulated independently. Thus 5'-deiodination appears sensitive, but 5-deiodination insensitive, to the pathophysiological states described, supporting the hypothesis that T_4 metabolism is diverted from an activating to inactivating pathway in these states (Chopra et al., 1975a; Vagenakis et al., 1975). The regulating mechanisms appear quite complex, as shown by the dietary studies. Thus peripheral generation of T_3 would appear to be governed by both carbohydrate and total caloric intake. What is more, alterations in the 5'-deiodination mechanism leading to decreased T_3 generation are not always associated with changes in the 5'-deiodinative clearance of rT_3 to 3,3'-T_2,

THYROID PHYSIOLOGY IN THE FETUS AND NEONATE

The pattern of circulating triiodothyronines in the near term sheep fetus is similar to that seen in severe illness and fasting in the human; T_4 concentration is similar to that seen in the adult animal, rT_3 is elevated and T_3 concentration is very low (Chopra, Sack and Fisher,

1975b). While the T_3 production rate is low in late gestation, $PRrT_3/PRT_4$ is little different to that in adult sheep. Although no direct measure of peripheral deiodination *in vivo* was made in the fetus, Chopra et al. (1975b) calculated that thyroid gland secretion wholly accounts for fetal T_3 production whereas rT_3 is principally derived by peripheral metabolism of T_4, as in the adult.

During late gestation in the fetal sheep, the circulating concentrations of both T_4 and rT_3 decline slowly toward term, increase to a maximum within one to two days of birth and gradually fall to adult levels during the neonatal period. On the other hand, circulating T_3 concentration rises gradually during the last six to eight days of gestation and within several hours of birth rises dramatically above adult levels (Klein, Oddie and Fisher, 1978; Nwosu, Kaplan, Utiger and Delivoria-Papadopolous, 1978). Similar changes occur in the human in the post-partum period (Chopra, Sack and Fisher, 1975c). While the rise in T_4 and rT_3 after birth was most likely due to the surge in TSH at this time, Sack, Beaudry, Delamater and Fisher (1976) concluded that the post partum T_3 increase was unrelated to this surge. Not only did T_3 concentration increase to a maximum earlier than the concentrations of TSH, T_4 and rT_3, but there was a concurrent rise in PRT_3/PRT_4. In contrast, the $PRrT_3/PRT_4$ remained similar to that in the adult sheep throughout late gestation, parturition and the neonatal period (Chopra et al., 1975b).

These observations suggest there is no active 5'-deiodination pathway in the fetus during most of the

gestational period. While this pathway appears in the peripartum stage, the facility to deiodinate T_4 to rT_3 is evident much earlier in gestation. Of particular interest is the apparent lack of any quantitative changes in this latter pathway postpartum. Fetal sheep iodothyronine metabolism has been proposed as a model for studying regulation of peripheral T_4 metabolism in the human. The changes in T_4 metabolism peripartum strongly attest to the presence of two independent deiodinating activities. What is more, taken together with the observed increase in $PRrT_3/PRT_4$ and reduced T_3 production rate during fasting (Suda et al., 1978a), the question is raised as to whether production of rT_3 is determined simply by the availability of T_4.

PROPERTIES OF THE PERIPHERAL DEIODINATING ACTIVITIES

The studies *in vivo* point to the sequential monodeiodination of thyroxine. The deiodinative pathways involved can be organized into the two groups, 5'-deiodination and 5-deiodination. Although monodeiodination of ^{131}I-T_4 to ^{131}I-T_3 *in vitro* was originally demonstrated in kidney slices in 1954 (Albright et al., 1954), it is only the recent numerous experiments which have clarified the mechanisms of T_4 metabolism and their regulation. Visser (1978) has reviewed a number of these reports.

1. Tissue Activities

T_4 is readily metabolized to T_3 in homogenates of liver (Visser, van der Does-Tobe, Docter and Hennemann, 1975) and kidney (Chopra, 1977); muscle, heart, spleen, brain and intestine have lower activity, while lung tissue

homogenates display no activity (Chopra, 1977). Formation of T_3 has also been shown in whole cell preparations including cultured fibroblasts (Refetoff, Matalon and Bigazzi, 1972), leukocytes (Woeber and Maddux, 1978) and lymphocytes (Holm, Lemarchand-Beraud, Scazziga and Cuttelod, 1975). 5-deiodination of T_4 to rT_3 has been demonstrated in liver homogenates (Cavalieri, Gavin, Bui, McMahon and Hammond, 1977; Hoffken, Kodding and Hesch, 1977), in leukocytes (Woeber and Maddux, 1978) and in dispersed cells of rat renal tubules (Heyma, Larkins, Stockigt and Campbell, 1978a). While rT_3 is rapidly degraded by deiodination in those tissues in which it has been shown to be produced, T_3 degradation is much slower and has been studied in cultured chick heart embryo cells (Dickstein, Schwartz, Gordon and Gross, 1978) and liver homogenates (Visser, Fekkes, Docter and Hennemann, 1978b). To date, only 3,3'-T_2 has been defined as a product of both T_3 and rT_3 deiodination *in vitro*. Furthermore, 3,3'-T_2 was shown to be degraded to 3'-T_1 in liver homogenates (Sorimachi and Robbins, 1977).

2. Enzymatic Nature

The enzymatic nature of these processes is well documented. Tissue homogenate deiodination of T_4 to the triiodothyronines is inhibited by heating to 56°C for 30 minutes. The activity is proportional to, and saturable with, the substrate T_4; is proportional both to temperature and the concentration of homogenate; and is inhibited by mercurous ion (Hg^+), an enzyme inhibitor (Visser, van der Does-Tobe, Docter and Hennemann, 1976; Cavalieri et al.,

1977; Chopra, 1977). The deiodinating activities have been termed deiodinases.

3. Subcellular Location

There is still uncertainty as to the exact subcellular location of the enzymes. Cavalieri et al. (1977) claimed to have separated the activities into a 5-deiodinase rich cytoplasmic fraction and a 5'-deiodinase rich membrane fraction. However, 5'-deiodinase metabolism of T_4 to T_3 has since been observed in rat liver cytoplasm (Yamada, Kaplowitz and Chopra, 1978). Furthermore, both activities have been demonstrated in the subcellular microsomal fraction (Hesch, Brunner and Soling, 1975; Woeber and Maddux, 1978; Kohrle, 1978). More recent studies also question the cytoplasmic location and suggest the activities are primarily associated with the plasma membrane (Leonard and Rosenberg, 1978), the endoplasmic reticulum rich microsomal fraction (Visser et al., 1976) or both (Maciel, Ozawa and Chopra, 1979). Kohrle (1978) has attempted to separate the two activities by solubilizing specifically the T_4-5'-deiodinase from beef liver microsomes. Unfortunately, the solubilized deiodinase turned out to be nonspecific in that it also degraded T_4 to rT_3 and metabolized other iodothyronines. Yamada et al. (1978) have claimed to have extracted 5'-deiodinase from rat liver cytosol but have, however, made no comment on its 5-deiodinase activity.

4. Reduced-Thiol Requirement

Both the 5-deiodinase and the 5'-deiodinase require the presence of reduced-thiol groups for optimum

activity in homogenates, microsomes and cytosol preparations (Visser et al., 1976, 1978b; Cavalieri et al., 1977). Confirmation of the thiol requirement was provided by the inhibitory action of the thiol-blocking agents p-chloromercuriphenyl-sulphonic acid, mercurous and argentous ions (Visser et al., 1976) and N-ethylmaleimide (Chiraseveenuprapund, Buergi, Goswani and Rosenberg, 1978). Glutathione (GSH) has been proposed as the intracellular source of thiol groups and was recently shown to be a substrate for the cytoplasmic T_4 to T_3 reaction (Yamada et al., 1978). Dithiothreitol (DTT) is most commonly used as a source of reduced-thiol *in vitro*. Requirement for reduced sulphydryl does vary and it has been shown that the 5'-deiodinase pathways, T_4 to T_3 and rT_3 to 3,3'-T_2, are stimulated to a much greater extent than the 5-deiodination pathways, T_4 to rT_3 and T_3 to 3,3'-T_2 (Visser et al., 1978b).

5. Kinetics of the Deiodination Enzyme Activities

Most kinetic studies have been performed on the rat deiodinase activities and reveal that the 5'-deiodination pathways have greater enzymatic activity than the 5-deiodination pathways. Comparison and interpretation of the data is difficult as the enzyme activities have been investigated in whole cells, homogenates and microsomal preparations from several tissue sources in the presence of varying reduced-thiol concentration and pH. Estimates of the K_m for T_4-5'-deiodinase vary between 1.6 - 7.7 μmol/L (Chopra, 1977; Huffner, Grussendorf and Ntokalou, 1977; Chiraseveenuprapund et al., 1978; Kaplan and Utiger, 1978; Woeber and Maddux, 1978; Visser, Fekkes, Docter and Hennemann, 1979). The

rT_3-5'-deiodinase is very active and K_m values between 8 - 400 nmol/L have been reported (Chopra, Wu, Nakamura and Solomon, 1978; Hoffken, Kodding, Kohrle and Hesch, 1978; Kaplan and Utiger, 1978; Visser et al., 1978b).

There has been very little documentation of the kinetics of the 5-deiodination. In general, the values for K_m are not very much different to those reported for the T_4-5'-deiodinase activity; Visser et al. (1979) estimate K_m for T_4-5-deiodinase of 1.8 - 3.3 μmol/L and K_m for T_3-5-deiodinase of 4.7 - 7.8 μmol/L.

6. pH Dependence

The deiodinase activities are pH dependent with 5-deiodination of T_4 to rT_3 and T_3 to 3,3'-T_2 demonstrated to be greatest at pH 8 - 9 and 5'-deiodination of T_4 to T_3 and rT_3 to 3,3'-T_2 greatest at pH 6 - 7 (Cavalieri et al., 1977; Hoffken et al., 1977, 1978). In the only human tissue studied to date, Woeber and Maddux (1978) have observed that both 5'-deiodination of T_4 to T_3 and 5-deiodination of T_4 to rT_3 are optimal at pH 6.5.

Visser et al. (1979) have recently made a comprehensive study of deiodination kinetics in rat liver microsomes at various pH between the two optimum ranges reported, namely pH 6.5, 7.2 and 8.0. While K_m and V_{max} were found to vary independently and in a complex manner with pH, the 5'-deiodinase activities did tend to be greater at pH 6.5 - 7.2 and the 5-deiodinase activities were greatest at pH 8.0, confirming the earlier studies.

7. Iodothyronine Inhibition of Deiodination Enzyme Activities

It is now well documented that rT_3 is a competitive inhibitor of T_4-5'-deiodinase and T_4 is a competitive inhibitor of rT_3-5'-deiodinase activity (Chopra, 1977; Kaplan and Utiger, 1978; Visser et al., 1979). Although it is unclear whether rT_3 and T_4 act as inhibitors in patho-physiological conditions *in vivo* the K_i suggest inhibition may occur. Thus K_i for rT_3 inhibition of T_4-5'-deiodinase activity is similar to the K_m for rT_3-5'-deiodinase. Similarly K_i for T_4 inhibition of rT_3-5'-deiodinase activity is similar to the K_m for T_4-5'-deiodinase. Neither of these iodothyronines inhibits the 5-deiodinase activities.

8. How Many Deiodination Enzymes

It appears unnecessary at present to invoke the existence of a separate enzyme for each iodothyronine substrate. Instead, the properties of the deiodinase activities *in vitro* suggest there to be two enzymes, namely a 5'-deiodinase and a 5-deiodinase. This conclusion is supported by their different requirement for thiol containing compounds, by their different pH optima and by the different iodothyronine inhibition properties.

The mutual inhibition of rT_3-5'-deiodinase and T_4-5'-deiodinase by T_4 and rT_3 strongly infers these two activities to be effected by the one enzyme. Visser et al. (1979) have proposed that the interaction of the iodothyronine with the active site of 5'-deiodinase is compromised by the existence of a 5-iodine (as in T_4), thus explaining the very rapid metabolism of rT_3 by this enzyme. The fact that rT_3

has little effect on 5-deiodinase activity confirms the separate existence of a 5-deiodinase enzyme.

While the 5'-deiodination and 5-deiodination activities are likely to exist as separate enzymes, the studies on their intracellular location would suggest that they are very closely associated, most probably in the cell membrane and endoplasmic reticulum.

The studies *in vivo* on the production and clearance of the triiodothyronines in various pathophysiological states reinforce the hypothesis that two separate enzymes metabolize the iodothyronines. Again these studies provide no evidence that there is a different enzyme for each of the iodothyronine substrates.

DEIODINATION IN THE FETUS AND NEONATE

Studies of 5'-deiodination of T_4 in fetal sheep liver homogenates not only support the thiol requirement of the 5'-deiodinase, but also suggest this enzyme to be more sensitive to intracellular nonprotein sulphydryl concentration than the 5-deiodination of T_4 to rT_3 (Chopra, 1978a). Chopra found the fetal liver homogenate concentration of nonprotein sulphydryl and 5'-deiodinase activity to be low and this activity was increased to adult levels by addition of DTT. While no comparison of adult and fetal liver T_4 5-deiodination was made, the similar $PRrT_3/PRT_4$ in both the fetus and the adult suggested 5-deiodinase activity was relatively insensitive to the lowered intracellular nonprotein sulphydryl concentration in the fetus.

Thyroxine-5'-deiodinase activity in the fetal

and neonatal rat liver has been shown to be reduced due to both a low nonprotein sulphydryl concentration and a low concentration of the enzyme itself (Harris, Fang, Hinerfeld, Braverman and Vagenakis, 1979). While generation of T_3 in the homogenates of liver from fetal, neonatal and adult rats was stimulated by DTT, it was not until seven days post-partum that the stimulated activity was similar to that in the 60 day old adult. Although the concentration of enzyme at seven days appeared similar to that in the adult, the hepatic nonprotein sulphydryl concentration was still significantly lower than in the adult rat.

The conclusion by Chopra (1978a) that 5-deiodinase activity is less sensitive to the requirement for sulphydryl groups is supported in studies made by Visser et al. (1978b) on rat liver metabolism of iodothyronines *in vitro*. 5-deiodination of T_4 to rT_3 and T_3 to 3,3'-T_2 were stimulated by DTT but to a less extent than the 5'-deiodination pathways T_4 to T_3 and rT_3 to 3,3'-T_2.

EXPERIMENTAL ALTERATIONS OF THE PERIPHERAL DEIODINATING ACTIVITIES

1. Propylthiouracil

Propylthiouracil, which has long been used as an antithyroid drug in the treatment of thyrotoxicosis, causes a marked fall in circulating T_3 and a rise in rT_3 (Westgren, Melander, Wåhlin and Lindgren, 1977; Laurberg and Weeke, 1978; Siersback-Nielsen, Kirkegaard, Ragowski, Faber, Lumholtz and Friis, 1978). Clearance of ^{125}I-T_4 to ^{125}I-T_3 in whole rats was observed to be markedly depressed during

PTU treatment and the fractional ratio of T_3 deiodination was diminished (Oppenheimer et al., 1972b). Studies *in vitro* have subsequently shown PTU to partially inhibit both 5'-deiodinase and 5-deiodinase (Visser et al., 1975, 1978b; Cavalieri et al., 1977; Heyma et al., 1978a).

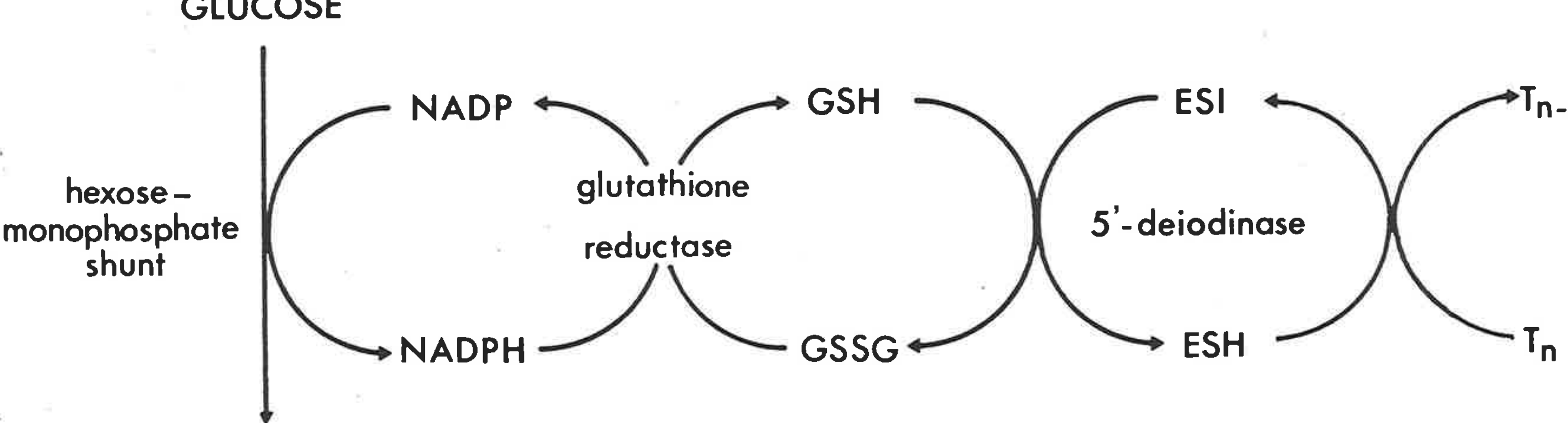
Studies on the T_4-5'-deiodinase activity have revealed PTU to be an uncompetitive inhibitor with respect to T_4 (Chopra, 1977) and a competitive inhibitor with respect to glutathione (Yamada et al., 1978). Visser (1980) and Leonard and Rosenberg (1980) have studied the interaction of labelled PTU with the enzyme in rat liver microsomal preparations. Labelled PTU formed mixed disulphides with the enzyme, inhibiting the enzyme activity in the process and providing evidence for the existence of a reduced sulphydryl group in the active site of the active enzyme. It was postulated that deiodination is effected through a two step process at the active site: (i) the transfer of an iodine atom from the iodothyronine to the enzyme with the formation of an enzyme-sulphenyl iodide intermediate complex (E-SI), and (ii) the subsequent reduction of the sulphenyl iodide by a thiol containing cofactor with the formation of the active reduced enzyme (E-SH). The specific reaction of thiouracils with sulphenyl iodides to form mixed disulphides (Visser, 1980) supports the proposed mechanism (Figure 1.3).

The lower sensitivity of the 5-deiodinase to both PTU and reduced intracellular glutathione concentration (Visser et al., 1978b) not only confirms the competition between PTU and glutathione, but also explains the reciprocal

Figure 1.3 Proposed mechanism of action of 5'-deiodinase and the relationship of this activity to glucose metabolism

Adapted from Yamada et al. (1978), Visser (1980) and Leonard and Rosenberg (1980)

T_n	iodothyronine with at least one iodine substituent in the outer, phenolic ring
T_{n-1}	5'-deiodinated metabolite of T_n
ESH	5'-deiodinase in the active reduced state
ESI	sulphenyl iodide enzyme intermediate formed during the 5'-deiodination reaction
GSH	reduced glutathione
GSSG	oxidised glutathione
NADP	oxidized nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate


GLUCOSE
hexose –
monophosphate
shunt
NADP
NADPH
glutathione
reductase
GSH
GSSG
5'-deiodinase
ESI
ESH
T_{n-1}
T_n

changes in the circulating triiodothyronines; the resulting greater inhibition of the clearance of rT_3 compared to its production explains the raised rT_3, and the decreased peripheral production of T_3 leads to a fall in circulating T_3.

2. Iodine Containing Drugs

Several iodine containing drugs, namely the radio-contrast agents iopanoic acid (Telepaque) and sodium iopodate (Oragraffin) (Burgi, Wimpfheimer, Burger, Zaunbauer, Rosler and Lemarchand-Beraud, 1976; Wu, Chopra, Solomon and Bennett, 1978; Beng, Wellby, Symons, Stuart and Marshall, 1980), and the antiarrhythmic agent amiodarone (Burger, Dinichert, Nicod, Jenny, Lemarchand-Beraud and Vallotton, 1976), cause a reduction in circulating T_3 and a rise in rT_3. Studies *in vitro* show these compounds to inhibit 5'-deiodinative metabolism of T_4 to T_3 and clearance of rT_3 to 3,3'-T_2 (Balsam, Ingbar and Sexton, 1978a; Chopra, 1978b; Kaplan and Utiger, 1978). Iopanoic acid and sodium iopodate are competitive inhibitors of 5'-deiodinase (Chopra, 1978b) suggesting that they act by interfering with the trans-iodination step, either by binding to the active site or by formation of an enzyme-sulphenyl iodide intermediate. No data is available on their action on 5-deiodinative metabolism of T_4 to rT_3. On the other hand, Balsam et al. (1978a) have demonstrated that 5-deiodination of T_4 to rT_3 is unaffected by amiodarone. The reciprocal changes in the circulating triiodothyronines during amidarone treatment can therefore be explained by reduced peripheral production of T_3, reduced clearance of rT_3 and unaltered peripheral production of rT_3 and clearance of T_3.

3. Propanolol

The β-adrenergic blocker propanolol, used to alleviate some of the symptoms of thyrotoxicosis, causes reciprocal changes in the circulating triiodothyronines (Verhoeven, Visser, Docter, Hennemann and Schalekamp, 1977). These changes are explained by reduced peripheral conversion of T_4 to T_3, reduced clearance of rT_3, unaltered production of rT_3 and normal clearance of T_3 (Lumholtz, Siersbaek-Nielsen, Faber, Kirkegaard and Friis, 1978b; Lumholtz, Kirkegaard, Faber, Busch-Sorensen, Siersbaek-Nielsen and Friis, 1978c. While Chiraseveenaprapund et al. (1978) have shown propanolol to have no effect on metabolism of T_4 to T_3 in rat kidney homogenates, Heyma, Larkins and Campbell (1978b) demonstrated an unequivocal reduction of T_4 to T_3 conversion in isolated intact rat renal tubules. They concluded that the drug action was due not to its β-adrenergic blocking property, but perhaps to its membrane stabilizing effects.

4. Glucocorticoids

The glucocorticoid, dexamethasone, causes reciprocal changes in the circulating triiodothyronines by lowering T_3 (Duick, Warren, Nicoloff, Otis and Croxon, 1974) and increasing rT_3 (Chopra, Williams, Orgiazzi and Solomon, 1975d). Although low serum T_3 was found during long term endogenous hypercortisolism in untreated Cushing's subjects, no significant change in rT_3 was demonstrated (Duick and Wahner, 1977). The mechanism of action of dexamethasone is uncertain but it is known not to act directly at the level of the deiodinating enzymes. Whereas T_4 to T_3 conversion is

low in homogenates of livers from dexamethasone treated rats, conversion in normal liver homogenates is unaltered after direct addition of dexamethasone (Huffner and Knopfle, 1976; Kaplan and Utiger, 1978).

5. Fasting

Studies with fasted rat liver homogenates suggest the decreased peripheral production of T_3 and clearance of rT_3 seen *in vivo* to be due to a selective reduction in 5'-deiodinase activity (Balsam et al., 1978a; Harris, Fang, Vagenakis and Braverman, 1978; Kaplan and Utiger, 1978). Harris et al. (1979) were able to restore 5'-deiodinase activity in homogenates of liver from two day starved rats by adding DTT. They concluded that while enzyme concentration was unaltered, activity was diminished during starvation by the absence of an essential cofactor. The nonprotein sulphydryl concentration in the starved rat liver homogenates was, in fact, about half that in fed rats. Similarly, Balsam, Ingbar and Sexton (1979) were able to restore full 5'-deiodinase activity to microsomal preparations from liver of two day starved rats by adding cytosol from fed rat liver homogenates (but not from starved rats) or glutathione to these microsomal preparations.

With longer fasting, more extensive changes in T_4-5'-deiodinase activity takes place. Kaplan (1979) found that a reduction in glutathione concentration in the liver of three day fasted rats does not completely account for the reduction in T_4-5'-deiodinase activity. Balsam et al. (1979) noted a similar difference between three and two day starved

rats. What is more, they found that the decline in enzyme concentration with longer fasting could be alleviated by maintaining the animals on T_4 and concluded that this phenomenon was secondary to hypothyroidism associated with starvation.

Thyroxine-5'-deiodinase in the rat kidney appears resistant to changes during starvation. Balsam, Sexton and Ingbar (1978b) first observed that while T_4-5'-deiodinase was significantly diminished in homogenates of liver from fasted thyroidectomized rats, no such change was evident in kidney homogenates. It has subsequently been shown that, in contrast to the liver, glutathione concentration in the kidney is not reduced during 2 - 3 day fasts (Balsam et al., 1979; Kaplan, Tatro, Breitbart and Larsen, 1979). That alterations in the circulating triiodothyronines do occur during starvation attests to the importance of the liver in T_4 metabolism.

The effects of fasting on 5-deiodinative metabolism are less well documented. The increase in $PRrT_3/PRT_4$ observed during fasting in man by Suda et al. (1978a) may have been due simply to an increased availability of T_4 in the peripheral tissues caused by a reduction in 5'-deiodination to T_3. Gavin, Bui, McMahon, Hammond and Cavalieri (1978) have demonstrated that the cytosolic T_4-5-deiodinase activity increased, while cytosolic rT_3-5'-deiodination in rat liver was unaffected during starvation. This is a particularly important observation as it represents the first clear demonstration of regulated 5-deiodinase activity. These changes would reinforce the alterations seen in 5'-deiodin-

ation in microsomal preparations from starved rat liver in leading to reduced T_3 and elevated rT_3 concentrations in the serum. The increased activity of the cytosolic T_4-5-deiodinase during starvation is difficult to reconcile with the demonstration that this activity is thiol-dependent *in vitro* (Cavalieri et al., 1977) and that rat liver cytosolic thiol concentration is reduced during starvation. Unfortunately, no other documentation of this effect has been reported.

While Balsam et al. (1979) found that deiodination of T_3 was reduced in microsomal preparations from liver of fasted rats, the products of deiodination were not identified making it unclear as to whether a reduction in 5'-deiodinase or 5-deiodinase activity was being observed.

6. Glucose and Thyroxine Metabolism

Experimental data from rat studies confirm the importance of glucose in the regulation of T_4 metabolism as seen in the human dietary studies. While there is reduced conversion of T_4 to T_3 in rat liver homogenates from streptozotocin induced diabetic rats, T_4-5'-deiodinase activity is normal in those diabetic rats treated with insulin (Balsam et al., 1978a). This study highlights the importance of adequate intracellular glucose concentration. A similar conclusion can be drawn from studies in which refeeding starved rats with carbohydrate or protein caused a return of serum T_3 concentration and liver T_4-5'-deiodinase activity to prefasting levels, whilst refeeding with lipid had no effect on either circulating T_3 or liver 5'-deiodinase

activity (Harris et al., 1978; Burger, Berger, Wimpfheimer and Danforth, 1980). Both carbohydrate and protein feeding stimulate insulin secretion, whereas a lipid diet does not, again confirming the requirement for a sufficient intracellular glucose concentration.

The addition of glucose to primary hepatocyte cultures in glucose-free medium containing insulin, stimulated conversion of T_4 to T_3 and inhibited rT_3 production (Gavin, Bissell, Hammond and Cavalieri, 1978). This was a clear demonstration of the relationship between glucose metabolism and T_4 metabolism.

REGULATION OF THE DEIODINATIVE METABOLISM OF THYROXINE

In summary, T_4 is deiodinated by either an activating route to T_3, or by an inactivating route to rT_3. The triiodothyronines are then sequentially monodeiodinated allowing recovery of the iodine atoms. Although other pathways of metabolism exist, the deiodinative pathways would appear to account for most of the iodothyronine metabolism under normal physiological conditions. It has been postulated that only two enzymes, a 5'-deiodinase and a 5-deiodinase, effect the complete sequential deiodination of T_4 (Figure 1.2).

There is now considerable documentation of the regulation of 5'- deiodinase under a variety of pathophysiological and experimental conditions. On the other hand, there is little evidence for regulation of 5-deiodinase. The available data imply that the deiodinative metabolism of T_4 is controlled by regulation of the 5'-deiodinase, with

5-deiodinase being relatively unaffected.

1. Regulation of 5'-Deiodinase

The regulation of 5'-deiodinase appears most likely to occur through variations in the intracellular concentration of glutathione. Visser (1978) has proposed a mechanism by which 5'-deiodinase activity, through the intracellular concentration of GSH, is sensitive to glucose metabolism (Figure 1.3). The enzyme glutathione reductase, which maintains basal intracellular GSH concentration, requires reduced nicotinamide adenine dinucleotide phosphate (NADPH) for activity. Amongst the intracellular sources of NADPH in the liver and kidney are the conversion of malate to pyruvate by malate dehydrogenase and metabolism of glucose-6-phosphate via the pentose phosphate pathway (Lehninger, 1975).

Although Visser et al. (1975) and Chiraseveenaprapund et al. (1978) showed no stimulation of T_4 to T_3 conversion by reduced pyridine nucleotides in rat liver homogenates, recent evidence demonstrates stimulation of 5'-deiodinase by addition of NADPH generating systems to microsomal preparations from liver of starved rats (Balsam et al., 1979). Diamide, a specific inhibitor of GSH, blocked the stimulation of 5'-deiodinase by both NADPH generating systems and GSH, confirming the dependence of 5'-deiodinase activity on adequate intracellular NADPH concentrations.

A recent study on the effect of nicotinamide in starving rats is consistent with the mechanism proposed for

the link between glucose metabolism and 5'-deiodination (Burger et al., 1980). In starved rats, nicotinamide caused an elevation of blood glucose and reduction in circulating free fatty acids and ketones to levels seen in carbohydrate-refed starved rats. Despite these changes, circulating T_3 remained low. As nicotinamide is known to stimulate gluconeogenesis and glycogenolysis, the intracellular glucose concentration would be expected to have been similar to that in the refed animals (Talke, Maier, Kersten and Gerok, 1973). However, nicotinamide also oxidizes the pyridine nucleotides and would thus lead to the combination of normal or elevated intracellular glucose concentration, low NADPH and GSH concentration and low T_4-5'-deiodinase activity.

During starvation glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase of the pentose phosphate pathway, and malate dehydrogenase activities are reduced, resulting in lowered NADPH and GSH concentrations and 5'-deiodinase activity. Conversely glucose or amino acid intake cause a rise in NADPH and GSH concentrations and 5'-deiodinase activity.

2. Tissue Specific 5'-Deiodinase Activity

There appears to be a variation in the responses of the 5'-deiodinases in different tissues to changes in physiological conditions. Thus rat kidney 5'-deiodinase activity is resistant to starvation as, unlike the liver, kidney GSH concentration does not alter appreciably. It is not known whether these differences have any physiological meaning, but the reduction in circulating T_3 during

starvation would suggest that the liver is the predominant deiodinating organ. What is more, Silva et al. (1978) have shown that nuclear T_3 receptor occupancy in the liver and kidney of the rat is determined by the circulating concentration of T_3. This implies then that T_3 receptor occupancy in the kidney during starvation is determined principally by the liver 5'-deiodinase activity.

Thyroxine-5'-deiodinase in the rat anterior pituitary has been shown to be different to both the liver and kidney enzyme activities (Kaplan, 1980). The pituitary enzyme required higher concentration of DTT *in vitro* than the liver or kidney activities to attain maximum rate. In addition, the enzyme activity was not significantly affected by PTU but was inhibited by iopanoic acid. This would suggest that the transiodination step was similar to that of the liver and kidney enzyme activities but that the subsequent reduction of the pituitary enzyme was different. The lack of reaction with PTU indeed implies that a sulphenyl-iodide enzyme intermediate may not exist. The K_m for T_4 was 8.8 nmol/L, being considerably lower than the K_m values for the liver and kidney enzymes.

3. <u>Non-Thyroidal Illness</u>

There is at present no experimental information on the regulation of the 5'-deiodinase and 5-deiodinase during severe non-thyroidal illness. While the studies *in vivo* indicate diminished 5'-deiodinase activity it is unclear how this comes about in the cell. However, from the foregoing discussion it would appear that there are changes in glucose

metabolism which are in some way related to the severity of illness and which lead to reduced intracellular GSH concentration.

THYROID DISEASE

While the iodothyronine clearance studies *in vivo* quite clearly show an increased rate of conversion of T_4 to T_3 in untreated hypothyroidism and a reduced rate in thyrotoxicosis, the absolute amount of T_3 produced in the peripheral tissues is reduced or elevated respectively (Inada, Kasagi, Turata, Kazawa, Takayama, Torizuka, Fukase and Soma, 1975; Maguire, Dennehy and Cullen, 1976).

In contrast, Smallridge, Wartofsky, Desjardins and Burman (1978) have demonstrated that both the rate of conversion of T_4 to rT_3 ($PRrT_3/PRT_4$) and the absolute peripheral production rate of rT_3 are reduced in hypothyroidism and elevated in thyrotoxicosis.

Although there is no obvious relationship between the changes in peripheral production of the triiodothyronines during thyroid disease and the alterations observed during starvation and severe illness, T_4 metabolism to T_3 may be regulated so as to compensate for major alterations in thyroid gland secretion of hormone.

1. Studies *In Vitro*

Thyroxine-5'-deiodinase activity in homogenates of rat liver and kidney is significantly reduced within three to four weeks post thyroidectomy (Balsam et al., 1978b; Harris et al., 1979). While the activity could be stimulated

by DTT, it was not restored to control levels thereby suggesting a reduction in enzyme concentration. Similarly, very low 5'-deiodinase activity was evident in the hypopituitary hypothyroid dwarf mouse (Harris et al., 1979).

Pituitary T_4-5'-deiodinase in pituitary homogenates from hypothyroid rats has been shown to be 3 - 12 fold greater in activity than that in the control rat pituitary homogenates (Kaplan, 1980). This increase could be accounted for in terms of an increase in the relative and absolute number of thyrotrophic cells in the pituitaries of hypothyroid rats (Surks and DeFesi, 1977). That is, the specific activity of the enzyme in the thyrotrophic cells themselves may have decreased as has been shown in the rat liver and kidney.

As no data is available on 5'-deiodinase activity in man it is not known whether the changes in peripheral T_3 production rate observed during thyroid disease are due simply to altered availability of T_4, whether enzyme activity is affected through changes in concentration as in the rat, or whether a combination of both phenomenon is operating.

Virtually nothing is known about the activity of 5-deiodinase in thyroid disease.

SUMMARY

The elaboration of the pathways of T_4 monodeiodination and their regulation has radically altered the traditional concepts of the regulation of thyroid hormone activity. It is now clear that the regulation of thyroid hormone action is effected not only at the hypothalamic-pituitary-thyroid gland level, but also at the target cell level.

Thyroid hormone action at the target cell is mediated by T_3 derived from the circulation. In turn the circulating T_3 is derived from two sources: that secreted by the thyroid gland and controlled by the hypothalamic-pituitary axis, and that generated by 5'-deiodination of T_4 in the peripheral tissues. While there is a certain level of thyroid hormone action ensured in the target cell by virtue of thyroid gland section of T_3, the target cell can to a certain degree regulate the action of T_3 by regulating conversion of T_4 to T_3. It does appear, however, that regulation of the peripherally derived T_3 component is mediated principally by the liver.

It is of some importance that the hypothalamic-pituitary-thyroid gland axis itself may be sensitive to factors varying deiodinase activities. Thus the pituitary, in monitoring circulating T_3 and T_4 concentration will be sensitive to changes in T_4-5'-deiodinase activity through variations in plasma T_3. What is more, both the pituitary gland and thyroid passes 5'-deiodinase activities; in the pituitary gland, nuclear T_3 receptor occupancy might then be altered by changes in conversion of T_4 to T_3, and in the thyroid gland, the ratio of secreted T_4 may be varied by similar changes in 5'-deiodinase activity.

Although rT_3 may be simply an inactive product of T_4 metabolism, circulating rT_3 concentration is a good indicator of 5'-deiodinase activity. While fluctuations in T_3 are due to variations in both thyroid gland secretion or peripheral production, rT_3 reflects only peripheral metabolism.

A clarification of what factors influence peripheral metabolism in non-thyroidal illness may make the measurement of serum rT_3 concentration a particularly valuable clinical tool in the diagnosis of thyroid disease in the presence of severe illness.

CHAPTER 2

MEASUREMENT OF THE SERUM IODOTHYRONINES

2.1 EARLY ANALYTICAL TECHNIQUES

The unique iodine content of the iodothyronines was the basis for the original estimates of their concentration in serum (Barker, Humphrey and Soley, 1951). This method also exploited the nearly complete binding of the iodothyronines to circulating proteins, and was termed the serum protein bound iodine, or PBI test. The technique involved the precipitation of the serum proteins and thorough washing of the precipitate to remove inorganic iodide. The organically bound iodine was released at high temperature and measured by the ceric sulphate-arsenious acid reaction of Sandell and Kolthoff (1937).

Considering the higher iodine content of T_4 and the fifty fold greater concentration of T_4 than T_3, the PBI reflected only the concentration of T_4. The method also had an inherent lack of specificity. Thus PBI was elevated by circulating iodoprotein, thyroglobulin, iodotyrosines and in particular by iodinated radiographic contrast media and a variety of other iodine containing drugs. Iodoprotein and iodotyrosines could be removed by washing butanol extracts of serum protein precipitates with alkali (Man, Kydd and Peters, 1951). The butanol-extractable iodine procedure did not however overcome the problems of contaminating iodine containing drugs and radiographic contrast media.

Several subsequent developments enabled the develop-

ment of assays for T_4 which considerably diminished contaminating iodine containing drug interference and incorporated a simpler determination of organically bound iodine. Firstly, Bowden, MacLagan and Wilkinson (1955) demonstrated that the iodothyronines directly decolorised the ceric sulphate-arsenious acid reagent, thus alleviating the necessity for exotic methods to release the bound iodine. Secondly, Ingbar, Dowling and Freinkel (1957) observed that T_4 could be extracted from serum in high concentration by ion-exchange chromatography. The serum was deproteinized by passage through the ion-exchange column and the bound T_4 subsequently eluted. From these observations Pileggi, Lee, Golub and Henry (1961) developed the T_4-by-column technique incorporating the extraction of T_4 from serum with an anion-exchange resin and direct determination of the iodine content of the subsequently eluted T_4 by the ceric sulphate-arsenious acid technique without prior digestion. This method offered the advantages outlined and was suited to the routine clinical laboratory.

The demonstration that T_3 was present in the circulation and had a greater metabolic potency than T_4 (Gross et al., 1952) evoked considerable interest in the specific determination of the thyroid hormones. The initial approach to this problem was to extract the iodothyronines from serum and separate T_3 and T_4 on ion-exchange resin (Galton and Pitt-Rivers, 1959; Lerner, 1963), by filter paper chromatography (McGreer, Robertson and McGreer, 1961), Sephadex G-25 filtration (Mougey and Mason, 1963), or thin layer chromatography (West, Wayne and Chavre, 1965). The

iodine content of the separated T_3 and T_4 was measured directly by the ceric sulphate-arsenious acid reaction. Unfortunately this method was limited by the relative insensitivity of measuring iodine and was too insensitive to be of practical use in measuring physiological concentrations of T_3.

2.2 SPECIFIC AND SENSITIVE ANALYTICAL TECHNIQUES FOR MEASUREMENT OF THE THYROID HORMONES IN SERUM

COMPETITIVE PROTEIN BINDING ASSAYS

The specific and sensitive determination of the iodothyronines began with the development of the competitive protein binding assay for T_4 (Ekins, 1960). Thyroxine was extracted from serum with acidified butanol and added with ^{131}I-T_4 to a reference serum. After incubation the TBG bound ^{131}I-T_4 (the tracer bound) and albumin bound ^{131}I-T_4 (the free tracer) were separated by electrophoresis and the ratio of counts in the albumin zone and counts in the TBG zone (F/B) plotted against the T_4 concentration of the standards. This was an elaborate technique and it was Murphy and Pattee (1964) who subsequently developed a competitive protein binding assay for T_4 which was more suited to routine laboratory use. The T_4 in ethanol extracts of serum was assayed by measuring the extent to which ^{131}I-T_4 was displaced from a standard serum thyronine binding protein mix after addition of the extract. This approach eliminated the interference by iodine containing drugs and radiographic contrast media. By separating T_3 and T_4 in the serum extracts, Nauman et al. (1967) provided a modification which enabled

the sensitive and specific determination of T_3. Both T_3 and T_4 were firstly purified from the ethanol extract by gel filtration through Sephadex LH-20 and separated on thin layer chromatograms. The T_3 recovered was assayed by measuring the displacement of ^{131}I-T_3 from the thyronine binding protein mix after addition of the extracted T_3.

The work up procedures required to make this technique specific were involved and laborious, making them inherently imprecise and unattractive for general routine analysis. Furthermore, there were problems in ensuring complete separation of T_3 and T_4 and deiodination of T_4 occurred during the purification and separation steps.

GAS LIQUID CHROMATOGRAPHY

The development of the gas liquid chromatography of the N,O-dipivalyl-methyl derivatives of the iodothyronines provided a potentially very useful assay in that a variety of circulating iodothyronines and iodotyrosines could be separated and measured during chromatography (Hollander, 1968). The iodoamino acids were extracted from serum by dialysis at low pH and the N,O-dipivalyl-methyl derivative was purified from the dialysate by anion exchange chromatography and passed through a gas chromatograph with an electron capture detector.

While this technique enabled the specific determination of the serum thyroid hormones and in particular provided a sensitive assay of serum T_3, the extensive work up procedure made it unsuitable for routine clinical use.

RADIOIMMUNOASSAY

The development of specific T_4 and T_3 antisera (Brown, Ekins, Ellis and Reith, 1970; Chopra, Nelson, Solomon and Beall, 1970) provided a breakthrough in measuring circulating iodothyronines. The techniques subsequently developed for the specific and sensitive radioimmunoassay of T_3 and T_4 have been used to assay virtually every possible postulated iodothyronine metabolite of T_4.

1. Production of Specific Antisera

Chopra et al. (1970) chose their antisera from those produced following injection of human thyroglobulin in rabbits. This procedure resulted in some animals producing antisera specific for T_3 and others specific for T_4. The procedure of Brown et al. (1970) ensured the production of antisera specific to T_3 by immunizing animals with T_3 covalently bound to poly-L-lysine to render the T_3 immunogenic. Other proteins which have been used are bovine or human serum albumin (Lieblich and Utiger, 1972; Larsen, 1972) and haemocyanin (Burger, Sakoloff, Staeheli, Vallotton and Ingbar, 1975).

2. Synthesis of High Specific Activity Radioiodothyronines

In 1963, Greenwood, Hunter and Glover introduced the chloramine T oxidative radioiodination of tyrosine residue in protein. This procedure specifically introduced radioiodine into the carbon atoms adjacent to the 4-hydroxyl group. Although this method was used for the production of high specific activity ^{131}I-iodotyrosines (Reith and Tampion,

1963), the majority of ^{131}I-iodothyronines were produced by diazotization and subsequent radioiodination (Harrington and Pitt-Rivers, 1944) or by the iodine chloride iodination procedure of Shiba and Cahnmann (1962). These methods yielded tracer of relatively low specific activities.

In 1973, Weeke and Orskov reported the chloramine T oxidative radioiodination of 3,3'-diiodothyronine yielding ^{125}I-T_3 of very high specific activity. This method was universally applicable to radioiodinating iodothyronines in the outer phenyl ring, and together with the availability of high affinity antisera, made possible very specific and sensitive radioimmunoassays.

3. Inhibition of Serum Binding Proteins

Given these reagents, the greatest problem in developing simple assays was the binding of T_3 and T_4 to the serum thyronine binding proteins. This was initially overcome by assaying ethanol extracts of serum (Chopra, Solomon and Ho, 1971a), serum deproteinized on Sephadex (Surks, Schadlow and Oppenheimer, 1972; Patel and Burger, 1973), or hormones extracted on Dowex 50W (Hesch, Hufner and Von Muhlen, 1972).

With a view to simplifying these assays to allow direct assay of thyroid hormones in serum, a number of agents were tested as thyroid hormone binding inhibitors. These were either known to raise the percent dialyzable T_4, or were structural analogues of the iodothyronines. Malkus and Donabedian (1974) have reviewed the use of a variety of inhibitors, the most commonly used being merthiolate (Gharib,

Ryan, Mayberry and Hockert, 1971), sodium salicylate (Larsen, 1972) and ammonium-8-anilino-naphthalene-1-sulphonate (ANS) (Chopra, 1972). The most important criterion for an effective inhibitor is for it to completely inhibit binding of the thyronines being measured at a concentration which does not substantially affect iodothyronine binding to the antibody. High pH barbital buffer has been shown to inhibit binding of thyroid hormones to TBPA (Ingbar, 1963), and is usually used as the assay buffer. However, this may not be necessary where binding proteins are effectively inhibited by other blockers (Chopra, Solomon and Beall, 1971b).

Of particular importance to the development of direct radioimmunoassays is the provision of serum based standards. This has been achieved by adding given amounts of thyroid hormone to serum completely deficient in thyroid hormones. Athyreotic sheep serum (Chopra et al., 1971b); human serum stripped of thyroid hormone by activated charcoal (Larsen, 1972), and anion exchange resins (Chopra, 1972; Burger et al., 1975); or heat inactivated serum (Sterling and Milch, 1974) have been used.

These developments have provided T_3 and T_4 assays which are straightforward, precise and eminently suited to general routine use. There is now good agreement on the physiological concentrations of T_3 and T_4 in serum, these results being consistent with those derived using the unrelated technique of gas liquid chromatography (Hollander, 1968).

2.3 ANALYSIS OF IODOTHYRONINE METABOLITES OF T_4 IN SERUM

REVERSE T_3

In 1974, Chopra reported the radioimmunoassay of rT_3 in ethanol extracts of serum, demonstrating the general applicability of these techniques to the measurement of the iodothyronines. The demonstration of elevated rT_3 in a number of patients with non-thyroidal illness (Chopra, 1974), and the discovery that circulating rT_3 was virtually totally derived from peripheral monodeiodination of T_4 (Chopra, 1976) generated intense interest in rT_3 and the peripheral metabolism of T_4. rT_3 was shown to circulate bound to serum proteins in a manner similar to T_3 and T_4 (Chopra et al., 1975a; Ratcliffe, Marshall and Ratcliffe, 1976). The original assays using serum extracts were soon replaced by direct estimations of rT_3 in serum using the protein binding inhibitors ANS and merthiolate (Table 2.1).

DIIODOTHYRONINES AND MONOIODOTHYRONINES

Assays have now been developed for each of the diiodothyronines (Wu, Chopra, Nakamura, Solomon and Bennett, 1976; Kirkegaard, Faber, Friis, Lumholtz and Siersbaek-Nielsen, 1978; Meinhold and Schurnbrand, 1978), and 3'-T_1 (Smallridge, Wartofsky, Green, Miller and Burman, 1979). The assay of 3,5-T_2 poses an interesting problem in that the chloramine T oxidative radioiodination procedure is not suitable. Meinhold and Schurnbrand (1978) prepared the ^{3}H-acetyl-3,5-T_2 derivative. Sorimachi and Cahnmann (1977) have prepared high specific activity ^{125}I-3,5-T_2 by radio-

iodinating DIT, conjugating with diiodo-hydroxy-phenyl pyruvic acid (providing the outer ring) and removing the 3'- and 5'-^{127}I in the presence of hydroiodic and hypophosphorous acids to produce the ^{125}I-3,5-T_2. A simpler technique producing tracer of lower specific activity is the production of ^{125}I-3,5-T_2 from 3,5-T_2 by ^{125}I exchange at the positions ortho to the 4-hydroxyl group in the presence of chloramine T (Kochupilla and Yalow, 1978). A similar approach would be required to synthesize ^{125}I-3-T_1.

The assays for rT_3, the diiodothyronines and for 3'-T_1 highlight the problem of measuring very low concentrations of iodothyronines. There is no consensus of opinion on the serum concentration of any of these metabolites. The variations in published concentrations may be related to the different reagents used, particularly antisera and standards.

TABLE 2.1 Published procedures for the radioimmunoassay of reverse T_3

		Standard	Immunogen	T_4 X/R	Buffer	pH	Inhibitor	% Serum	Separation	Concentration (nmol/L)
Extraction										
Chopra	1974	dl-rT_3 W-L	dl-rT_3-HSA	0.06%	0.07M Barb	8.6			DAB	0.63 ± 0.16
Meinhold et al.	1975	l-rT_3 Henning	l-rT_3-BSA	0.025%	0.08M Barb	8.6			DAB	0.28 ± 0.18
Corcoran et al.	1976	l-rT_3 Henning	dl-rT_3-BSA	N.S.						0.31 ± 0.16
Donaldson et al.	1976	l-rT_3 Henning	l-rT_3-Glob	0.13%	0.05M Barb	8.6			DCC	0.60 ± 0.14
Faber et al.	1978	l-rT Henning	rT_3-albumin	0.03%	0.07M Phos.	7.4			Sephadex G25	0.74 ± 0.14
Direct										
Griffiths et al.	1976	l-rT_3 Henning	l-rT_3-BSA	0.025%	0.05M Barb	8.6	0.022% ANS	12.5	DAB	0.68 ± 0.16
Huffner et al.	1976	l-rT_3 Henning	l-rT_3-BSA	0.05%	0.05M Phos.		0.023% ANS	7.7	DAB	0.32 ± 0.14
Kodding et al.	1976	l-rT_3 Henning	l-rT_3-BSA	0.05%	0.08M Barb	8.4	ANS		DCC	0.25 ± 0.12
Nicod et al.	1976b	l-rT_3 Cahnmann	l-rT_3-hemocyanin	0.06%	0.06M Barb	8.8	0.038% ANS	12.5	QAE-Seph A-25	0.70 ± 0.15
Vagenakis et al.	1975	l-rT_3 Jorgensen								0.56
Ratcliffe et al.	1976	l-rT_3 Henning	l-rT_3-BSA	0.14%	0.05M Barb	8.6	0.050% ANS	20	DAB	0.27 ± 0.06
Burman et al.	1977	l-rT_3 Cahnmann	l-rT_3-BSA	0.06%	0.20M Borate	8.5	0.060% ANS	20	NH_4SO_4	0.93 ± 0.19
Burrows et al.	1977	l-rT_3 Henning		0.022%						0.24
Fisher et al.	1977	l-rT_3 Jorgensen	l-rT_3-BTg	0.01%	Barb		ANS			0.64 ± 0.22
Gavin et al.	1977	l-rT_3 Jorgensen	l-rT_3-BSA	0.062%			ANS		DAB	0.50 ± 0.12
Kaplan et al.	1977	l-rT_3 Cahnmann	dl-rT_3-BSA	0.04%	0.10M Phos.	7.5	0.10% ANS	42	DAB	0.36 ± 0.12

TABLE 2.1 (continued)

Laurberg et al.	1977								0.16 ± 0.04
Visser et al	1977	l-rT_3 Henning	l-rT_3-BSA	0.043%	0.055 Barb	8.6	ANS	DAB	
Premachandra	1978	l-rT_3 Henning	rT_3-albumin	0.04%					0.13 ± 0.05
Tagaki et al.	1978	l-rT_3 Cahnmann			0.1M Borate	8.6	ANS	PEG	0.43 ± 0.14
Roti et al.	1979	l-rT_3 Cahnmann	l-rT_3-BSA	0.04%	Barb		0.038% ANS	Charcoal	0.69 ± 0.13

Abbreviations:

X/R	Cross-reaction
W-L	Warner Lambert
PEG	Polethylene Glycol
Barb	Barbital
Phos	Phosphate
Glob	Gamma Globulin
DCC	Dextran Coated Charcoal
Seph	Sephadex
BTg	Bovine Thyroglobulin

CHAPTER 3

THE ANALYSIS OF SERUM 3,3',5'-TRIIODOTHYRONINE

3.1 INTRODUCTION

The demonstration of increased serum rT_3 concentration in patients with moderate to severe non-thyroidal illness (Chopra et al., 1975a) raised the possibility that the estimation of serum rT_3 concentration might be of value in diagnosing thyroid disease during severe illness. While the low serum T_3 characteristically seen during severe illness might suggest hypothyroidism where this is suspected clinically, an accompanying elevated rT_3 would imply that thyroid gland secretion of T_4 was adequate. That is, it could be concluded that hypothyroidism was not present, but that peripheral metabolism of T_4 was impaired (Chapter 1 (1.3)).

The aim was to establish an assay for serum rT_3 to assess the diagnostic usefulness of measuring this iodothyronine in evaluating thyroid disease, particularly where thyroid disease was suspected in patients with moderate to severe non-thyroidal disease.

This chapter describes in detail the development of this technique. While several assays for rT_3 had been published at the time of beginning this project, these employed ethanol extraction procedures, T_4 cross-reaction was significant, and they did not agree on the serum concentration in euthyroid subjects (Chopra, 1974; Meinhold, Wenzel and Schurnbrand, 1975). It was decided to establish

an assay to measure directly the concentration of rT_3 in serum and to investigate the problems associated with the accurate determination of serum rT_3 levels and the cross-reaction by T_4.

3.2 SYNTHESIS OF ^{125}I-rT_3

^{125}I-rT_3 was synthesized by radioiodination of 3,3'-T_2 using a modification of the chloramine T oxidative iodination procedure of Weeke and Orskov (1973). Unreacted iodide was removed by elution of the reaction mixture through Sephadex LH-20 (Williams, Freeman and Florsheim, 1969).

MATERIALS AND METHODS

1. Reagents

Sodium iodide (^{125}I) for iodination was purchased from the Radiochemical Centre (U.K.) as 2 mCi in 20 µL of NaOH pH 7 - 11.

3,3'-T_2 was kindly donated by Henning GmbH (Germany). 3,3'-T_2 for iodination was stored as a 1 µg/ 10 µL solution in 50% ethanol at 4°C.

All reagents were AR grade and generally available except where stated. Chloramine T (5 mg/2.5 mL) and sodium metabisulphite (0.5 mg/mL) were dissolved in 0.05 M phosphate buffer, pH 7.4, just prior to use.

2. Gel Filtration Systems

The iodination reaction mixture was eluted through either a high pH Sephadex G-25 filtration system or, in most

cases, through Sephadex LH-20 with an ethyl acetate based eluant mixture.

The aqueous system (Kjeld, Kuku, Diamant, Fraser, Joplin and Mashiter, 1975) comprised a 0.8 x 10 cm column of Sephadex G-25 in 0.05 M K_2HPO_4, pH 7-7.5. Before use the column was equilibrated with 0.05 M K_2HPO_4, pH 12. The reaction mixture was eluted with this buffer pumped through using a Technicon (U.S.A.) peristaltic pump at 40 mL/h. 40 drop (approximately 1½ mL) fractions were collected with an LDTM fraction collector (Paton Industries, South Aust.). Each fraction was neutralized with one drop of 6 M HCl. After use the column was washed with 0.05 M K_2HPO_4, pH 7-7.5. Each fraction was counted for 1 s in a Mini-Assay type 6-20 portable gamma counter (Mini-Instruments, U.K.).

The non-aqueous eluting solvent was ethyl acetate/ methanol/water/NH_4OH (400:100:36:4) (Williams et al., 1969). The Sephadex LH-20 was prepared by soaking in the eluting solvent for 3 h and the gel was then washed three times in methanol/2 N NH_4OH (450:50), twice in diethyl ether, and twice in eluting solvent. The washed gel was stored in eluting solvent at 4°C until used. A 1 x 40 cm column of Sephadex LH-20 was poured into a solvent resistant Pharmacia (Sweden) SR 10/50 gel filtration column. Solvent resistant polytetrafluoroethylene (PTFE) tubing was used throughout the system. This tubing was brittle and to prevent collapse of the tubing running through the peristaltic pump, this region of tubing was contained within a 20 cm length of peristaltic pump tubing (Technicon, U.S.A.). The internal diameter of this latter tubing was selected to give as tight

a fit around the PTFE tubing as possible, so that, after the passage of a pump roller bar, this outer flexible tubing tended to force open the PTFE tubing. Fractions of 100 drops (approximately 1 mL) were collected into 5 mL polypropylene tubes using the LDTM automatic fraction collector and subsequently counted in the Mini-Assay counter.

3. Iodination Reaction

As iodide is so readily taken up and concentrated by the thyroid gland, the entire iodination and clean up procedure was carried out in a fume hood using surgical gloves. The ^{125}I content of the thyroid gland was measured before and after each iodination procedure with a Phillips remote probe and associated gamma counter, providing a guide to how effective the precautionary measures were.

The reaction was carried out in a vial similar to that in which $Na^{125}I$ was routinely received. The vial had a volume of 500 µL and tapered internal walls. The reaction procedure is summarized in Table 3.1. The rubber seal was removed and any accumulated $^{125}I_2$ allowed to escape. A 10 µL (1 mCi) aliquot was withdrawn with a 25 µL Pierce (U.S.A.) syringe and transferred to the reaction vial. The pH was neutralized with 12.5 µL of 0.5 M phosphate buffer, pH 7.4, introduced into the radioiodine solution with the same syringe. To this was added 1.9 nmole (10 µL) of 3,3'-T_2, the solution mixed, and 89 nmole of chloramine T added to start the reaction. After 20 s the reaction was stopped with 658 nmole sodium metabisulphite. Eluting solvent was added to fill the vial, the contents transferred

TABLE 3.1 Oxidative radioiodination procedure

	This study	Weeke and Orskov (1973)	Kjeld et al. (1975)	Kochupilla and Yalow (1978)
Na iodide (mCi)	1	5	2	0.5
Phosphate buffer (μL)	12.5	50	25	20
Iodothyronine (nmole)	2	3.1	1.6	1.6
Chloramine T (nmole)	89	319	355	186
Reaction time (sec)	20	15	20	45
Metabisulphite (nmole)	658	1260	1315	276
$\frac{\text{Metabisulphite}}{\text{Chloramine T}}$	7.4	4.0	3.7	1.5
$\frac{\text{Chloramine T}}{\text{Iodothyronine}}$	44.5	103	222	116
Final reaction volume (μL)	75	245	180	41

to the filtration column and eluted with the appropriate eluting solution.

An equal volume of ethanol was added to the desired aqueous fractions and stored at room temperature in a lead lined wooden container. The ethyl acetate fractions were allowed to dry down in the fume cupboard and stored in 50% aqueous ethanol.

Once the iodination procedure was established for routine use, it was found the elution profile and specific activity were reasonably consistent. Each iodination was thus checked by simply measuring the % B_o/T under the usual assay conditions. Any recurrent iodination problems were investigated with a thin layer chromatographic profile of the eluted peaks and a check on specific activity.

4. Thin Layer Chromatography

Where necessary, the eluted peaks were characterized by thin layer chromatography using a method developed by Maerschel (1973). 8.8 g of cellulose MN 300 (Machery-Nagel, Germany), 2.2 g of Kieselguhr 60HR silica gel (U.S.A.), and 60 mL ion free water were mixed at high speed for 30 s in a domestic blender to form a homogeneous slurry. The slurry was deaerated under reduced pressure and poured onto 20 x 20 cm glass plates using a thin layer chromatogram spreading apparatus (Quickfit, U.K.) set at 25 microns. The plates were dried overnight and stored covered until required.

A 10 μL aliquot of the eluted peak to be examined was spotted onto a chromatographic plate along with ^{125}I-rT_3,

^{125}I-T_3 and ^{125}I-T_4 (The Radiochemical Centre, U.K.) as markers. The plate was developed to 15 cm in 1 M acetic acid/ methanol (2:5) and dried.

To locate the radiolabelled components, an RP/S X-Omat X-ray plate (Kodak, Aust.) was placed in direct contact with the chromatogram and developed for an appropriate period of time, usually overnight.

5. Determination of Specific Activity

Specific activity was determined by the self-displacement method (Figure 3.5). In one series of tubes, a standard curve ranging from 0 to 2.56 nmol/L rT_3 was set up in the usual manner, each containing a constant concentration of tracer, p*. That is, the total concentration of labelled and unlabelled rT_3 ranged from [p* + 0] to [p* + 2.56] nmol/L. A second series of tubes contained no unlabelled rT_3. In these tubes, tracer was present in concentrations increasing from p* to approximately 100p*. The % B/B_o for each tube was then calculated and plotted against the amount of ligand in each tube.

So as to be able to compare the two series of tubes, the amount of ligand in each tube was corrected to [total ligand - p*]. In this way, the displacement by cold ligand alone in the first series of tubes (0 → 2.56 nmol/L) could be directly compared to the self-displacement by labelled ligand in the second series of tubes (0 → [100p* - p*] nmol/L). The specific activity was determined by equating that concentration of tracer in the second series of tubes giving the same % B/B_o as a given concentration of unlabelled rT_3 in

the first series of tubes.

Specific activity was calculated by equating that concentration of tracer (point a, Figure 3.5) causing the same relative displacement as a given concentration of unlabelled rT_3 (point b, Figure 3.5),

Specific activity

$$= \frac{\text{counts at point a/min/tube}}{\text{concentration of unlabelled } rT_3 \text{ at point b (nmol/L)}} \times 3.875 \times 10^{-3} \text{ pCi/pg}$$

The specific activity was calculated at several points on the standard curve and the values averaged.

RESULTS AND DISCUSSION

1. Oxidative Iodination

Chloramine T is a commonly used oxidizing agent in the iodination of aromatic compounds (Bradfield, Orton and Roberts, 1928). Although the mechanism of iodination is not entirely clear, it is thought that in aqueous solution, chloramine T oxidizes I^- to I_2, which is considered to be the iodinating species (Mayberry, 1972). The presence of the hydroxyl substituent on the outer phenolic ring of the iodothyronines directs substitution at the 3'- position and 5'- position. This reaction is thus ideally suited to the specific labelling of the outer ring in the synthesis of ^{125}I-rT_3, leaving the inner tyrosyl ring monoiodinated. The elution profile (Figure 3.1) and thin layer chromatographic profile of eluted peaks (Figure 3.2) verify the specificity of the reaction. ^{125}I-rT_3 and ^{125}I-3,3'-T_2 are the major products of the reaction. The presence of ^{125}I-3,3'-T_2

suggests that under the conditions of this iodination procedure, isotopic exchange is as important a reaction as substitution. Mayberry (1972) has suggested that exchange occurs through a similar mechanism to the substitution reaction, occurring only in the presence of the oxidizing agent.

The ratios of chloramine T to iodothyronine and iodide to iodothyronine were less than suggested by Weeke and Orskov (1973) (Table 3.1). These modifications limited the specific activity to about 1200 pCi/pg, which was quite adequate for use in the assay, and reduced autolysis during storage. The low concentration of free $^{125}I^{-}$ remaining suggested the iodide concentration to be limiting. A higher specific activity product would likely have been obtained by simply increasing the concentration of iodide, although increased chloramine T would be required when this became limiting.

2. Gel Filtration

The crosslinked dextran gels strongly adsorb aromatic substances, particularly those which are substituted (Gelotte, 1960; Porath, 1960). This was the basis for the separation of iodide, the iodotyrosines and the iodothyronines on Sephadex G-25, the iodotyrosines and iodothyronines being eluted well after V_t (total volume of the gel column) (Lissitzky, Bismuth and Rolland, 1962). Retention of the iodoamino acids was subsequently found to depend on both the degree of substitution (Mougey and Mason, 1963) and the position of the iodine atom substituents (Blasi and de Masi,

1967). The demonstration of pH dependent elution characteristics (Mougey and Mason, 1963) provided the basis for the development of a number of aqueous systems for the resolution of the iodoamino acids. At low pH, MIT and DIT were effectively separated from each other and iodide, whereas T_3 and T_4, which were eluted later, were not well resolved. At high pH, T_3 and T_4 were well resolved, whilst the iodotyrosines tended to elute with iodide.

Nauman et al. (1967) first demonstrated the retarded elution of T_3 on Sephadex LH-20 using a 10% aqueous solvent. Williams et al. (1969) subsequently developed a less polar eluting system which provided a very good separation of the iodoamino acids. This ethyl acetate/methanol/2 N aqueous NH_4OH (400:100:40) elution system was adopted for the separation of the radioiodination reaction products. Although this system required the use of a solvent resistant gel filtration system, it had the particular advantage that eluted fractions were readily dried down for storage in other solvents or for analysis by thin layer chromatography. Drying down for storage was simply achieved by evaporation overnight in a fume hood whereas concentration of aqueous solutions must be carried out *in vacuo* with the attendant problem of equipment contamination.

The retardation of the iodothyronines on Sephadex LH-20 was quite different to that on Sephadex G-25. The reaction products ^{125}I-3,3'-T_2 and ^{125}I-rT_3 were eluted with a ratio of elution volume to total gel volume (V_e/V_t) of 0.66 ± 0.06 and 1.83 ± 0.32 respectively in 11 gel filtrations using the Sephadex LH-20 system (Figure 3.1).

In contrast, there was much stronger adsorption of these products in the aqueous system. The V_e/V_t of ^{125}I-3,3'-T_2 and ^{125}I-rT_3 averaged 6.5 and 17.3 respectively in two filtrations (Figure 3.3). Despite the lower V_e/V_t, ^{125}I-rT_3 was quite effectively separated from the other products in the non-aqueous system. Analysis of the pooled ^{125}I-rT_3 fractions by thin layer chromatography showed minimal contamination by $^{125}I^-$ or ^{125}I-3,3'-T_2 (Figures 3.2 and 3.3).

3. Structural Integrity of ^{125}I-rT_3

^{125}I-rT_3 exhibited displacement parallel to that of unlabelled standard rT_3 (Figure 3.4) demonstrating the purity of the ^{125}I-rT_3 product. Specific activity ranged between 1200 - 1800 pCi/pg. This ensured a sensitivity of 0.02 nmol/L which was quite adequate for the assay of physiological levels of rT_3.

Four batches of a commercial preparation of ^{125}I-rT_3 (The Radiochemical Centre, U.K.) (Figure 3.4) were found to have a specific activity of 500 - 700 pCi/pg. Although there was an attendant reduction in sensitivity in those assays in which this tracer was used, sensitivity was adequate for routine use.

4. Storage

^{125}I-rT_3 underwent rapid changes on storage in 50% aqueous ethanol (Figure 3.1). Gel filtration on the day following separation of the reaction components showed other iodinated material to be already present. By 44 days, ^{125}I-rT_3 represented only 22% of the radioactivity present in

the sample. Despite these changes, the tracer preparations were useful for 3 - 4 weeks without repurification.

Deiodination did not appear to be a major problem during storage of ^{125}I-rT$_3$. While 44 day old ^{125}I-rT$_3$ exhibited little self-displacement (Figure 3.6), on repurification the ^{125}I-rT$_3$ showed specific activity equivalent to that of the same tracer on the day of synthesis. That is, the filtration system purified the ^{125}I-rT$_3$ from accumulating impurities. Despite the evidence suggesting a low rate of deiodination during storage, there was a pronounced accumulation of unidentified iodinated material eluting near the void volume. Kochupilla and Yalow (1978) have reported the elution of unidentified iodinated material during separation of radioiodination reaction mixtures. In the 44 day old preparation of ^{125}I-rT$_3$ in the study reported here, similar material accounted for 50% of the radioactivity present.

Jirousek (1979) has described three types of reactions which take place in aqueous solutions of ^{125}I-substituted molecules. The primary reaction of ^{125}I-decay results in disruption of the C-I bond. Jiang and Welch (1977) have identified the 3-hydroxyl-tyrosine derivative following storage of ^{125}I-MIT and suggested that hydroxyl substitution was a predominant reaction after disruption of the C-I bond. The second type of reaction is the alteration of the molecule secondary to gamma bombardment and the third, the tertiary process of interaction of the molecule with the radiolysis products of water: H$\cdot$, $\cdot$OH and hydrated electrons.

From its elution characteristics, the unidentified iodinated material would appear to have a relatively high molecular weight. Jiang and Welch (1977) identified reaction products of intramolecular nucleophilic attack by the amino group of 3-hydroxytyrosine at the 6-position on the aromatic ring. Although no mention was made of the possibility of intermolecular nucleophilic attack at the same position, this may be a particularly important reaction in the case of the 3'-hydroxythyronines, in which intramolecular attack of the phenyl ring by the amino group would be sterically unlikely. Extensive intermolecular reactions of this type might give rise to high molecular weight products. It appears surprising however that extensive reactions of this type would take place in the presence of the free radical scavenger, ethanol. In the presence of this scavenger, the concentration of $H^{\bullet}$ and $^{\bullet}OH$ would be expected to be kept to a minimum. Furthermore, disruption of the C-^{125}I bond would lead to the formation of both the 3'-hydroxy and 3'-ethoxy thyronine derivatives. The latter would not be expected to significantly activate the 6'-position on the phenyl ring. The formation of iodinated large molecular weight polymers would also infer that a considerable amount of the ^{125}I-rT$_3$ in storage was actually in the diradioiodinated form, which would seem unlikely.

Despite these alterations during long term storage, ^{125}I-rT$_3$ stored in 50% aqueous ethanol was found to be useful in the assay for 3 - 4 weeks.

Figure 3.1 Elution of reaction mixture post radio-iodination of 3,3'-T_2 through Sephadex LH-20

Column : 1 x 40 cm

Eluting solvent : ethyl acetate/methanol/water/NH_4OH

Rate : 10 mL/h

Fraction : 100 drops (approx. 1 mL)

(a) Elution of Reaction Products

Fractions 46 - 71 were dried, reconstituted in ethanol, pooled and stored at room temperature. Aliquots were eluted at 1 and 44 days post synthesis.

(b) Elution of ^{125}I-rT_3 One Day Post Synthesis

66% of total radioactivity added to column eluted as ^{125}I-rT_3.

(c) Elution of ^{125}I-rT_3 44 Days Post Synthesis

22% of total radioactivity added to column eluted as ^{125}I-rT_3.

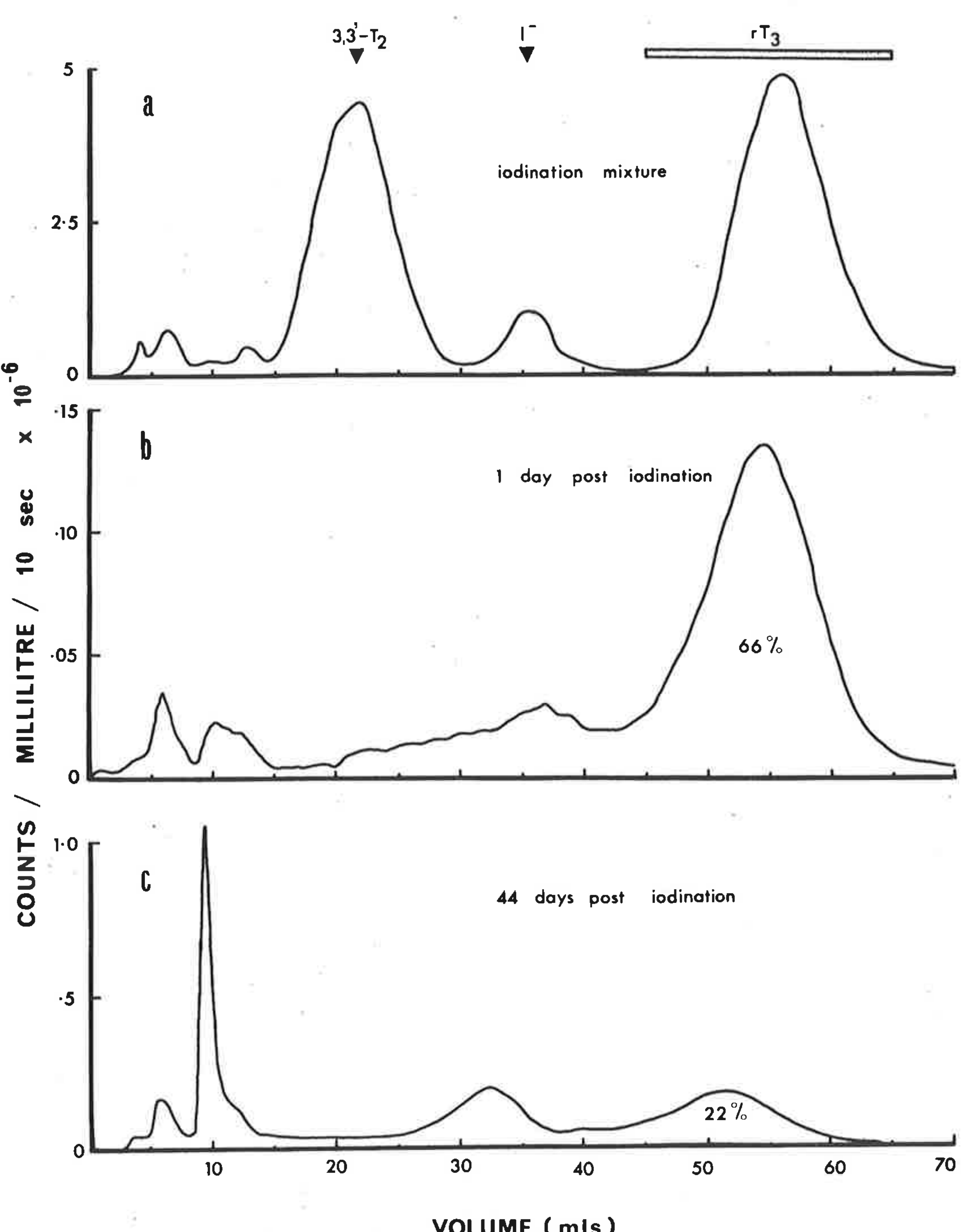

3,3'-T2
I⁻
rT3
a
iodination mixture
b
1 day post iodination
66%
c
44 days post iodination
22%
COUNTS / MILLILITRE / 10 sec x 10⁻⁶
5
2·5
0
·15
·10
·05
1·0
·5
10
20
30
40
50
60
70
VOLUME (mls)

Figure 3.2 Thin layer chromatogram of the fractions eluted from the 3,3'-T_2 radioiodination reaction mixture

^{125}I-T_3, ^{125}I-T_4 and ^{125}I-rT_3 (The Radiochemical Centre, U.K.) were run as markers.

Fractions eluting at 56, 35 and 22 mL in Figure 3.1a were chromatographed.

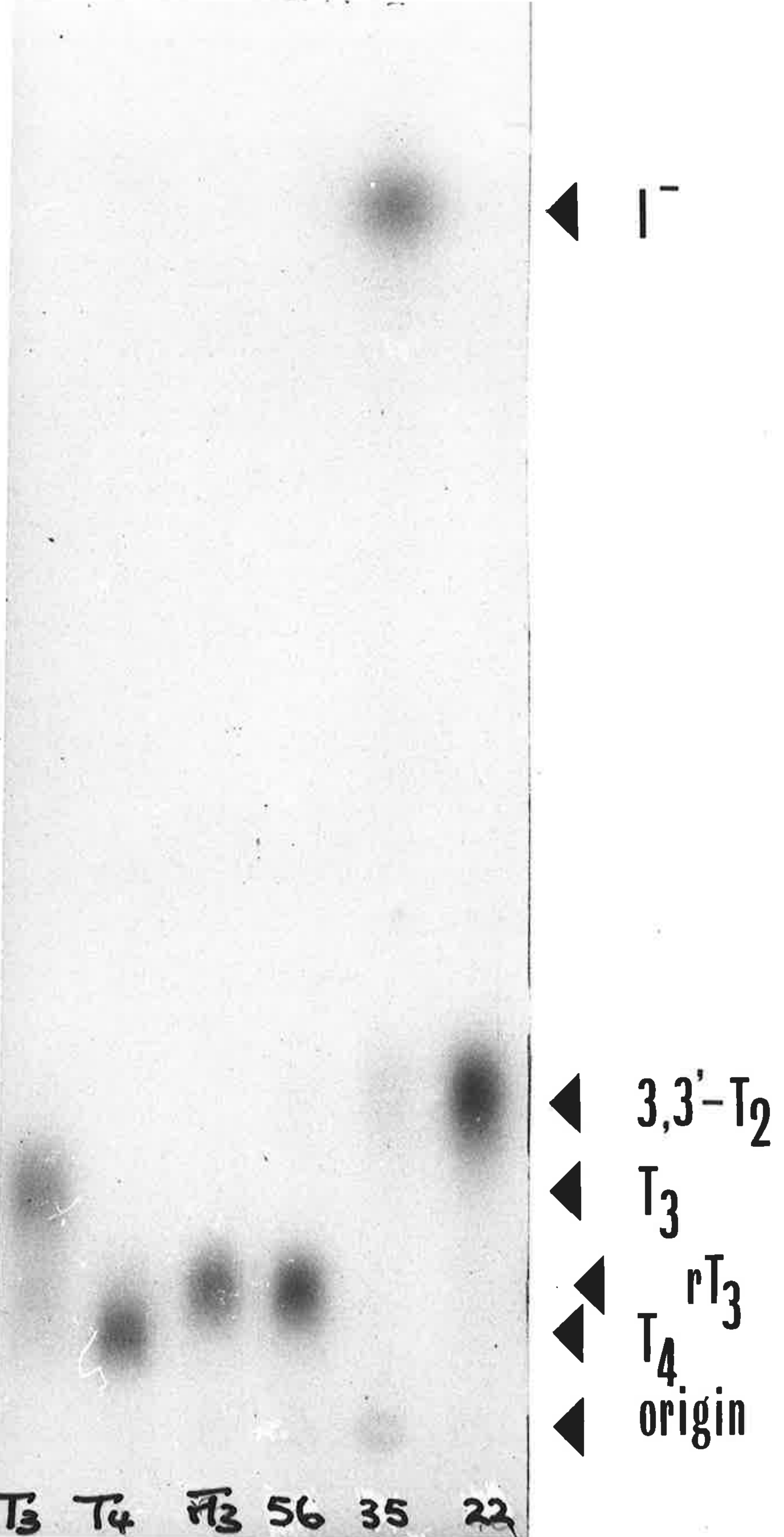

T3
T4
rT3
56
35
22
I⁻
3,3'-T2
T3
rT3
T4
origin

Figure 3.3 Elution of reaction mixture post radioiodination of 3,3'-T_2 through Sephadex G-25

(a) Sephadex G-25 Elution Profile

Column 0.8 x 10 cm

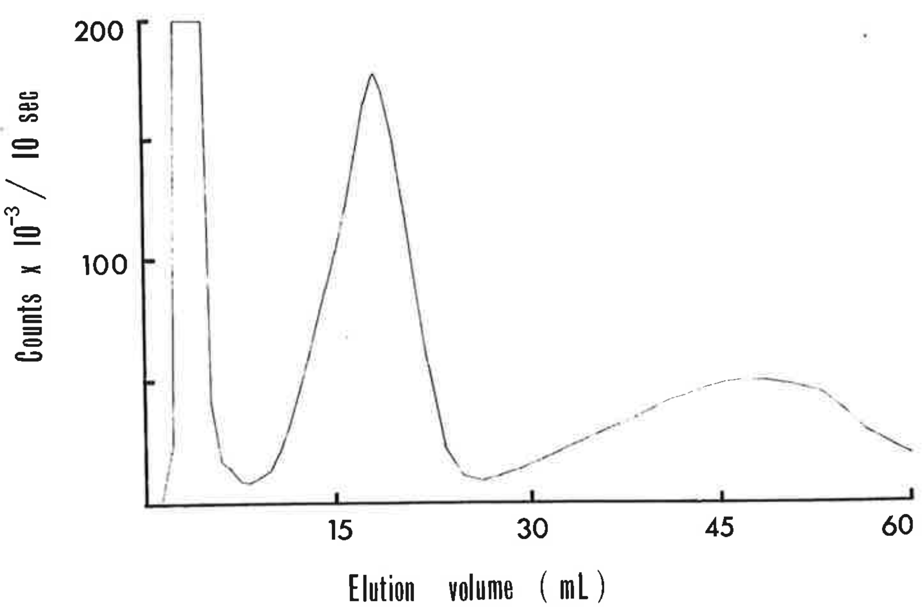
Eluting Buffer : 0.05 M K_2HPO_4, pH 12

Rate : 40 mL/hr

Fractions : 40 drops (approx. 1½ mL)

(b) Thin Layer Chromatography of Products Eluted at 24 mL and between 40 - 55 mL (fractions eluting between 40 - 55 mL were pooled and are labelled rT_3 on the chromatogram)

^{125}I-T_3 and ^{125}I-T_4 (The Radiochemical Centre) were run as markers


200
100
Counts x 10^{-3} / 10 sec
15
30
45
60
Elution volume (mL)

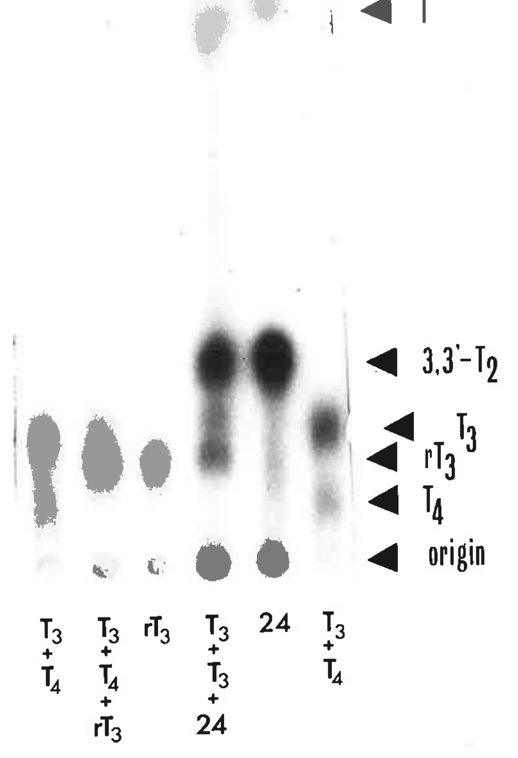

I$^-$
3,3'-T$_2$
T$_3$
rT$_3$
T$_4$
origin
T$_3$ + T$_4$
T$_3$ + T$_4$ + rT$_3$
rT$_3$
T$_3$ + T$_3$ + 24
24
T$_3$ + T$_4$

Figure 3.4 Structural integrity of ^{125}I-rT_3

Laboratory preparation of ^{125}I-rT_3:

●———● Displacement by unlabelled rT_3

○———○ Self-displacement

^{125}I-rT_3 from The Radiochemical Centre, U.K.:

▲———▲ Displacement by unlabelled rT_3

△———△ Self-displacement

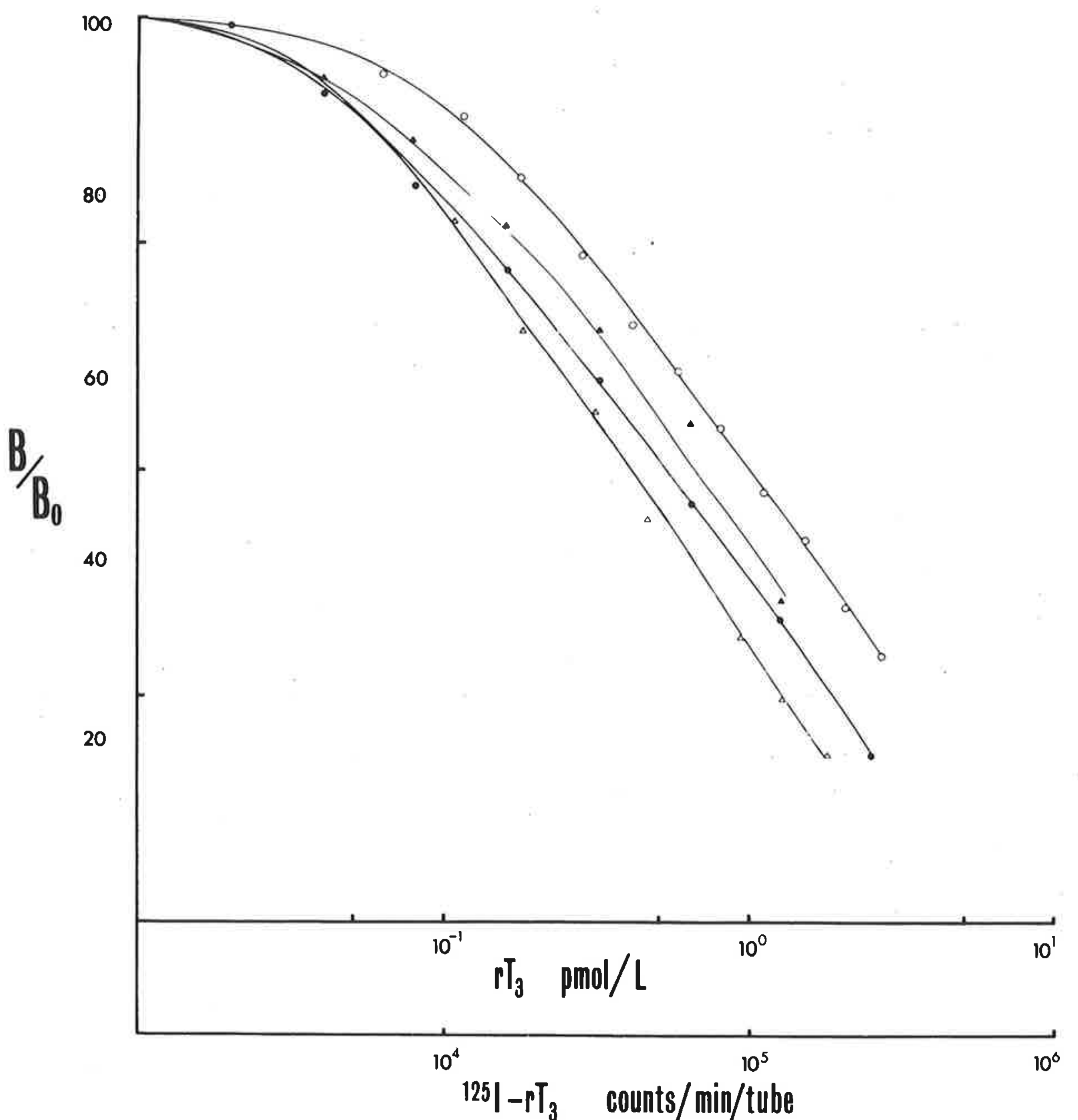

100
80
60
40
20
B/B0
10^{-1}
10^{0}
10^{1}
rT3 pmol/L
10^{4}
10^{5}
10^{6}
125I-rT3 counts/min/tube

Figure 3.5 Determination of specific activity

Specific activity was determined as outlined in the text (Chapter 3.1, Materials and Methods)

The upper abscissa represents the concentration of unlabelled rT_3.
The displacement ($\%B/B_o$) at each concentration is represented.

The lower abscissa represents the concentration of labelled rT_3 in each tube in a second series of tubes in which no unlabelled rT_3 is present.
The counts per tube plotted are net counts/tube.
Net counts = (total counts/tube) - (counts/tube in the series of tubes containing standards).
The displacement ($\%B/B_o$) at each concentration is represented.

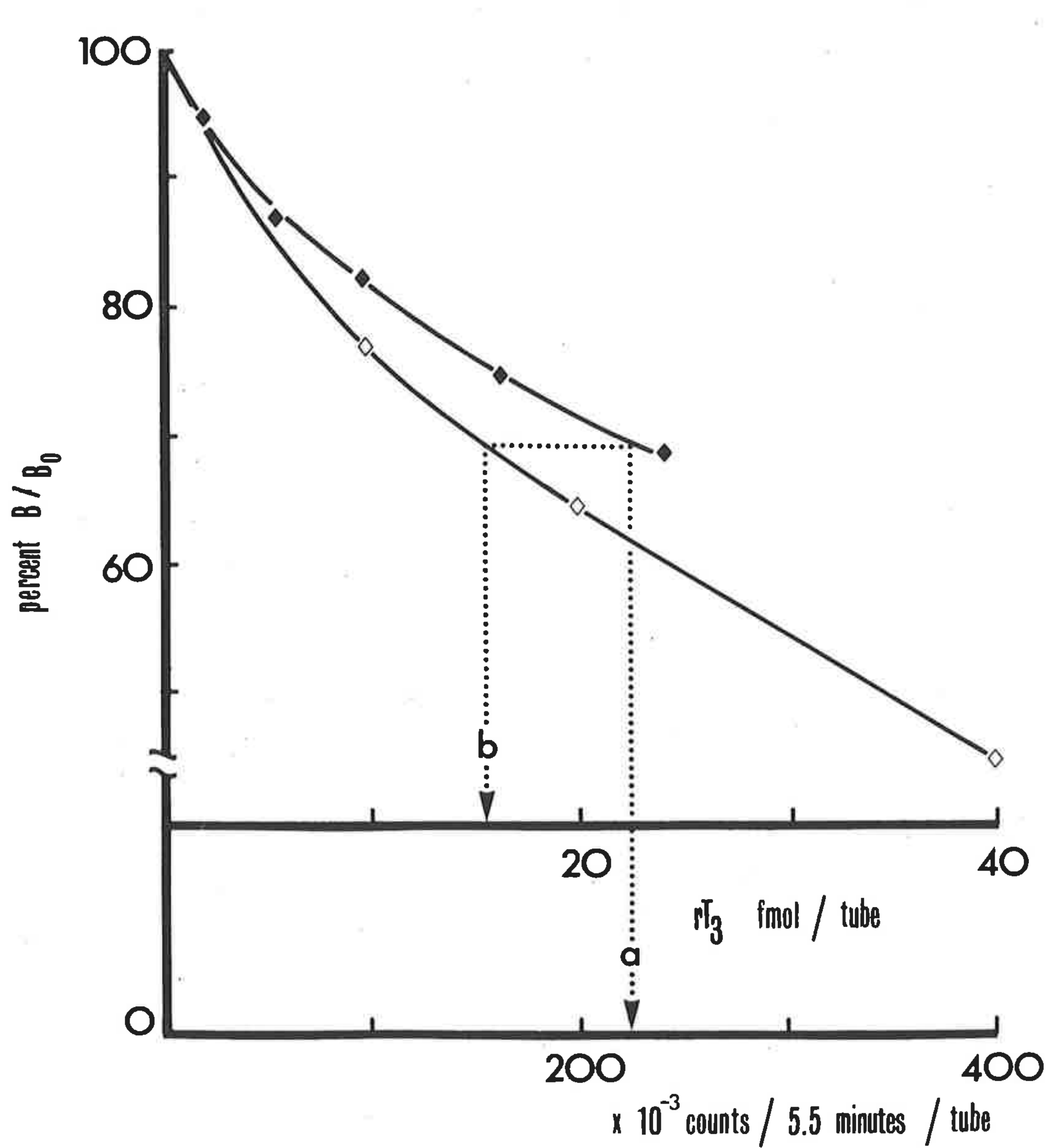
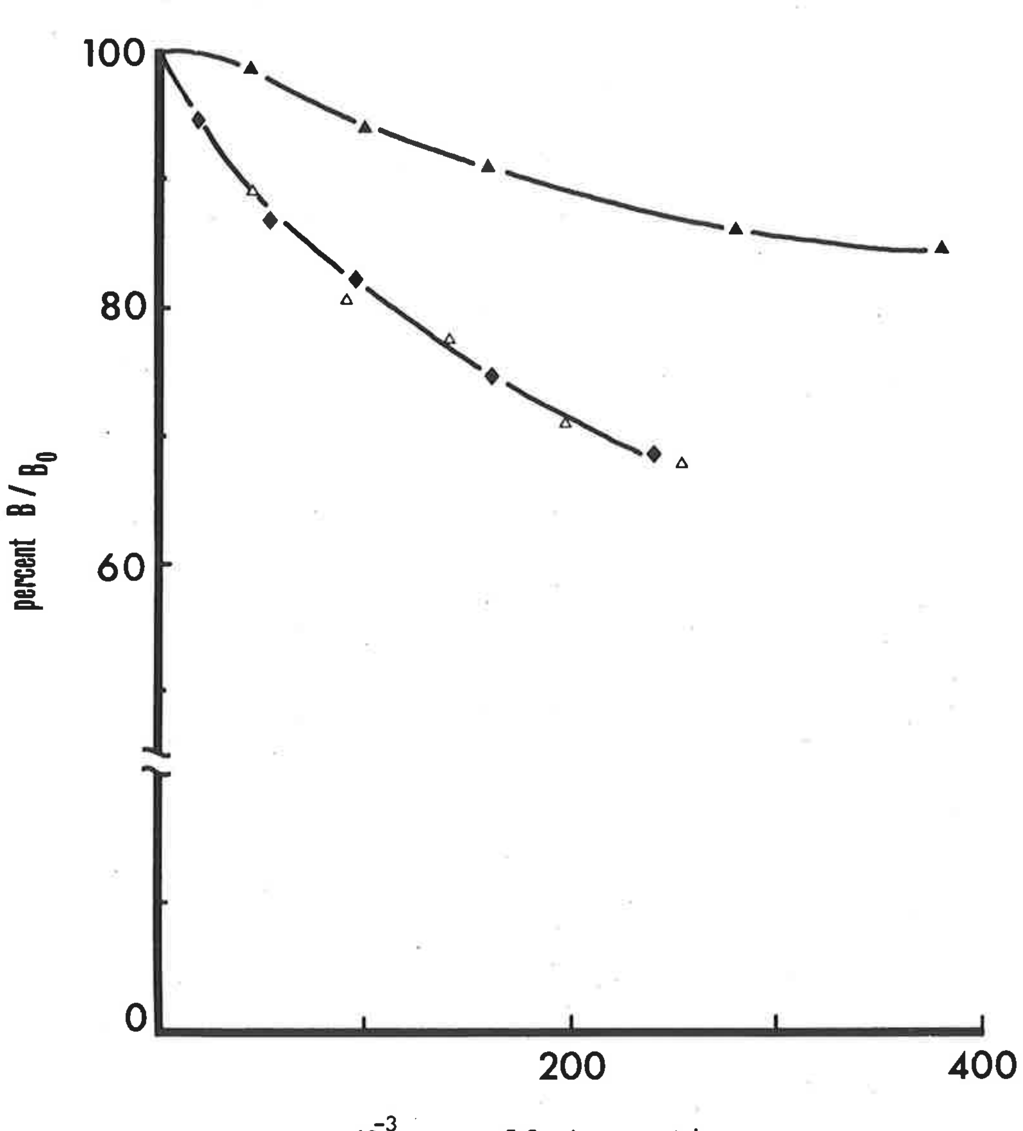

100
80
60
0
percent B / B0
b
a
20
40
rT3 fmol / tube
200
400
x 10^-3 counts / 5.5 minutes / tube

Figure 3.6 Effect of aging on specific activity

▲——▲ Self-displacement by ^{125}I-rT_3 stored for 44 days (Figure 3.1)

◆——◆ Self-displacement after Sephadex LH-20 Filtration of ^{125}I-rT_3 stored for 44 days (Figure 3.1C)

△——△ Self-displacement by Freshly Prepared ^{125}I-rT_3


100
80
60
0
200
400
percent B / B0
10-3 counts / 5.5 minutes / tube

3.3 PRODUCTION OF ANTISERUM TO BSA-rT_3

MATERIALS AND METHODS

Antiserum was produced by conjugating rT_3 to bovine serum albumin (BSA) (Goodfriend, Levine and Fasman, 1964) to render the iodothyronine immunogenic and immunizing rabbits with the conjugate using the multiple site intra-dermal technique (Vaitukaitis, Robbins, Nieschlag and Ross, 1971).

All reagents were of analytical grade and were generally available unless otherwise stated. The L-rT_3 was donated by Henning GmbH (Germany), DL-rT_3 was donated by the Warner-Lambert Research Institute (U.S.A.), and T_3, T_4 and 3,5-T_2 were purchased from Sigma (U.S.A.).

1. Conjugation

Iodothyronine, either 5 mg L-rT_3 or 5 mg DL-rT_3 was dissolved in 0.5 mL of dimethyl formamide. If necessary, several drops of 0.025 M NaOH were added to ensure stabiliz-ation. The efficiency of conjugation was assessed by adding 5000 cpm ^{125}I-rT_3. This solution was added with mixing to 1.5 mL ion free water containing 10 mg BSA. To this mix was added dropwise 100 mg of 1-ethyl-3(3-dimethyl-amino-propyl)-carbodiimide HCl (Sigma, U.S.A.) in 0.5 mL ion free water. The reaction was allowed to proceed for 30 min at room temperature and twice dialyzed at 4°C against 1000 volumes of ion free water. The dialysand was freeze dried and stored at 4°C until used.

2. Immunization

Conjugate (3 mg) was dissolved in 2 mL normal saline and mixed to a thick paste with 4 mL Freund's Complete Adjuvant (Difco, U.S.A.). Four mongrel rabbits were used. Two were prepared for multiple site intradermal immunization by shaving a 4 x 150 cm strip along each side of the spine. 1 mg (2 mL) of conjugate mixture was injected intradermally with a 26 gauge needle into about 20 sites along each side of the spine. Rabbit 1 received BSA-L-rT_3 and rabbit 2, BSA-DL-rT_3. Both rabbits were boosted with 200 µg of conjugate each by the same route at 54 days post primary immunization.

Rabbits 3 and 4 were injected intramuscularly into each of the forelimbs and hindlimbs, with 1 mg BSA-DL-rT_3 into the former and 1 mg BSA-L-rT_3 into the latter rabbit. Rabbit 3 was boosted intramuscularly with 200 µg conjugate on day 32, 100 µg on day 54, 150 µg on day 70, 140 µg on day 84 and 170 µg on day 102. The rabbit died on day 113.

Similarly, rabbit 4 was boosted with 200 µg conjugate on day 32, 200 µg on day 54, 100 µg on day 70, 120 µg on day 84 and 130 µg on day 102.

The rabbits were bled at 3 weeks post primary immunization and every week thereafter from a major ear vein.

3. Assessment of Antisera

Each bleed was titrated by assessing the binding of ^{125}I-rT_3 to the antisera diluted from 1/100 to 1/1,000,000

under normal conditions of assay (Table 3.2). Rabbit 2 showed the best response by day 63 post primary immunization and subsequent antisera produced were studied in more detail for suitability in a routine assay.

These antisera were assessed for affinity and sensitivity by titrating between 1/5000 and 1/200,000 dilution in both the absence of unlabelled rT_3 and in the presence of either 775 fmole/tube (equivalent to a serum rT_3 concentration of 7.75 nmol/L using the protocol of Table 3.2) for antisera produced on days 63, 70, 77 and 84; or 200 fmole/tube (equivalent to a serum concentration of 2.00 nmol/L) for antisera produced on days 91 and 99.

Binding in the presence of unlabelled rT_3 was expressed as a percent of binding in the absence of standard (B/B_o) at each dilution for a particular antiserum and plotted against B_o at that dilution (Figure 3.8). By plotting the relative displacement at each dilution in this manner, a rapid and convenient visual assessment of the optimum dilution of each bleed could be made.

After cross-reaction studies, the antiserum from rabbit 2 was selected for routine use. Blood (15 mL) was taken on both days 99 and 102, and the serum separated and pooled (referred to in the text as 99/102). Serum (50 µL) was aliquoted into 2 mL ampoules, freeze-dried, nitrogen blown over the dried aliquot, and the ampoules sealed. These were stored at 4°C.

RESULTS AND DISCUSSION

1. Conjugation

Calculation of the percent ^{125}I-rT_3 remaining in the freeze-dried dialysand suggested that 22% of the L-rT_3 and 28% of the DL-rT_3 was conjugated. This degree of conjugation corresponded to 11 molecules of L-rT_3 and 14 molecules of DL-rT_3 per molecule of albumin. The extent of conjugation was similar to that achieved in a pilot run in which 21% of ^{125}I-rT_3 in the conjugation reaction mixture was eluted in the void volume on Sephadex G-25. The exhaustive dialysis confirms that the residual hapten in the dialysand was covalently attached to the albumin.

2. Immunization

Neither route of immunization appeared to be unequivocally superior (Figure 3.7). Thus, using the multiple site intradermal technique, one rabbit (R1) gave the poorest response of all four rabbits, and the other (R2) gave the best response.

Despite the poor response in R1, the very good response in R2 vindicated the proposal of Vaitukaitis et al. (1971) that the multiple site intradermal procedure offered a method in which relatively good antisera could be raised with a good chance of success using only very small amounts of immunogen. It was suggested that the intradermal route would tend to prolong clearance of antigen due to reduced blood flow through this tissue. Furthermore, antigen would be expected to be released at different rates and times from

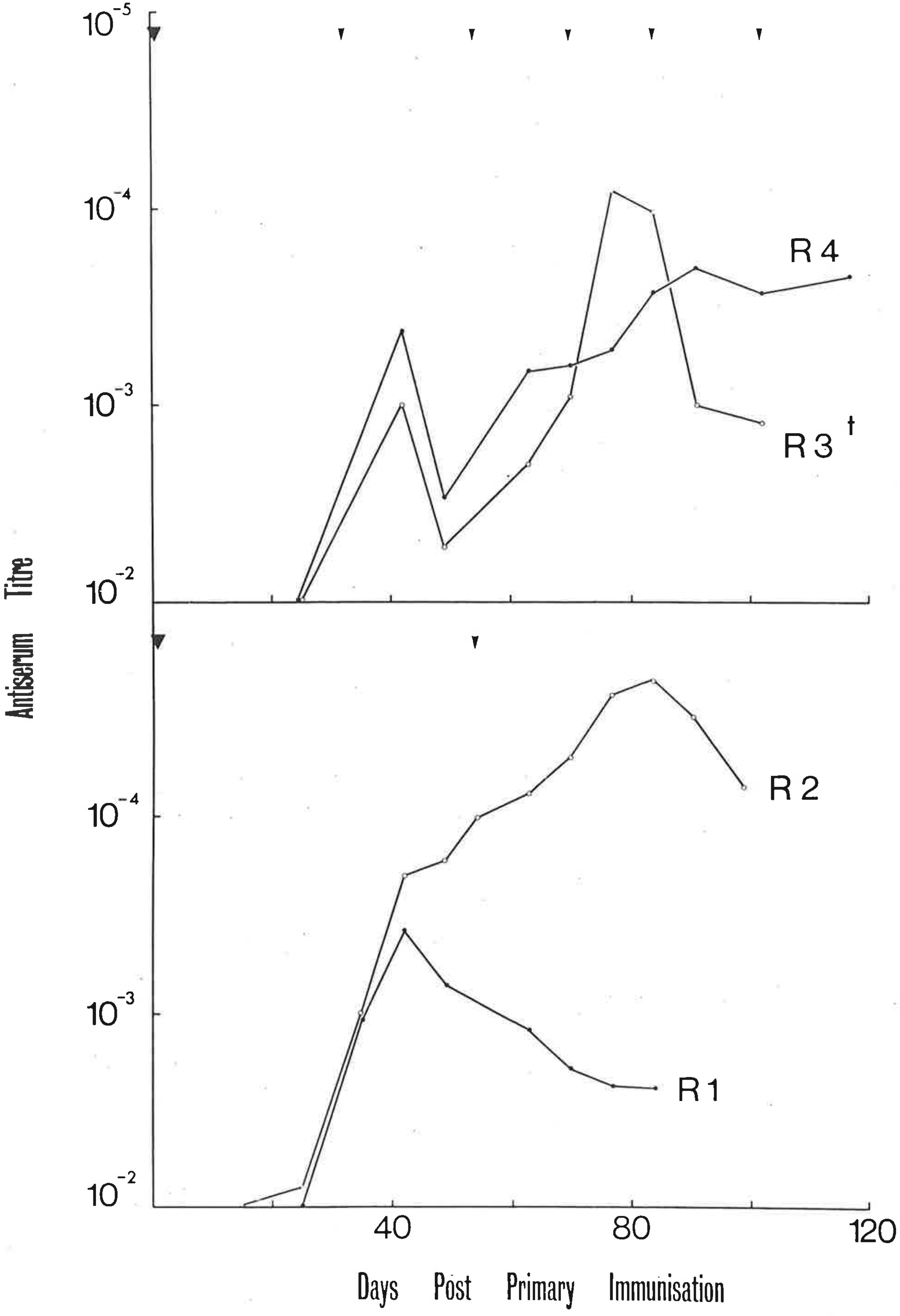
Figure 3.7 Response of rabbits to immunization with rT_3-BSA conjugate

Antiserum titre in rabbits 2, 3 and 4 was determined as that dilution of antiserum binding 50% ^{125}I-rT_3

Antiserum titre in rabbit 1 was determined as that dilution of antiserum binding 10% ^{125}I-rT_3

▼ primary immunization

▼ booster



10^{-5}
10^{-4}
10^{-3}
10^{-2}
R 4
R 3 †
Antiserum Titre
10^{-4}
10^{-3}
10^{-2}
R 2
R 1
40
80
120
Days Post Primary Immunisation

the multiple sites, providing a series of small stimuli or boosts to the immune system over a prolonged period of time.

While the use of booster injections does appear to have maintained the titre of antiserum in rabbit 3 and 4, the value of a booster after a primary immunization by the multiple site intradermal technique appears questionable.

The antisera produced by rabbit 2 were considered to be of sufficient affinity to be assessed for use in a rT_3 radioimmunoassay.

3. Assessment of Antisera

The B/B_o plot very conveniently and clearly pin-pointed those rabbit antiserum dilutions which looked most suitable as starting points for optimisation studies (Figure 3.8).

The B_o at any given dilution increased with increasing titre from day 63 to a maximum at day 84. As expected, for any given antiserum, there was an initial steep decrease in B/B_o as antiserum dilution was increased. At intermediate dilutions, the graph reached a point of inflection reflecting the approach to maximum displacement of tracer as the optimum antiserum dilution was reached. For example, on day 84, the antiserum dilution range 1/20,000 to 1/40,000 was considered to give the best sensitivity in the presence of the tracer concentration used. By increasing antiserum dilution, no further significant displacement was achieved and B_o began to fall markedly. Of particular interest was the observation that the point of inflection appeared to be relatively invariant with regard to antiserum

dilution; as the titre increased between days 63 and 84, the point of inflection moved to higher B_o and lower B/B_o, but remained in the 1/15,000 to 1/30,000 dilution range.

The displacement dose of 775 fmole/tube (equivalent to 7.75 nmol/L serum rT_3 concentration) was high and caused nearly maximum displacement in the higher titre antisera. It was thus decided to assess later antisera with a lower dose of rT_3 (22 fmole/tube; Figure 3.8) in the hope of providing a more sensitive pointer of suitable antiserum dilutions.

With a lower displacing dose, the point of inflection moved to higher antiserum dilutions (day 91 antiserum), confirming that the 775 fmole displacing dose was too high to reflect accurately the sensitivity at concentrations of rT_3 closer to those measured in serum.

Of particular interest was the change in displacing characteristics between days 91 and 99. Although there was a fall in B_o from day 91 to 99 correlating with the drop in titre, displacement by 200 fmole rT_3 at 1/20,000 and 1/30,000 antiserum dilution increased between these two days.

While a drop in either titre or binding affinity between these two days would be expected to lower B_o, a drop in both binding affinity and titre may lead to conditions where sensitivity was actually increased at a given tracer concentration (Feldman and Rodbard, 1971).

A comparison of the data for rabbit 4 on day 91 (Figure 3.9) with that of rabbit 2 on day 99 (Figure 3.8), showed that although they had similar B_o at 1/20,000 and 1/50,000 antiserum dilution, rabbit 4 gave similar displace-

Figure 3.8 Binding and displacement characteristics of antisera obtained from rabbit 2

The graphs illustrate the relative displacement of tracer by 200 (top panel) or 775 (bottom panel) fmol rT_3/tube at indicated dilutions for antisera of days 63 to 99

B/B_o and B_o represent percent relative displacement and percent tracer bound respectively

The reciprocal of the (antiserum dilution x 10^3) are shown in the circles

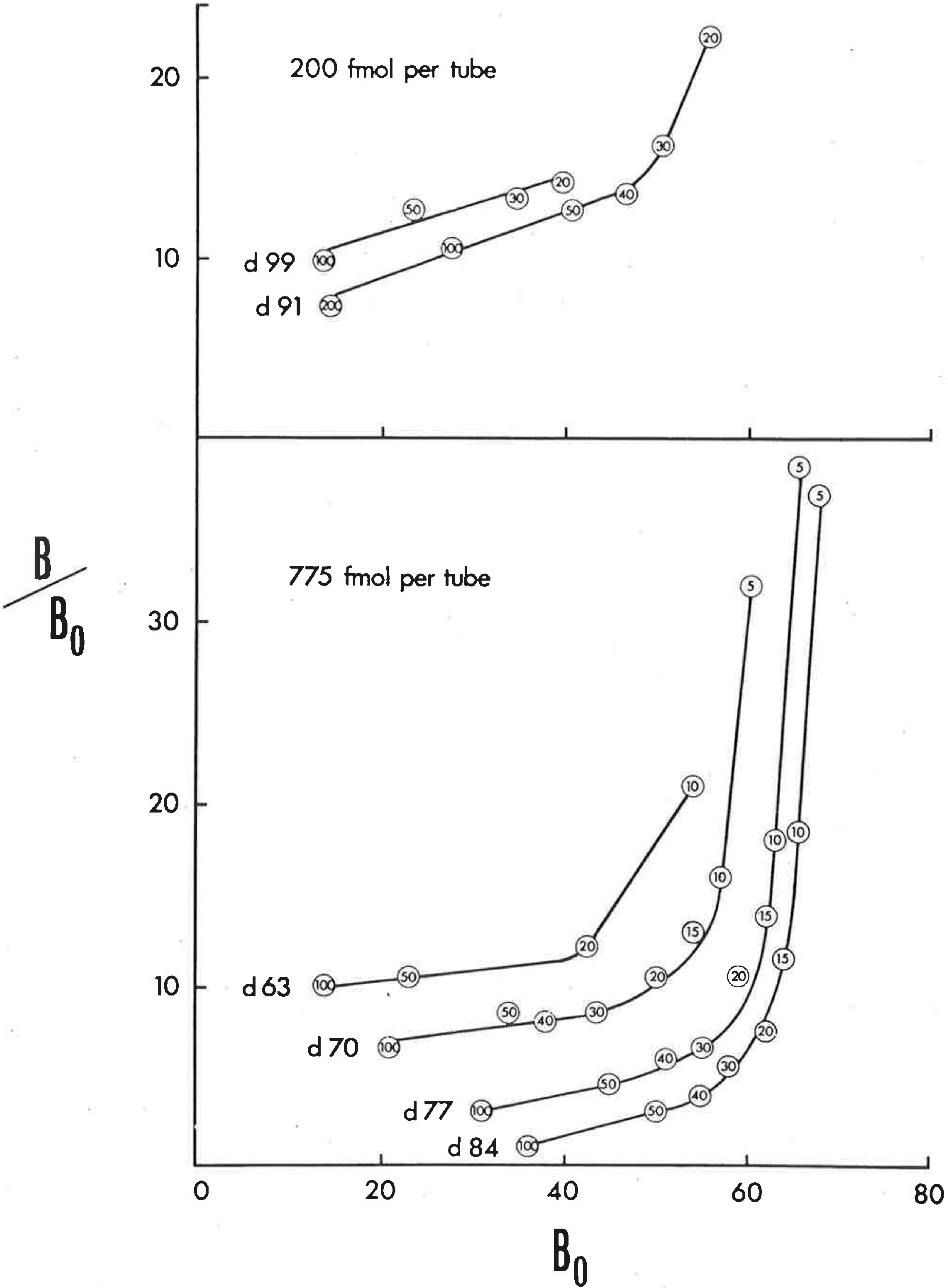
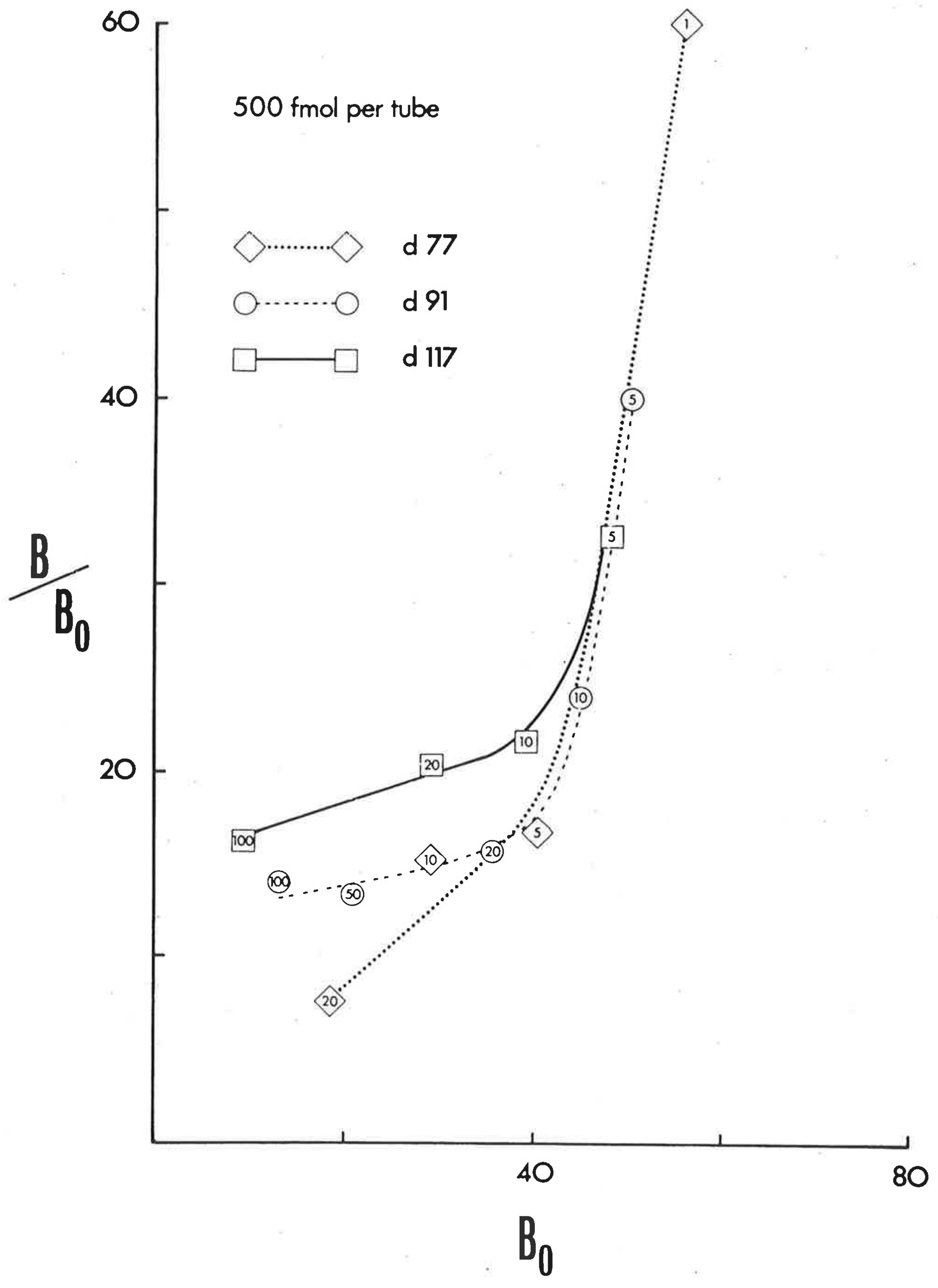

200 fmol per tube
d 99
d 91
775 fmol per tube
d 63
d 70
d 77
d 84
B/B0
B0
0
20
40
60
80
10
20
30

Figure 3.9 Binding and displacement characteristics of antisera obtained from Rabbit 4

See legend of Figure 3.8 for details


60
40
20
40
80
500 fmol per tube
d 77
d 91
d 117
B
B_0
B_0
1
5
5
10
10
20
20
100
5
10
20
100
50
20

ment with 500 fmole rT_3 as rabbit 2 gave with 200 fmole rT_3. This pattern is consistent with the antisera of rabbit 4 being of poorer quality in having lower affinity and higher binding capacity.

4. Cross-Reaction Studies

Of the iodothyronines tested, 3,3'-T_2 showed the greatest cross-reaction with the rabbit 2 antiserum of days 99/102 (Figure 3.10). However the interference was negligible at physiological concentrations. The 3,5-iodinated iodothyronines, T_3 and 3,5-T_2, showed negligible cross-reaction, while T_4 showed intermediate cross-reaction, suggesting that binding to the antiserum was unfavourable for the 3,5-configuration (T_3, 3,5-T_2) except in the presence of the 3',5'-configuration (T_4).

Iodothyronines with only a monoiodinated tyrosyl or inner ring (3-configuration) bound with greater affinity than the diiodinated tyrosyl ring compounds. Binding affinity increased with increasing substitution of the phenyl or outer ring (3,3',5'T_3 > 3,3'-T_2).

Unfortunately, no 3',5'-T_2 was available for assessment but from the above discussion, the 3',5'-configuration would be expected to bind strongly to the antiserum. Faber, Friis, Kirkegaard and Siersbaek-Nielsen (1978) have recently reported 0.34% cross-reaction of 3',5'-T_2 which, although high, would not cause significant displacement of ^{125}I-rT_3 at the highest reported mean estimates of 3',5'-T_2 concentration (0.14 nmol/L; Chopra, Geola, Soloman and Maciel, 1978).

Although T_4 cross-reaction was low, the 400 fold greater concentration of T_4 resulted in a significant displacement of $^{125}I\text{-}rT_3$ at physiological concentrations of T_4. As the cross-reaction could account for 1/3 to 1/2 of the measured rT_4 concentration, it was necessary to correct for cross-reaction due to the T_4 in each sample.

Looking at T_4 cross-reaction in rabbit 2 antisera other than that of days 99/102, there was a marked fall in affinity for T_4 between days 49 and 54 (Figure 3.11). The absence of a sharp increase in titre at this time would suggest that a species of antibody with higher specificity but similar affinity has become predominant, highlighting the importance of monitoring not only the titre, but also cross-reaction with T_4 at each bleed.

5. Antiserum Characteristics

A Scatchard analysis of the displacement of $^{125}I\text{-}rT_3$ from the antiserum of rabbit 2, days 99/102 under the conditions of normal assay procedure (Table 3.2) revealed two binding sites (Figure 3.12). There was a very high affinity ($K_1 = 4 \times 10^{10}$ L/mol) site with a binding capacity of 160 nmol/L, and a lower affinity ($K_2 = 3.2 \times 10^8$ to 1.4×10^9 L/mol) binding site with a considerably higher capacity between 1.6 to 7.2 µmol/L.

3.4 THYROID HORMONE FREE SERUM

PREPARATION

Serum free of thyroid hormones was prepared by charcoal extraction. Approximately 1×10^5 cpm (0.08 nmole)

Figure 3.10 Cross-reaction characteristics of rT_3 antiserum rabbit 2 (days 99/102)

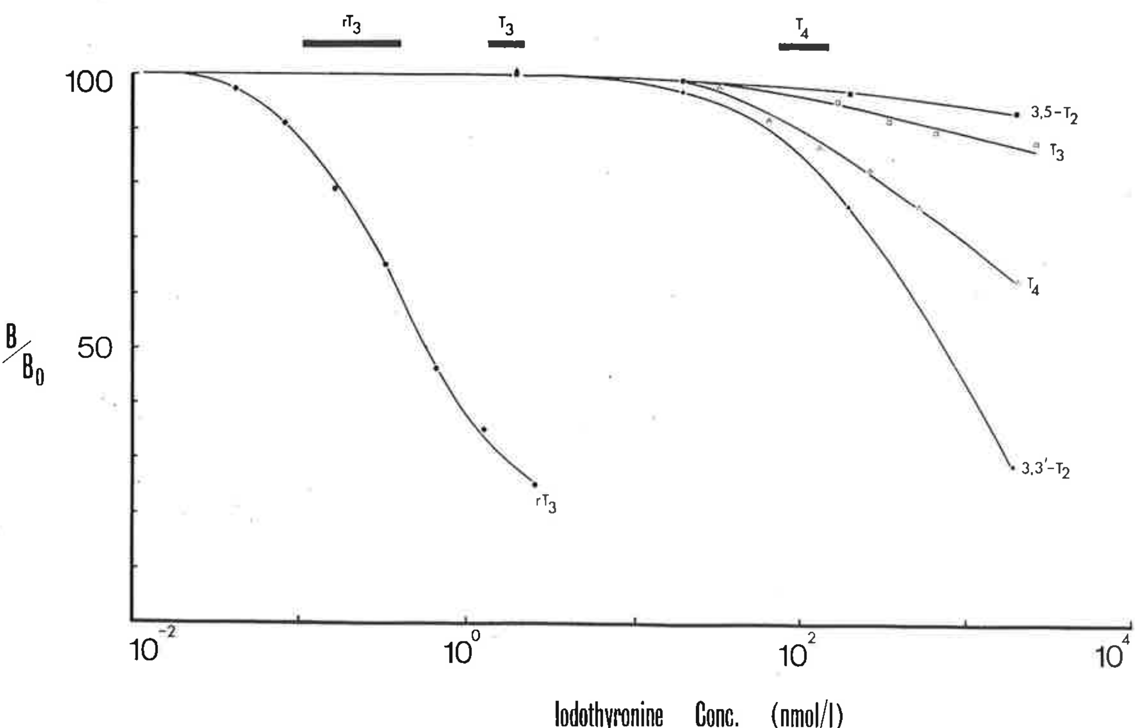
The normal euthyroid reference ranges for rT_3, T_3 and T_4 are represented as bars.

	Mean euthyroid conc. (nmol/L)	% cross-reaction at mean conc.	% cross-reaction at B/B_o = 0.95	% cross-reaction at B/B_o = 0.50
T_3	1.7	N.D.	0.03	-
3,3'-T_2	0.14	N.D.	0.15	0.08
3,5-T_2	0.1	N.D.	0.01	-

N.D. - no detectable displacement

Source of mean euthyroid concentrations: T_4 and T_3 (laboratory) 3,3'-T_2 (Wu et al., 1976), 3,5-T_2 (Meinhold and Schurnbrand, 1978).

T_4 concentration (nmol/L)	% cross-reaction $\pm$ 1 S.D.	Equivalent rT_3 concentration (nmol/L)
50	.072 ± .033	.036
75	.069 ± .023	.052
100	.065 ± .018	.065
200	.057 ± .014	.114



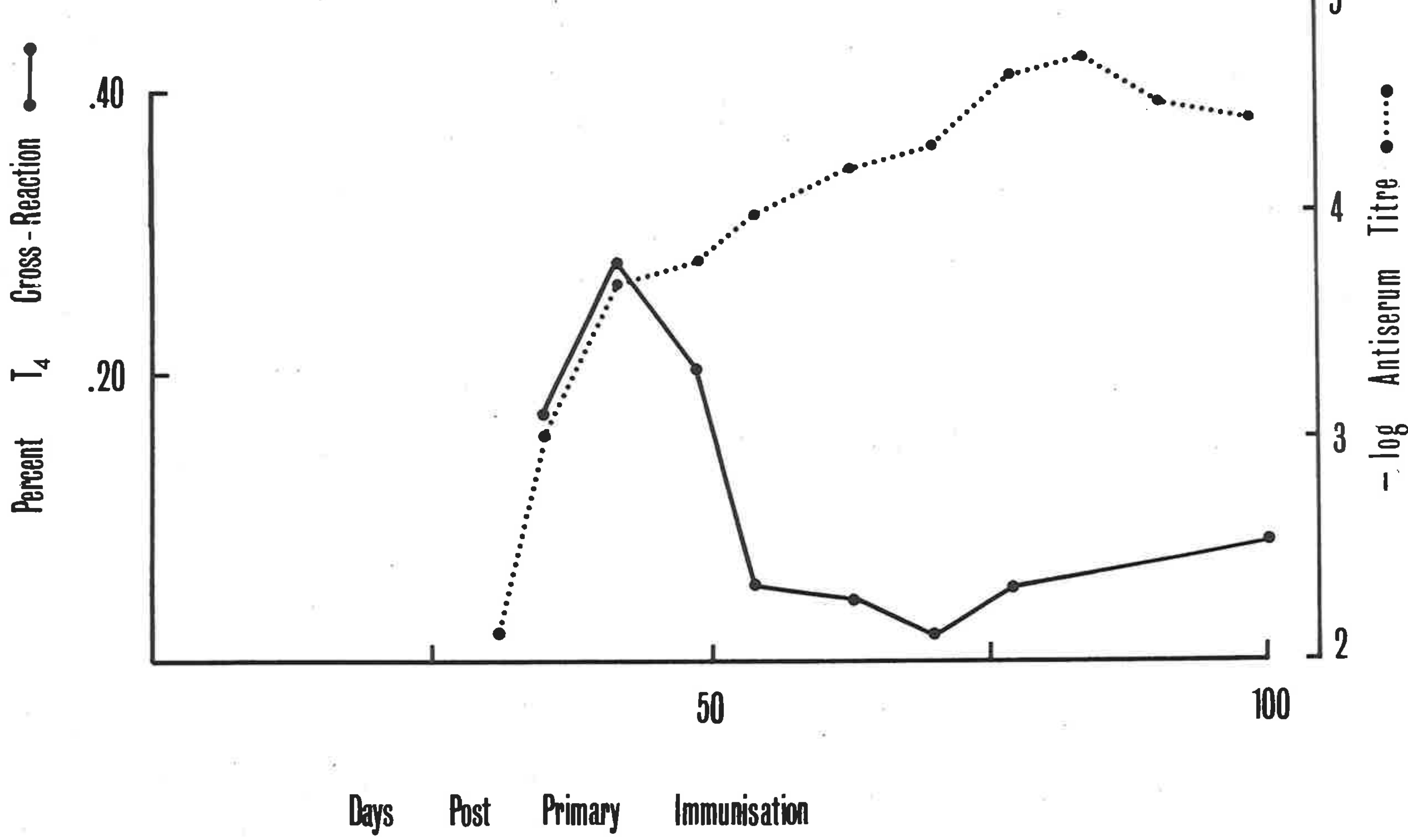
rT3
T3
T4
100
50
B/B0
3,5-T2
T3
T4
3,3'-T2
rT3
10^-2
10^0
10^2
10^4
Iodothyronine Conc. (nmol/l)

Figure 3.11 Affinity of antiserum produced by rabbit 2 for T_4

A retrospective study of antiserum produced by rabbit 2 between days 35 and 99

●——● Affinity for T_4 assessed by measuring displacement of ^{125}I-rT_3 by 100 nmol/L T_4

●·····● Antiserum dilution at which 50% ^{125}I-rT_3 is bound is expressed as (- log antiserum dilution). That is dilutions 1/100 to 1/100,000 ≡ 2 to 5.



Percent T_4 Cross-Reaction
.40
.20
50
100
Days Post Primary Immunisation
5
4
3
2
-log Antiserum Titre

Figure 3.12 Scatchard analysis of antiserum - rabbit 2 (days 99/102)

Antiserum used at 1/20,000 dilution. Values quoted for q are corrected for this dilution

▲······▲ Binding in the absence of thyroid hormone free serum

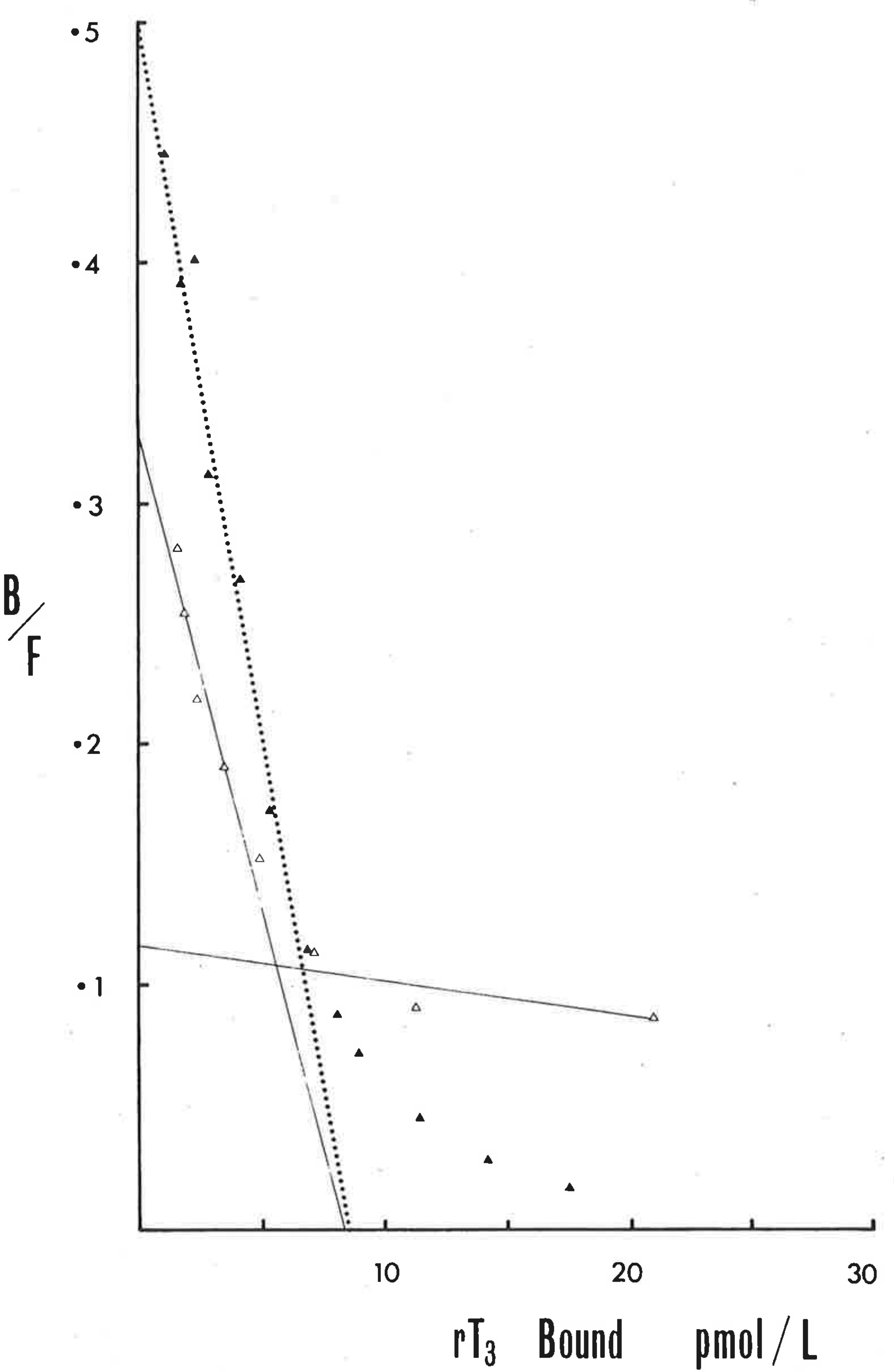
$K = 6.0 \times 10^{10}$ L/mol

$q = 166$ nmol/L

△——△ Binding in the presence of thyroid hormone free serum

$K_1 = 4.0 \times 10^{10}$ L/mol (higher affinity site)

$q_1 = 120$ nmol/L



·5
·4
·3
·2
·1
B/F
10
20
30
rT3 Bound pmol / L

$^{125}I-rT_3$ were added to 100 mL pooled serum and mixed at room temperature for 30 min. A 1 mL aliquot was put aside and 10 g activated Norit A charcoal (BDH, U.K.) added. After mixing overnight at 4°C the suspension was centrifuged at 2000 rpm for 30 min to remove most of the charcoal and the remainder removed by passing the supernatant through a prefilter and a series of filters (Millipore, U.S.A.) of pore size decreasing to 0.22 micron. A 1 mL aliquot was counted and the extraction considered satisfactory if more than 99% of the $^{125}I-rT_3$ had been removed.

The effect of this procedure on other serum constituents was checked by analysis of serum on a Technicon AutoAnalyzer 16 (Technicon, U.S.A.) before and after ten consecutive separate extractions.

EFFECT OF EXTRACTION PROCEDURE ON OTHER SERUM CONSTITUENTS

The complete extraction of thyroid hormones from serum caused significant changes in 12 of the 16 serum constitutents measured (Table 3.3).

Of the electrolytes, only bicarbonate and phosphate concentration were significantly altered. Dilution of a sample of extracted serum showed phosphate concentration to be > 10 mmol/L. It was likely that most of the bicarbonate was expended in buffering the phosphate introduced during the extraction.

All the lower molecular weight constituents measured (glucose, urea, creatinine, uric acid, bilirubin) were reduced in concentration. Creatinine, uric acid and bilirubin were reduced to concentrations below the normal

TABLE 3.3 Changes in serum constituents during preparation of thyroid hormone free serum

	Serum pool concentration ± 1 S.D.	Concentration after extraction ± 1 S.D.	P	Normal range
Electrolytes				
Na	141 ± 2	138 ± 3	N.S.	137-144 mmol/L
K	4.8 ± .6	4.6 ± .5	N.S.	3.5-5.4 mmol/L
Cl	106 ± 3	103 ± 2	N.S.	94-108 mmol/L
HCO_3^-	23 ± 5	.7 ± .5	< .001	20-28 mmol/L
Ca	2.3 ± .4	2.2 ± .4	N.S.	2.1-2.6 mmol/L
Metabolites				
Glucose	6.3 ± 2.1	4.1 ± 1.4	< .001	3.1-8.3 mmol/L
Urea	6.1 ± .8	4.1 ± .5	< .001	2.8-7.1 mmol/L
Uric acid	405 ± 68	98 ± 64	< .001	190-510 mmol/L
Creatinine	87 ± 19	10 ± 7	< .001	50-110 µmol/L
Bilirubin	7.8 ± 2.6	1.7 ± .7	< .001	2.17 mmol/L
Phosphate	.93 ± .12	> 5		.77-1.30 mmol/L
Enzymes				
Alkaline phosphatase	85 ± 29	16 ± 10	< .001	25-90 U/L
Aspartate transaminase	37 ± 12	15 ± 10	< .001	0-50 U/L
Lactate dehydrogenase	233 ± 138	53 ± 36	< .001	120-250 U/L
Protein				
Total protein	71 ± 3	65 ± 3	< .001	64-82 g/L
Albumin	42 ± 3	38 ± 2	< .001	33-49 g/L

range. It is unlikely that changes in these constituents would cause differences in tracer binding between tubes containing stripped serum and those with samples being assayed. Bilirubin and creatinine undergo marked physiological variation without disturbing thyroid hormone binding to the serum proteins.

Total protein and albumin concentration were lowered by 8 - 9%, being well within normal physiological variation and so unlikely to cause problems in the assay. The large reduction in the activities of the enzymes assayed was most likely due to the removal of components from the serum necessary for activity in the *in vivo* test system.

3.5 INHIBITION OF rT_3 BINDING TO THYROID HORMONE BINDING PROTEINS

Like the thyroid hormones, rT_3 circulates bound to the thyronine binding proteins. Tabachnick and Giorgio (1964) showed rT_3 to be bound to albumin with an affinity similar to that of T_4 and more recently, Snyder, Cavalieri, Goldfine, Ingbar and Jorgensen (1976) calculated the association constant of rT_3 binding to TBG to be 8×10^9; this was four times greater than the T_3 binding affinity and half that of T_4. Chopra et al. (1975a) reported 0.26% of serum rT_3 was dialyzable which suggested similar binding affinity of rT_3 and T_3 (0.25% dialyzable) for the serum binding proteins.

The earliest assays overcame the problems of protein binding by measuring rT_3 in ethanol extracts of serum (Chopra, 1974; Meinhold et al., 1975). These were soon followed by assays employing the conventional iodothyronine

binding protein inhibitors, ANS or merthiolate (Table 2.1). The direct assays gave concentrations similar to the extraction methods validating this simpler approach.

Success in using thyronine binding protein inhibitors depend on their ability to displace rT_3 from the binding protein at concentrations which do not significantly alter rT_3 binding to the antiserum. That this does occur in practise reflects the broader binding specificity of the binding protein sites compared to that of the antisera.

It was decided to use ANS as this had been shown to be a more effective inhibitor of T_3 and T_4 binding than salicylate or merthiolate in terms of concentration required to inhibit serum protein binding. The optimum ANS concentration was determined for both the charcoal and double antibody assays.

MATERIALS AND METHODS

Sodium 8-anilino-1-naphthalene-sulphonate (Sigma, U.S.A.) was used in these studies. Other features of the assay are described in the assay protocol (Table 3.2; ^{125}I-rT_3, 10,000 cpm/tube or 3.2 pmol/L). Charcoal was used at a final concentration of 0.6% (w/v) (Norit A activated, BDH, U.K.).

RESULTS AND DISCUSSION

1. Optimisation of ANS in the Charcoal Assay

(a) Effect of ANS in the absence of antiserum - in the absence of ANS, ^{125}I-rT_3 would be expected to be partitioned between the hormone free serum binding proteins, buffer protein

(human serum albumin), and charcoal. A small fraction of tracer may remain unbound to either charcoal or binding protein; this would comprise both intact ^{125}I-rT_3 and damaged radioactive material which binds to neither charcoal, binding protein or antibody. That is,

$$F + B_p + B_c = 100 \quad (3.1)$$

where F is the percent of tracer not bound to either serum protein or charcoal

B_p is the percent of tracer bound to serum and buffer protein

B_c is the percent of tracer bound to charcoal.

At the lowest concentration of ANS (0.002%, w/v; 62 µmol/L), 75% of ^{125}I-rT_3 was bound to charcoal (Figure 3.13). The ANS was at a concentration 2.5×10^6 times that of the ^{125}I-rT_3 (3.2 pmol/L) and would be expected to be bound to both the serum binding proteins and charcoal. With increasing ANS concentration, there was a small increase in the fraction of ^{125}I-rT_3 bound to the charcoal, which was interpreted as a displacement of tracer from the binding proteins. Charcoal binding fell at concentrations of ANS greater than 0.08% (2.5 mmol/L) suggesting that the concentrations of charcoal binding sites was becoming limiting and that ANS was displacing ^{125}I-rT_3 from these sites. The 15 - 20% of ^{125}I-rT_3 not bound to charcoal at 0.06 to 0.08% ANS would represent either tracer bound to unblocked protein binding sites or most likely, tracer free in solution.

(b) Effect of ANS in the presence of antiserum - in the presence of antiserum, ^{125}I-rT$_3$ would be expected to be partitioned between serum binding proteins, antiserum and charcoal binding sites. That is,

$$F' + B'_p + B_{ab} + B'_c = 100 \quad (3.2)$$

where F' percent of tracer not bound to antibody, serum protein or charcoal

B'_p percent of tracer bound to serum and buffer proteins in the presence of antiserum

B_{ab} percent of tracer bound to antibody

B'_c percent of tracer bound to charcoal in the presence of antiserum.

With increasing ANS concentration, charcoal binding fell slightly to a nadir at 0.010% ANS. It was not clear why this should have occurred, particularly as ANS was shown to displace tracer from charcoal only at much higher concentrations. At concentrations of ANS between 0.020 and 0.080% charcoal binding increased suggesting either that the antibody binding sites had become saturated, or that ANS was displacing ^{125}I-rT$_3$ from both serum binding proteins and antibody binding sites. A Scatchard analysis of antibody binding at 0.025% ANS revealed an antibody binding capacity of 8 pmol/L (Figure 3.12), suggesting that at the concentration of tracer used (3.2 pmol/L), binding site concentration was not limiting with regard to tracer concentration.

The net binding of tracer to antibody at any particular ANS concentration was calculated as the difference between binding of ^{125}I-rT$_3$ to charcoal in the presence and

absence of antiserum.

From equation 3.1,

$$B_c = 100 - B_p - F$$

and equation 3.2,

$$B'_c = 100 - (B_{ab} + B'_p) - F'$$

As a first approximation, $F = F'$,

then,

$$B_c - B'_c = (B'_p - B_p) + B_{ab} \qquad (3.3)$$

If $B'_p = B_p$, then,

$$B_{ab} = B_c - B'_c \qquad (3.4)$$

It was considered that this calculation of antibody bound tracer was likely to be an underestimate, particularly at low concentrations of ANS, where serum protein binding sites were incompletely blocked. The percent of ^{125}I-rT_3 bound to serum and buffer protein in the presence of antibody was likely to be either equal to or less than that bound in the absence of antiserum.

That is,

$$B'_p \leq B_p$$

or $$(B'_p - B_p) \leq 0$$

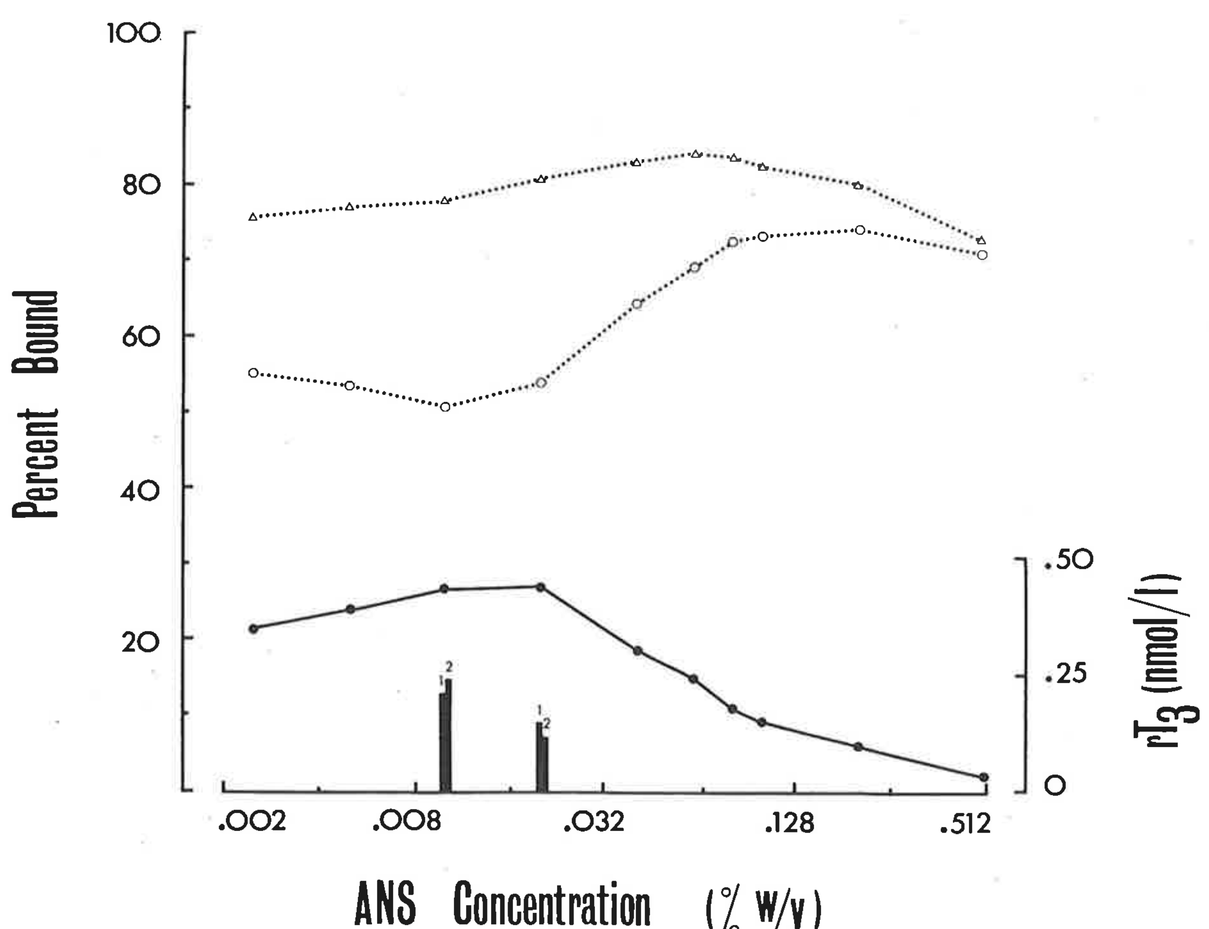
Figure 3.13 Effect of ANS on binding of ^{125}I-rT_3 to charcoal, serum protein and antiserum

△·····△ Percent ^{125}I-rT_3 binding to charcoal in absence of antiserum

○·····○ Percent ^{125}I-rT_3 binding to charcoal in presence of antiserum

●——● Net ^{125}I-rT_3 binding to antiserum

Two serum samples (1 and 2) were assayed at each concentration of ANS. Reverse T_3 was detectable at only .01 and .02% ANS (% w/v).



100
80
60
40
20
Percent Bound
.50
.25
0
rT_3 (nmol/l)
1
2
.002
.008
.032
.128
.512
ANS Concentration (% W/V)

From equation 3.3,

$$B_{ab} = (B_c - B_c') - (B_p' - B_p)$$

If $(B_p' - B_p) \leq 0$

then,

$$B_{ab} \geq (B_c - B_c') \quad (3.5)$$

Two test sera were also incubated with $^{125}I-rT_3$ and antibody at various ANS concentrations. Surprisingly, displacement of antibody bound $^{125}I-rT_3$ by endogenous rT_3 occurred only at 0.010% and 0.020% ANS. This may have reflected the relative insensitivity of the assay at low ANS concentration due to binding of endogenous rT_3 to both binding protein and antibody, and insensitivity at high ANS concentration due to displacement of tracer by ANS.

This phenomenon limits the optimum concentration of ANS for this antiserum in the charcoal assay. A concentration of 0.025% ANS was selected for routine use, being a compromise between maximum serum protein binding inhibition and minimum antibody inhibition.

2. <u>Optimisation of ANS in the Double Antibody Assay</u>

A similar procedure was adopted to optimize the concentration of ANS in the double antibody method (Figure 3.14). This system is simpler in that only two groups of binding sites are present; those of the buffer and serum binding proteins, and the antibody binding sites. Furthermore, the percent bound tracer is determined directly, it being assumed that the second antibody precipitates only the

rT_3 antibody.

Thus,

$$B_p + F + B_{ab} + BMISF = 100 \qquad (3.6)$$

where B_p is the percent of tracer bound to serum and buffer protein

F is the percent of tracer remaining unbound in solution

B_{ab} is the percent of tracer bound to antibody

BMISF is the percent of tracer bound to antibody but not precipitated by the second antibody (bound misclassified as free).

B_{ab} is determined directly as the percent of counts precipitated by second antibody and will be underestimated if BMISF, which was not measured, is significant.

The binding in the absence of ANS reflects the relative concentration and affinities of the binding proteins and antibodies. In the presence of increasing ANS concentration, binding to antibody rose as tracer was displaced from binding protein (Figure 3.14). The marked fall in binding at high ANS concentrations most likely reflected displacement of ^{125}I-rT_3 from antibody binding sites by ANS.

Binding to antibody in the absence of thyroid hormone free serum followed a similar pattern. It was of interest that the binding to antibody in the absence of serum protein exhibited a lower optimum ANS concentration range than in the presence of serum protein. This was taken to suggest that due to binding of ANS by serum protein, the effective

concentration as perceived by the antibody was lower than in the absence of serum. That is, a greater concentration of ANS must be added to inhibit antibody binding sites in the presence of serum.

Scatchard analysis of antibody binding characteristics at 0.025% ANS revealed a single high affinity site with a capacity of 8.3 pmol/L in the absence of hormone free serum. In the presence of hormone free serum a second site with lower affinity and much higher capacity was evident, suggesting the presence of incompletely blocked higher affinity serum binding protein (TBG and TBPA).

Binding of ^{125}I-rT_3 was also assessed in the presence of two test sera (not the same as those studied in the charcoal assay). Maximum displacement of tracer occurred at 0.01% ANS, well below the concentration at which maximum tracer binding occurred. Reduced displacement at low ANS concentration may reflect partial binding of endogenous rT_3 by the serum proteins. It was unclear why displacement should be suboptimal at highest B_o, but may reflect a suboptimal combination of tracer and antibody binding site concentration such that tracer concentration was greater than required for good sensitivity at this concentration of binding sites (Feldman and Rodbard, 1971).

The optimum ANS concentration range was taken to be that at which B_o was maximum, that is, 0.020 to 0.040%, to ensure maximum inhibition of serum protein binding sites. A concentration of 0.025% ANS was used routinely. Reported double antibody rT_3 assays (Table 2.1) have used 0.022 to

Figure 3.14 Effect of ANS on binding to serum protein and antiserum in the double antibody method

●——● Binding of ^{125}I-rT_3 to anti-rT_3 in the presence of thyroid hormone free serum.

Top panel:

○·····○ Binding of ^{125}I-rT_3 to anti-rT_3 in the absence of thyroid hormone free serum.

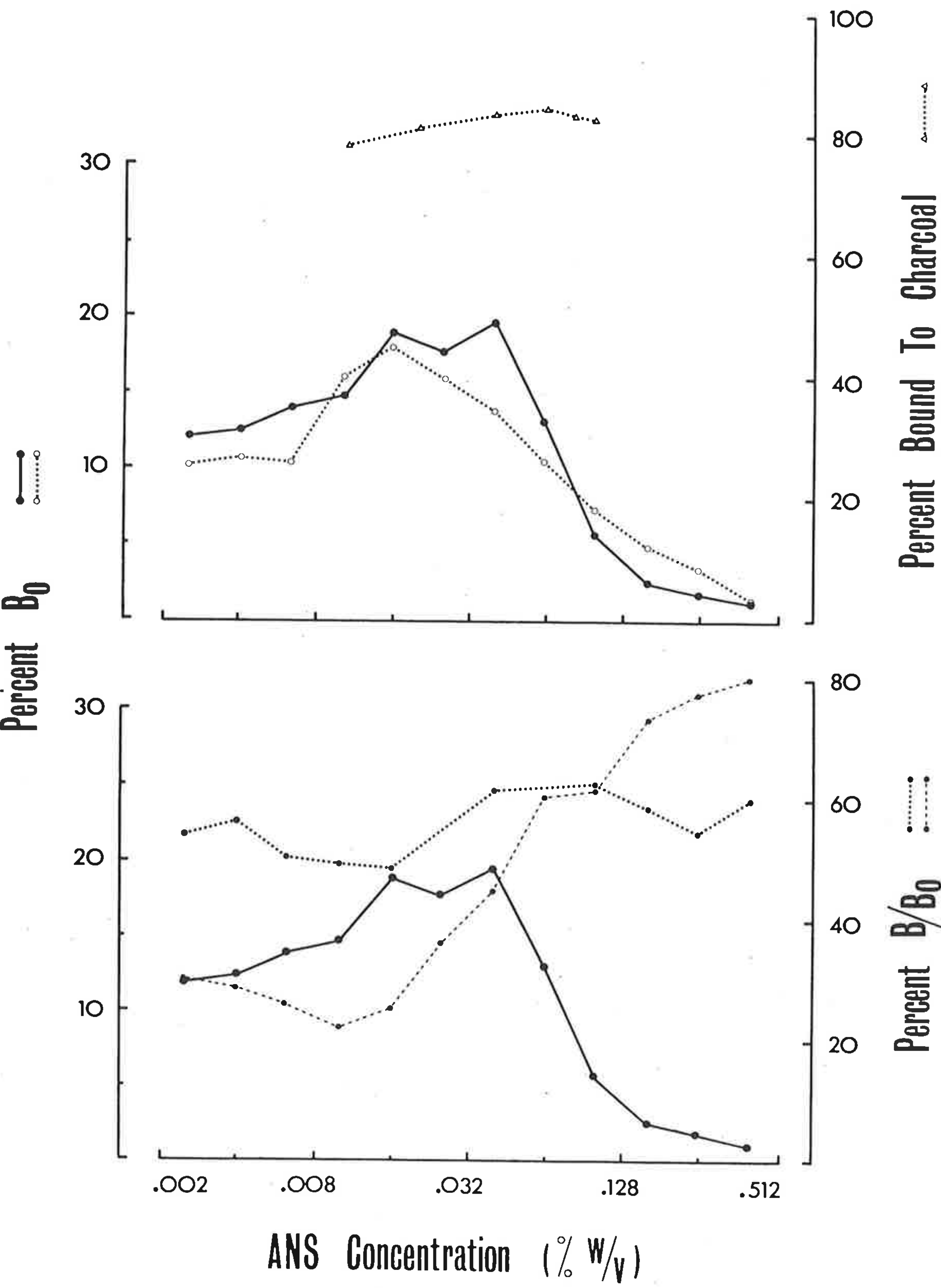
△·····△ Binding of ^{125}I-rT_3 to charcoal in the absence of antiserum and presence of thyroid free serum (from Figure 3.13: to demonstrate the binding of ^{125}I-rT_3 to serum proteins, even at high concentrations of ANS).

Bottom panel:

●·····● Relative displacement of ^{125}I-rT_3 by endogenous
●- - -● rT_3 in two serum samples.

Recovery of exogenous rT_3 was also determined. Data for the two sera were pooled -

% ANS	% Recovery $\pm$ S.D.	P (vs 0.025% ANS)
0.015	120 ± 11	< .05
0.020	111 ± 12	N.S.
0.025	110 ± 6	



Percent Bo
30
20
10
100
80
60
40
20
Percent Bound To Charcoal
30
20
10
80
60
40
20
Percent B/Bo
.002
.008
.032
.128
.512
ANS Concentration (% w/v)

0.038% ANS where serum is 8 - 12.5% of assay incubation volume, agreeing well with the optimum range reported here for a 10% serum assay.

3.6 SEPARATION TECHNIQUES

MATERIALS AND METHODS

Optimum charcoal was determined by measuring the percent ^{125}I-rT_3 bound to charcoal in the presence and absence of antiserum and calculating the percent bound to antibody using equation 3.4. These studies were carried out at 0.01%, 0.02% and 0.03% ANS in thyroid hormone free serum and in two unextracted sera of unknown rT_3 concentration.

Optimum second antiserum (Goat Anti-Rabbit Gamma Globulin, GARGG; Calbiochem, U.S.A.) dilution and normal rabbit serum concentration were determined by the conventional grid method at 37°C in the presence of thyroid hormone free serum. The temperature dependence and kinetics of both this and the reaction of ^{125}I-rT_3 with rabbit 2 antiserum (days 99/102) were studied. The latter study was performed using charcoal as a separant.

Recovery experiments were performed by adding 100 μL of serum of unknown rT_3 concentration and 100 μL of phosphate buffer, pH 7.4 alone, or 0.39, 0.77 and 1.16 nmol/L L-rT_3 (Henning GmbH, Germany) in phosphate buffer, pH 7.4, to each tube and preincubating for 30 min at room temperature before adding the other reagents (Table 3.2). Recoveries at each of these concentrations were pooled and the average calculated.

In the assessment of charcoal as a separant, a

random group of 28 serum samples from inpatients and outpatients were assayed at 0.30% and 0.60% charcoal.

All charcoal concentrations are expressed as % w/v in the assay tube.

RESULTS AND DISCUSSION

1. Charcoal Separation

In the absence of antiserum, adsorption of ^{125}I-rT_3 increased with increasing charcoal concentration to a maximum at 0.6 to 1.0% charcoal (Figure 3.15). The lack of further adsorption at these high concentrations of charcoal suggested that the remaining counts unbound were in a chemical form which would not adsorb to charcoal, for example, $^{125}I^-$. Alternatively, a fraction of the remaining counts may have also been bound to serum and buffer protein, suggesting that, even at 0.030% ANS, the binding protein sites were incompletely blocked. The increased adsorption with increasing ANS concentration was in agreement with the data presented in Figure 3.13.

The displacement of ^{125}I-rT_3 by unlabelled rT_3 in the presence of hormone free serum did not alter significantly with increasing charcoal concentrations (Figure 3.16). There was however an unexpected and considerable alteration in relative displacement in the two unextracted sera tested, the pattern of one of which is shown in Figure 3.16. Displacement increased with increasing charcoal concentration and this increased displacement was greatest at the lowest concentration of ANS. The second serum tested showed a similar pattern of displacement.

The pattern at 0.60% charcoal was similar to that reported in Figure 3.13 in which the greatest estimates of rT_3 in two serum samples (different to those used here) were at 0.010% ANS, being less at 0.02% ANS and undetectable at 0.040% ANS.

The measurement of recovery of L-rT_3 in a serum sample at 0.020% ANS confirmed this pattern of displacement (Figure 3.16, legend). Recovery was inadequate at concentrations below 0.57% charcoal. The measured mean concentration of rT_3 in 38 serum samples was lower when using 0.30% charcoal (0.16 $\pm$ 0.16 nmol/L) than when using 0.60% charcoal (0.82 $\pm$ 0.61 nmol/L; $P < 0.001$), further illustrating differences in measured rT_3 at differing charcoal concentrations.

Although it was unclear as to why this pattern should be evident, the data did suggest differences in charcoal adsorption of tracer between those tubes containing extracted serum and those containing unextracted serum. The observation of minimum changes in displacement at 0.030% ANS would suggest that, in unextracted serum, there was interference by unblocked protein binding sites at the lower ANS concentrations. The most obvious differences between charcoal extracted and unextracted serum were the very low concentration of lower molecular weight metabolites and lower, but normal, concentration of total protein and albumin in the extracted serum (Table 3.3). Although binding capacity of extracted serum was not checked, it would have been unlikely that there were any significant changes in TBG or TBPA concentration relative to albumin (all have

Figure 3.15 The use of charcoal to separate free and antibody bound ^{125}I-rT_3

Percent binding of ^{125}I-rT_3 to charcoal in the absence of antiserum

●——● 0.030% ANS

■·····■ 0.020% ANS

▲- - -▲ 0.010% ANS

Net percent binding of ^{125}I-rT_3 to antibody

○——○ 0.030% ANS

□·····□ 0.020% ANS

△- - -△ 0.010% ANS

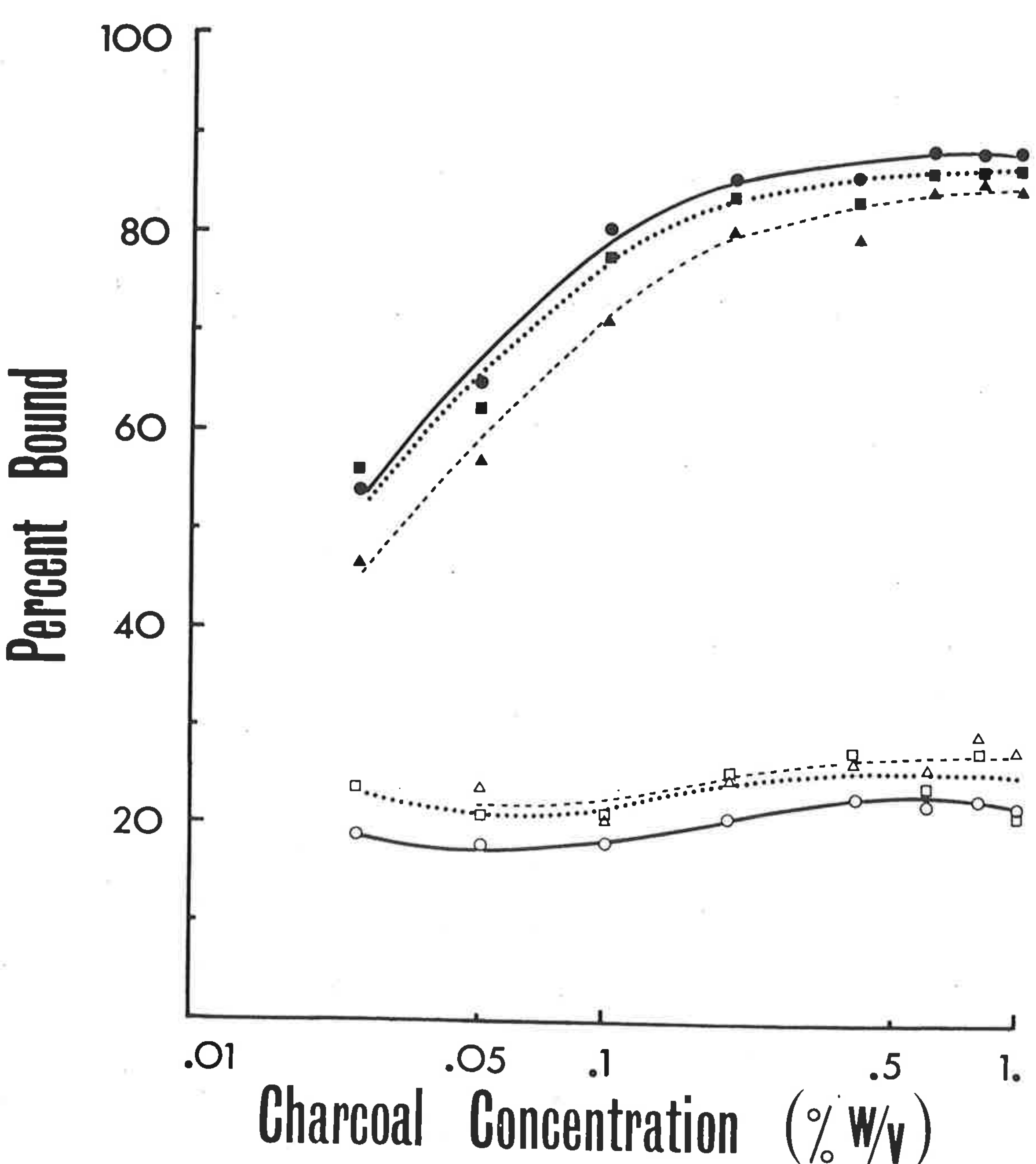

100
80
60
40
20
Percent Bound
.01
.05
.1
.5
1.
Charcoal Concentration (% W/V)

Figure 3.16 Relative displacement of $^{125}I-rT_3$ by exogenous and endogenous rT_3 at various charcoal concentrations

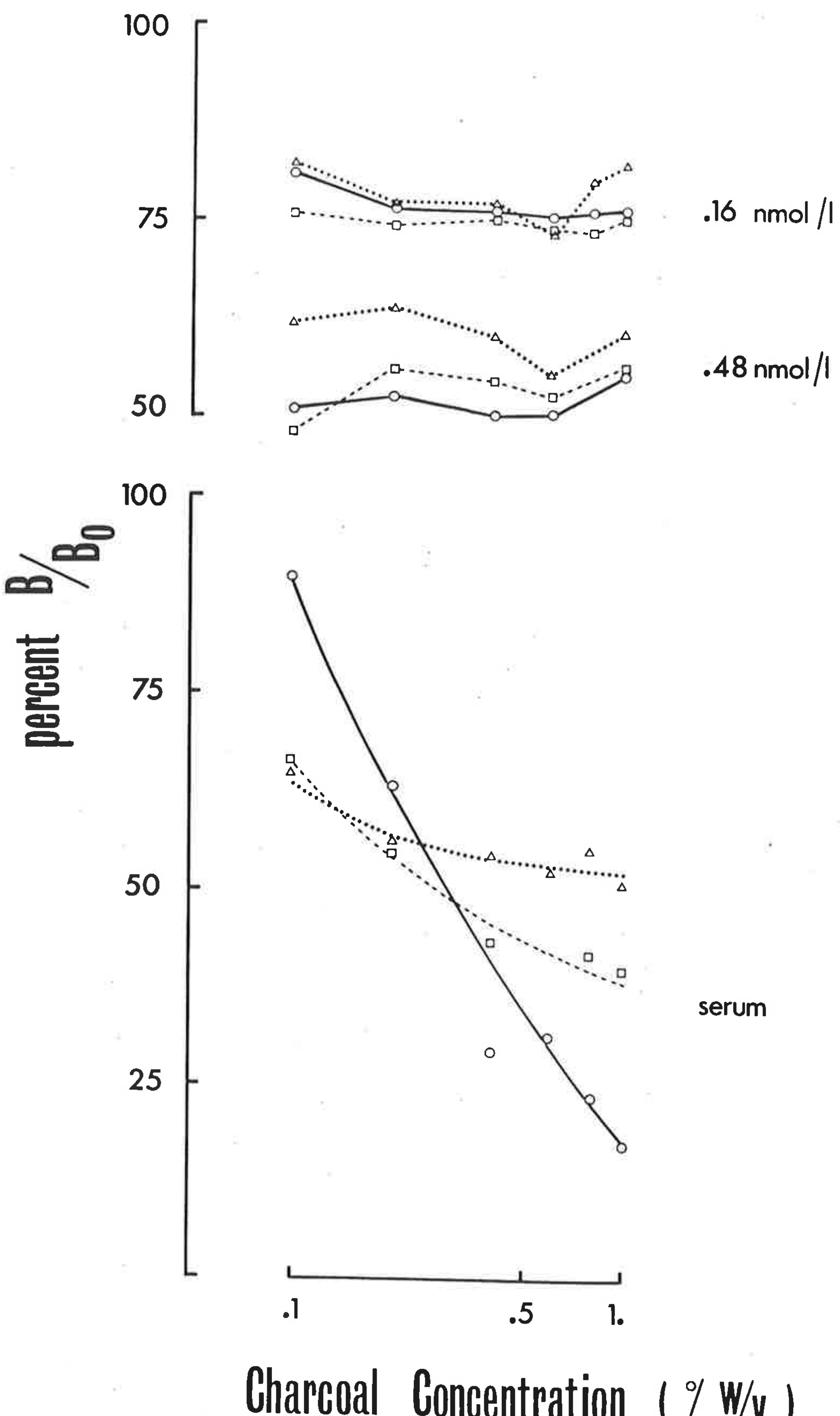
Top panel - relative displacement of $^{125}I-rT_3$ by 0.16 and 0.48 nmol/L rT_3 in thyroid hormone free serum at 3 different ANS concentrations

Bottom panel - relative displacement of $^{125}I-rT_3$ by endogenous rT_3 in a pooled serum sample at 3 different ANS concentrations

Displacement was assessed in the presence of 0.010% (O——O), 0.020% (□---□), and 0.030% (△·····△) ANS (%w/v)

Recovery at various charcoal concentrations
ANS concentration 0.020% ANS, unextracted serum was used

% Charcoal (w/v)	% Recovery ($\bar{x} \pm$ S.D.)
0.20	43 ± 14
0.30	62 ± 11
0.40	83 ± 6
0.45	72 ± 9
0.50	89 ± 8
0.53	91 ± 10
0.57	102 ± 12
0.60	104 ± 21


100
75
50
.16 nmol/l
.48 nmol/l
100
percent B/B0
75
50
25
serum
.1
.5
1.
Charcoal Concentration (% W/V)

similar molecular weight). Whether the differences in concentration of lower molecular weight metabolites was involved is unclear, but again, the very marked physiological fluctuation in concentration of these metabolites without noticeable affect on thyroid hormone concentration, would make it unlikely that they played a role.

2. Double Antibody Separation

Despite the slightly lower B/T, 1 unit of GARGG and 2 μL normal rabbit serum was chosen as the precipitating conditions at 37°C due to the formation of a larger pellet (Figure 3.17).

It was subsequently found that the precipitation reaction occurred very rapidly, being complete within 2 h, and was temperature labile with the greatest precipitation occurring at 4°C (Figure 3.18). That is, BMISF was substantial at higher temperatures. The precipitate formed at 4°C was temperature labile and could be partially solubilized by incubating at 37°C for 1 h, emphasizing the need for maintenance of low temperature right throughout the separation procedure to ensure maximum precipitation (Figure 3.19).

A check on the temperature dependence of the binding of ^{125}I-rT_3 to rT_3 antiserum (Figure 3.20) confirmed that the temperature lability was at the precipitation step.

The faster reaction between GARGG and the rT_3 antibody than between rT_3 and rT_3 antibody was surprising in view of the expectation that the reaction between a small ligand and antibody would be expected to be faster than that between two macromolecules.

From the first order law of mass action, the rate of formation of bound rT_3 is,

$$\frac{d[\text{anti-}rT_3.rT_3]}{dt} = k_1[\text{anti}.rT_3][rT_3] - k_2[\text{anti-}rT_3.rT_3] \qquad (3.7)$$

and the rate of formation of the GARGG.anti-rT_3 complex is,

$$\frac{d[\text{GARGG.anti-}rT_3]}{dt} = k_3[\text{GARGG}][\text{anti-}rT_3] - k_4[\text{GARGG.anti-}rT_3] \qquad (3.8)$$

Considering the reaction between rT_3 and rT_3 antibody to be reversible, the unimolecular dissociation reaction in equation 3.7 (k_2[anti-rT_3.rT_3]) would be expected to be extremely fast and would result in relatively slow net binding of rT_3. However, the formation of the GARGG.anti-rT_3 complex in equation 3.8 involves extensive intermolecular cross-linking and associated precipitation of the immunocomplex. Dissociation of this complex would be limited, with a resulting rapid net rate of formation of the immunocomplex.

If the term k_4[GARGG.anti-rT_3] were temperature dependent, with k_4 being greater at higher temperatures, precipitation at these temperatures may be less complete as $K = k_3/k_4$ would be reduced.

3.7 OPTIMISATION OF REAGENT CONCENTRATIONS

The aim was to develop a robust assay which had sensitivity sufficient to measure rT_3 concentration below the normal euthyroid range. At the time of establishing the assay, those assays published (Chopra, 1974; Meinhold et al., 1975; Table 2.1) assayed rT_3 in ethanol extracts of serum and reported euthyroid ranges which varied considerably.

Figure 3.17 Optimisation of precipitating reagents in the double antibody method

B/T represents percent tracer bound

Goat Anti-Rabbit Gamma Globulin (GARGG):

100 μL ≡ 1 unit (as defined by Calbiochem, U.S.A.)

Normal Rabbit Serum:

△——△ 0.5 μL/tube

□——□ 1 μL/tube

○——○ 2 μL/tube

After reaction of ^{125}I-rT_3 and anti-rT_3 (protocol Table 3.2), GARGG and NRS were added in the quantities indicated and incubated for a further 24 h at 37^oC.

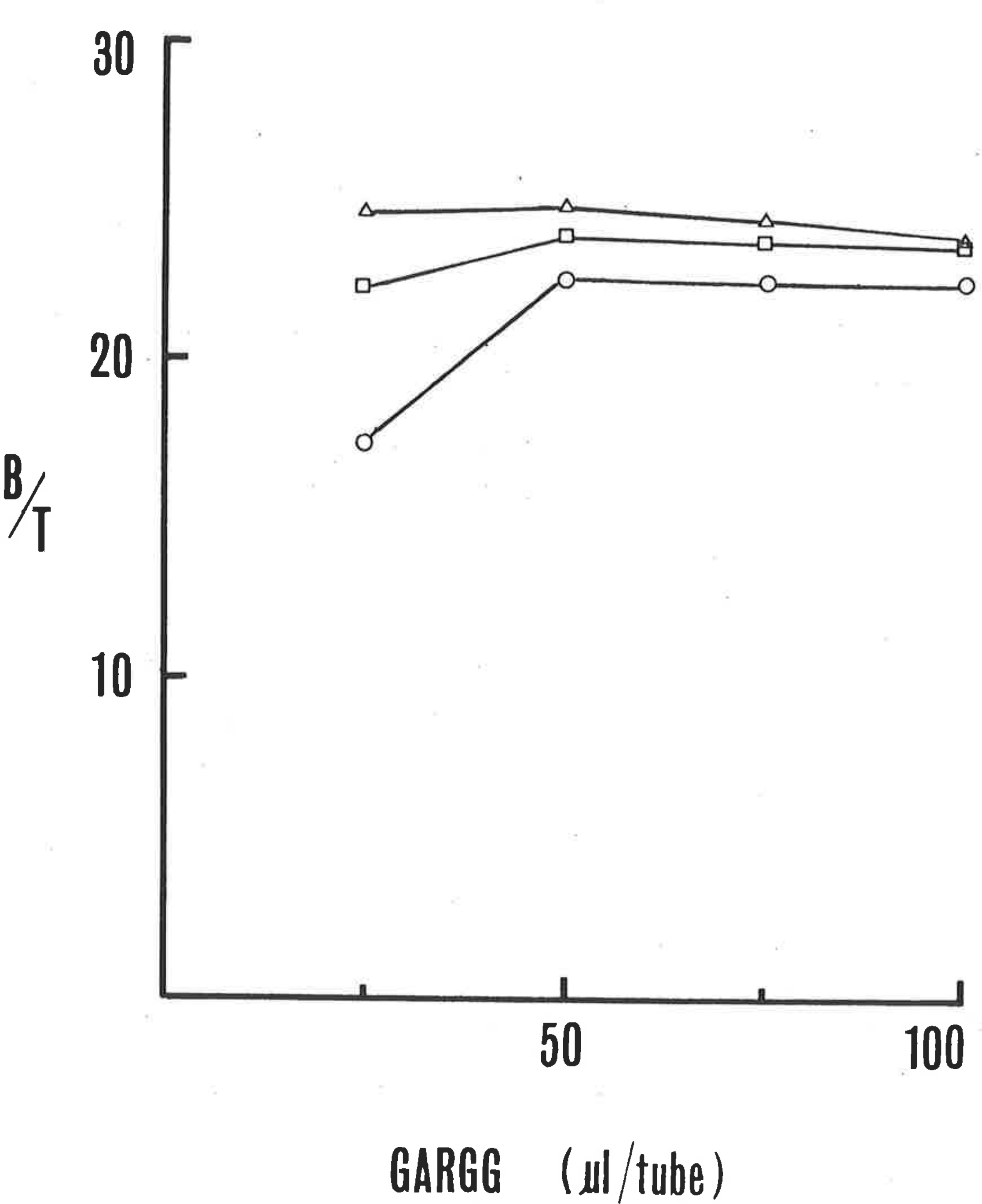

30
20
10
B/T
50
100
GARGG (µl/tube)

Figure 3.18 The effect of incubation time and temperature on double antibody separation

B/T represents percent tracer bound

After incubation of ^{125}I-rT_3 and anti-rT_3 (protocol Table 3.2), GARGG was added and incubated for various times at 4°, 20° or $37^{\circ}C$ before centrifugation at the same temperature.

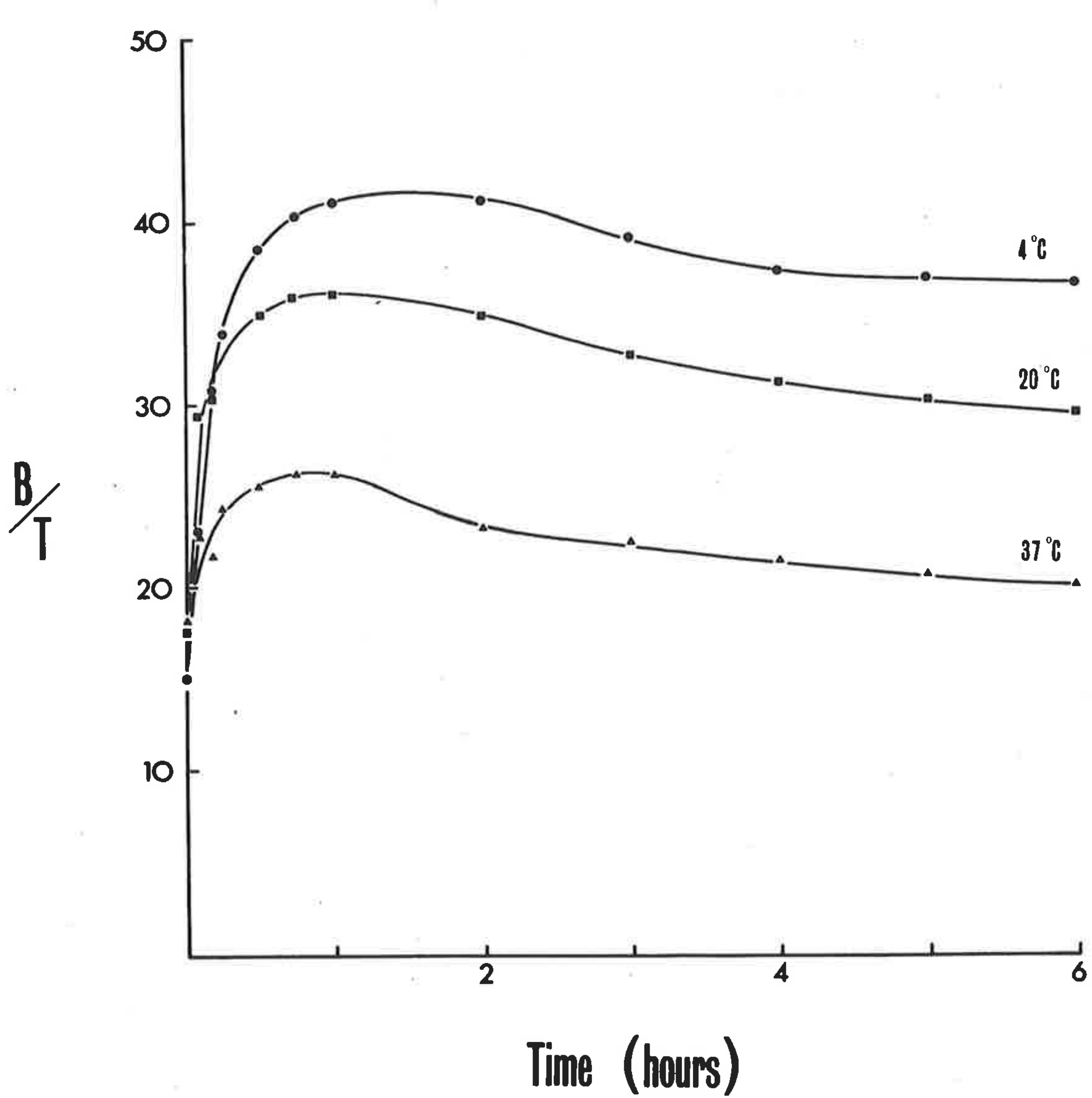

50
40
30
20
10
B/T
4 °C
20 °C
37 °C
2
4
6
Time (hours)

Figure 3.19 Temperature lability of the precipitated immunocomplexes in the double antibody assay

^{125}I-rT_3 was incubated with anti-rT_3 for 24 h (protocol Table 3.2) and bound tracer then precipitated by incubating with GARGG for various indicated periods of time at 4°C (♦——♦), the reaction being stopped by centrifugation at 4°C for 30 min. B/T represents percent tracer bound.

● Bound ^{125}I-rT_3 was precipitated with GARGG for various incubation times at 4°C (as above). Before centrifugation, the tubes were incubated for a further 1 h at 37°C and centrifuged at 37°C.

▲ As for (●), except that the tubes were incubated for a further 2 h at 37°C before centrifugation.

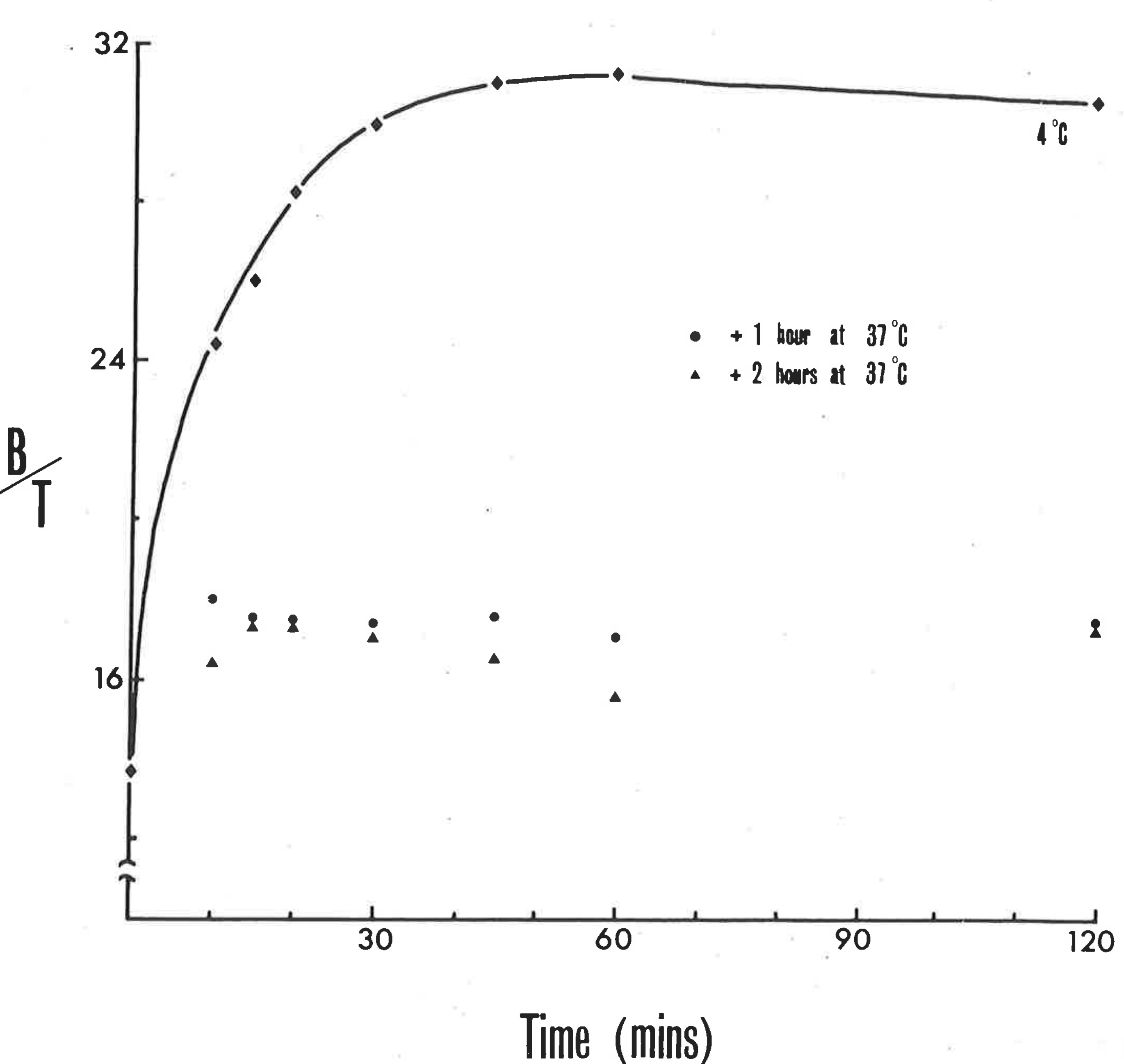
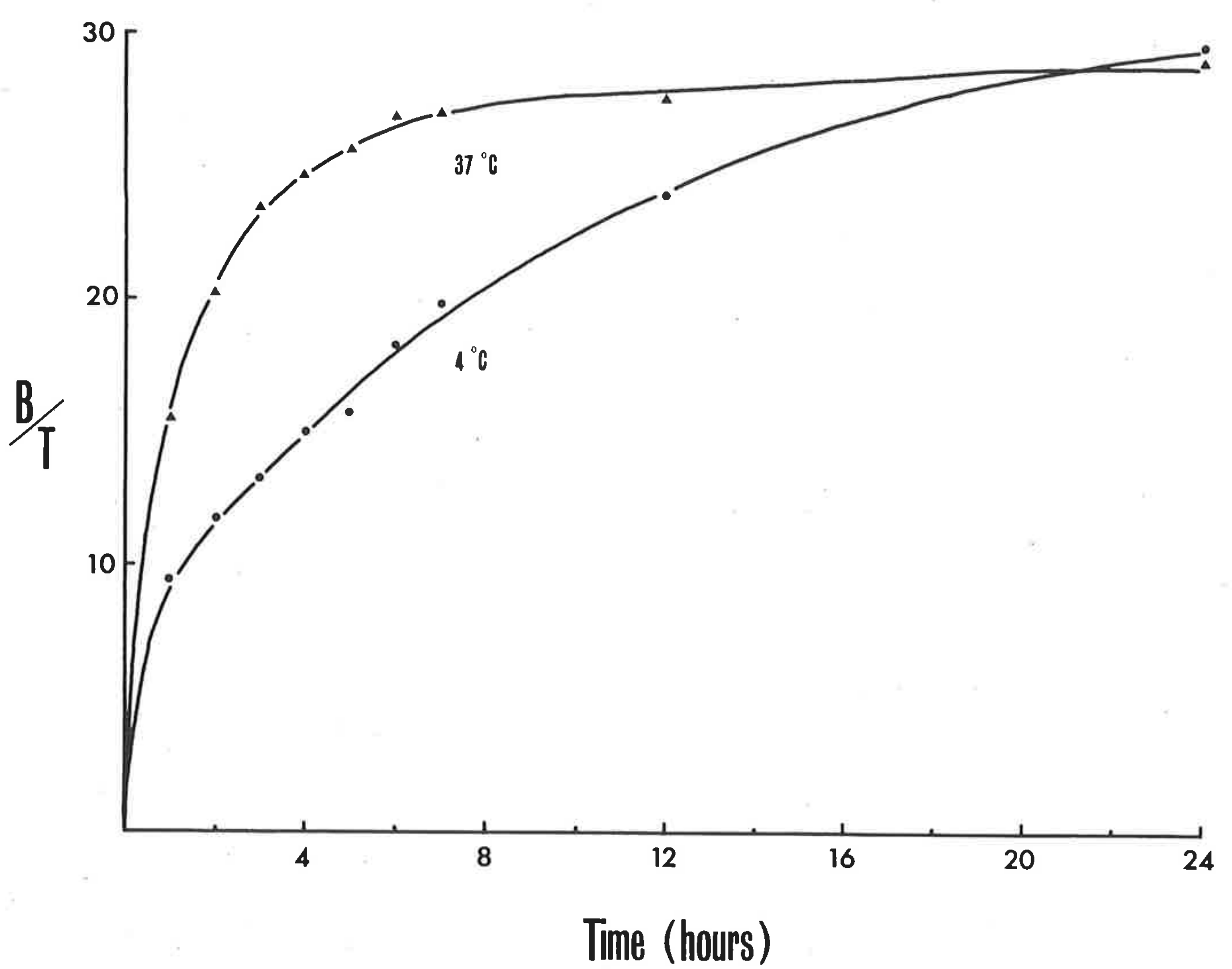

32
24
16
B/T
4°C
● + 1 hour at 37°C
▲ + 2 hours at 37°C
30
60
90
120
Time (mins)

Figure 3.20 $^{125}I-rT_3$ binding to anti-rT_3 at 4° and 37°C

Charcoal was used as the separant.

B/T represents net percent $^{125}I-rT_3$ bound to antibody.


30
20
10
B/T
37 °C
4 °C
4
8
12
16
20
24
Time (hours)

Preliminary analysis of several serum samples under suboptimum conditions suggested that measured concentrations would be similar to those of Meinhold et al. (1975) and it was decided to aim for a sensitivity of 0.01 - 0.02 nmol/L.

Two approaches to optimising the assay were used; the empirical approach, formalized by Yalow and Berson (1968) and Ekins, Newman and O'Riordan (1968), and reviewed by Zettner (1973), and the simplex optimisation technique (Long, 1969).

EMPIRICAL OPTIMISATION - METHODS

It was decided to begin with those dilutions of antiserum at the inflection point of the B/B_o vs B_o diagram (rabbit 2 (day 99/102); Figure 3.8). The displacement of 5,000, 10,000 and 20,000 cpm/tube ^{125}I-rT_3 at 1/10,000, 1/20,000 and 1/30,000 antiserum dilution was assessed and the combination giving the required sensitivity and good binding was selected. The studies were done using the double antibody method outlined in Chapter 3 (3.6).

1. Sensitivity

The sensitivity of the assays established was evaluated in several ways (Figure 3.21). The most commonly used method was the calculation of the least detectable concentration, p, from the standard deviation of the counts bound to antibody in the absence of unlabelled hormone ($B_o \pm \Delta B$) and the slope of the displacement curve between B_o and the counts bound, B, at the lowest standard concentration used, p (Figure 3.21a). Then,

$$\text{slope} = \frac{B - B_o}{p - 0}$$

Now the standard deviation of B_o is ΔB. Thus any $B_p < B_o - 2\Delta B$ can be considered to be significantly lower than B_o. The lowest hormone concentration giving rise to $B_{p'} < B_o - 2\Delta B$ is defined as the sensitivity of the assay, p'. This can be calculated from the slope as,

$$\text{slope} = \frac{B-B_o}{p-0} = \frac{B_{p'}-B_o}{p'-0} = \frac{(B_o-2\Delta B)-B_o}{p'} = \frac{-2\Delta B}{p'}$$

$$\text{That is,} \quad p' = \frac{-2\Delta B}{\text{slope}}$$

The second method used determined the sensitivity by experimental means (Figure 3.21b). A series of standards of concentrations ranging down to levels thought to be below the sensitivity were assayed in quadruplicate. The mean and standard deviation of the percent tracer bound at each concentration was calculated. The lowest concentration of hormone, p', giving rise to a mean percent tracer bound significantly lower than B_o was defined as the sensitivity.

The third method makes use of the precision profile and is described in the next section.

It should be noted that an assay of high sensitivity is defined in this text as one which enables the measurement of low hormone concentrations. In contrast, an assay of low sensitivity is one in which the least detectable concentration is higher.

2. Precision Profiles

The precision profile of an assay has been defined by Ekins (1973). The standard deviation (ΔB) of the mean response variable, B, (counts bound, counts free, percent bound, etc.) was calculated at each dose (concentration of unlabelled hormone) on the standard curve. Using the slope of the dose response curve at each dose, the precision at each dose (Δp) was calculated as $\Delta p = \Delta B/\text{slope}$. The precision profile, $\Delta p/p$ vs p, was then drawn (Figure 3.23).

The precision profile for the double antibody method was determined by pooling data from 20 consecutive assays, and for the charcoal method, from 8 consecutive assays. The within assay variation of the percent bound at each dose (ΔB) was calculated by the one way analysis of variance of the duplicate data from each assay.

The sensitivity of the assay as determined from the precision profile was the lowest dose with $\Delta p/p < 0.5$ (Figure 3.21c). This approach can be rationalized by considering Δp as the 1 S.D. variation in p (being derived from 1 S.D. variation of $B(\Delta B)$. That is $2\Delta p$ represents the 95% confidence range at p. Thus, the estimate with mean concentration, p, ranges from $p-2\Delta p$ to $p+2\Delta p$.

If $\Delta p/p \geq 0.5$
Then $2\Delta p \geq p$ and $p - 2\Delta p \leq 0$
in which case p is not distinguishable from zero.

But if $\Delta p/p < 0.5$
Then $2\Delta p < p$ and $p - 2\Delta p > 0$
in which case p is distinguishable from zero.

Figure 3.21 Measurement of sensitivity

p - standard concentration

B - fraction of tracer added which is bound to antiserum.

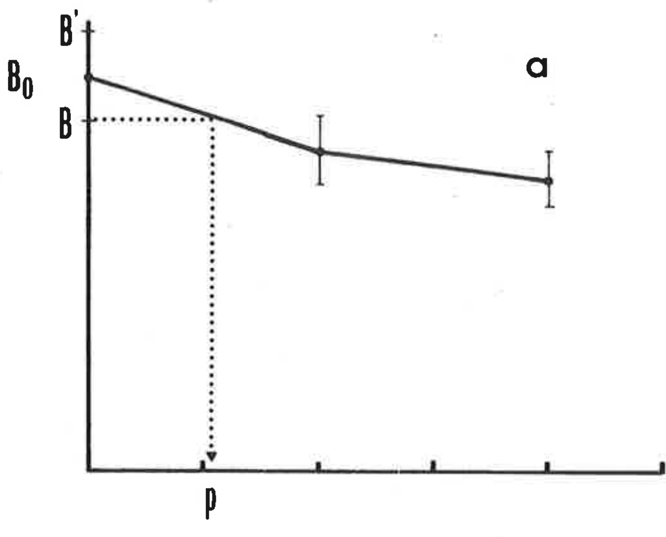
The 95% ranges of the fraction tracer bound at each p are marked.

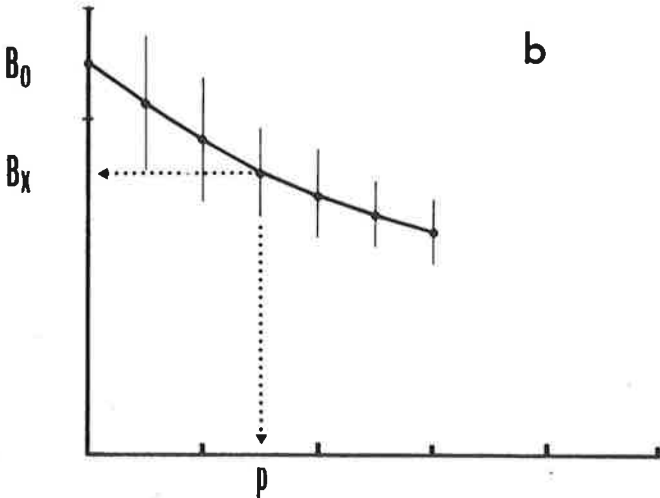
See text for details of sensitivity calculation.

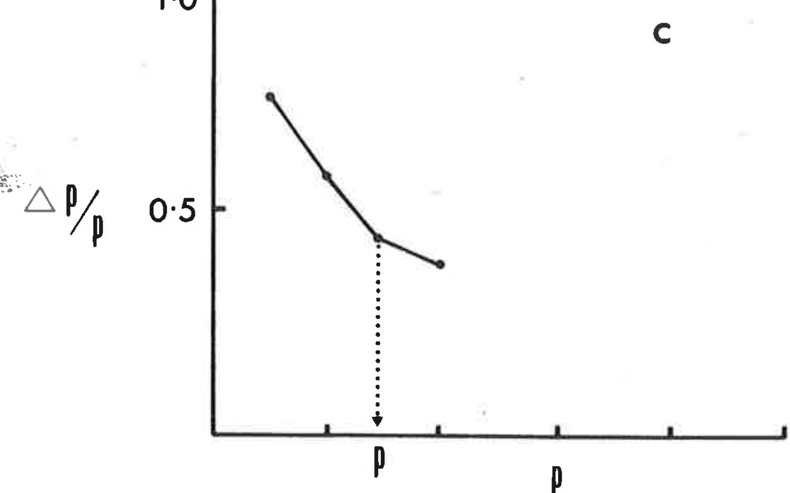
(a) Using the error in measurement of B_o and initial slope of the dose response (calculation)

(b) Smallest dose giving significant displacement of tracer (experimental measurement)

(c) Precision profile (experimental measurement)


B'
B_0
B
a
p


B_0
B_X
b
p


1·0
c
△p/p
0·5
p
p

EMPIRICAL OPTIMISATION - RESULTS AND DISCUSSION

Yalow and Berson (1968) emphasized those combinations of p* (tracer concentration) and q_o (antibody binding site concentration) which give rise to the steepest displacement slopes. These were attained by reducing tracer concentration to very low levels, at which point a value of q_o giving B/F = 0.5 (q_o = 0.5/K, where K is antibody binding affinity) was said to ensure maximum dose response at low concentration of standard.

Ekins et al. (1968) have pointed out that under these conditions, there is a tendency to poor precision which offsets the theoretical advantages of steep slope. They have stressed that the attainment of maximum sensitivity involves the concomitant search for best precision and steepest slope. Where high specific activity tracer and high affinity antibody is available, Ekins et al. (1968) have recommended the combination p* = 4K and q_o = 3/K (giving B/F = 1, or B_o = 50%) calculated using the one binding site, one ligand binding model derived from the law of mass action.

In this study a 1/20,000 dilution of antiserum and 10,000 cpm/tube (5 pmol/L) ^{125}I-rT_3 gave a sensitivity of 0.02 - 0.04 nmol/L and 30 - 35% B_o. Any further addition of ^{125}I-rT_3 caused self-displacement and an accompanying fall in sensitivity (Figure 3.4). Similarly, although a 1/10,000 dilution gave higher B_o, sensitivity was reduced. Scatchard analysis of the binding reaction established q_o = 6.1 pmol/L (Figure 3.12).

Expressed as 1/K units (K = 4 x 10^{10} mol/L; Figure 3.12), p* = 0.13/K and q_o = 0.24/K. These values are well below those recommended by Ekins et al. (1968). In fact, these recommendations would suggest an antiserum dilution of 1/2100 and tracer concentration of 100 pmol/L or 200,000 cpm/tube, leading to an assay of very low sensitivity. It is of particular interest that q_o is close to that recommended by Yalow and Berson (1968). The disagreement with Ekins' recommendation may be due to the very high specific activity of the tracer which would be expected to give rise to assays fitting better into Yalow and Berson's model of vanishingly small tracer concentration and finite but low q_o (= 0.5/K).

In the optimum assay reported here, p*/q_o = 0.54, which is close to p*/q_o = 1.3 suggested by Ekins. This may mean that although Ekins' model does not accommodate assays with very high specific activity tracer, the ratio p*/q_o to attain maximum sensitivity at the lower q_o is still of the same order as given by Ekins. That is, Yalow and Berson's model can be looked upon as an extrapolation of Ekins' model to those assays which do have vanishingly small tracer concentrations and which are now a reality due to the availability of very high specific activity tracer.

1. <u>Precision Profiles</u>

The precision profile of the double antibody assay showed an assay which regularly attained sensitivities between 0.02 - 0.04 nmol/L. Precision was consistent within and above the euthyroid reference range of 0.12 - 0.38 nmol/L.

SIMPLEX OPTIMISATION - METHOD

Long (1969) has formulated the simplex technique for optimising chemical systems. It was decided to test this technique for optimising sensitivity as an alternative to the empirical approach.

The simplest form of the simplex technique is the variation of two variables to give an optimum response. This is ideally suited to optimising radioimmunoassays in which the response, sensitivity, is determined largely by the two variables p^* and q_o. Of particular importance is that for any combination of p^* and q_o there can only be one value for sensitivity. Thus, by plotting the sensitivity (p') for every combination of p^* and q_o a response surface is formed, which in its simplest form has a depression in that region of p^* and q_o values giving best sensitivity (lowest p giving significant displacement). The simplex method provides a rational step by step selection of p^* and q_o values such that they move towards those combinations in the depression, that is toward those combinations giving highest sensitivity.

The selection of initial coordinates (values of p^* and q_o) is described in Table 3.4. Three combinations of p^* and q_o were selected according to a table supplied by Long (1969). These form the vertices of an equilateral triangle. A displacement curve with rT_3 concentrations of 0, 0.005, 0.01, 0.02, 0.04 and 0.08 nmol/L was run in quadruplicate at each vertex. The response to be optimised was sensitivity, and was assessed by finding the smallest dose

which caused a significant displacement of tracer (Figure 3.21b). That combination which gave the worst sensitivity was dropped and a new equilateral triangle constructed graphically, containing the 2 retained vertices and a new combination of p* and q_o values making up the third vertex (Table 3.4).

Once the pathway had begun, any newest vertex which turned out to have the worst sensitivity was retained and the next worst dropped. This prevented the regeneration of previous vertices. The pathway was complete once it had completed a circuit around the point of highest sensitivity.

Several of the points on the pathway were selected and their precision profiles assessed over 5 assays, each in triplicate.

SIMPLEX OPTIMISATION - RESULTS AND DISCUSSION

1. Selection of Initial Coordinates

The simplex pathway was followed graphically with q_o represented as the reciprocal of (antiserum dilution x 10^3) on the ordinate, and p* represented as cpm per tube on the abscissa (Figure 3.22a). The initial starting point (vertex A) was at p* = 20,000 cpm and q_o = 50 (1/50,000 antiserum dilution), which were deliberately selected to be quite different from the empirically derived optimum combination (p* = 10,000 cpm, q_o = 20). Vertex A was considered to be suboptimal as q_o was reduced and p* increased, a combination which was expected to reduce sensitivity.

A step size of p* = 20,000 cpm and q_o = 12.5 was

selected and the initial 3 vertices located as described in Table 3.4. The pathway followed is outlined in Figure 3.22 with the data on which the pathways decisions were made presented in Table 3.4.

2. The Simplex Pathway

Vertex A had the poorest sensitivity (0.08%) in the triangle ACB and a new vertex was generated at D (p* = 50,000 cpm, q_o = 62.5). As all the vertices in the new triangle had sensitivity 0.04 nmol/L, the vertex with the highest P value at 0.04 nmol/L (vertex B) was discarded, generating the next vertex, E (p* = 40,000 cpm, q_o = 75). In a similar manner, vertex C was discarded in the triangle EDC. Although the P values at 0.04 nmol/L at each vertex of FDE were similar, D was considered to display the poorest dose response as a hook effect was evident at 0.005 and 0.01 nmol/L rT_3. The new vertex, G, displayed a marked fall in B_o with an accompanying significant hook at 0.005 nmol/L rT_3 and very poor sensitivity. Although this may have simply reflected grossly suboptimal conditions, it was decided to use fresh tracer and repeat GFE.

The 3 vertices all exhibited markedly improved sensitivity, and it was subsequently found that the fresh tracer was of unusually higher specific activity (1830 pCi/pg) than the previous tracer (1120pCi/pg). It thus became necessary to express p* in terms of concentration of ^{125}I-rT_3, the 1/K coordinate system being adopted (Figure 3.22b). The conversion was calculated such that the cpm (Figure 3.22a) and 1/K (Figure 3.22b) axes were superimposable for the

1830pCi/pg tracer. Thus the vertices using the higher activity tracer were superimposable in the two coordinate systems (cpm and 1/K). As expected, the adoption of an abscissa in terms of concentration of tracer resulted in a shift of the vertices GFE to lower p*, causing a discontinuity in the pathway. Furthermore, using the previous tracer of lower specific activity, the pathway A → G appeared distorted in this coordinate system.

As the aim was to achieve maximum sensitivity, it was decided not to retrace the pathway, but to carry on from GFE as outlined in Table 3.4. The pathway finally rotated about point G, indicating this to be the region of best sensitivity. The completed sensitivity map is illustrated in Figure 2.22c.

The direction of the pathway was particularly surprising. The progression from a point (vertex A) which was considered to have too high a p^*/q_o (p^*/q_o = 0.97 compared to p^*/q_o = 0.54 for the empirically determined combination of p* and q_o) to even higher p^*/q_o was unexpected. The initial direction toward higher p^*/q_o was followed by a movement to lower p* and q_o, whilst maintaining constant p^*/q_o in the range 1.7 - 3.1. There was an accompanying fall in B_o from 9.7% at vertex A to 7.1% at E and 5.5% at G, well below the B_o of 30 - 35% for the empirically optimised assay.

At vertices G and E, tracer was present in concentration up to 3 times in excess of the antibody binding capacity. At these points, the least detectable dose (0.01 nmol/L) was less than the concentration of ^{125}I-rT_3

TABLE 3.4 Simplex optimisation

(a) Location of initial vertices
(refer to Table 1 in Long, 1969)

(i)

Vertex	Factor	
	variable 1	variable 2
A	0	0
B	1.0	0
C	0.5	0.866

variable	step size	units
1 tracer	20,000	cpm/3 min/tube
2 antiserum dilution	14.43	1/(antiserum dilution x 10^3)

(ii) Multiply the factor supplied in table by appropriate variable step size.

Vertex	(Factor x Step size)	
	tracer	antiserum
A	0	0
B	20,000	0
C	10,000	12.5

(iii) Add the (Factor x Step size) to the coordinates of the initial vertex (vertex A)

Vertex	Coordinates	
	tracer	antiserum
A	20,000	50
B	40,000	50
C	30,000	62.5

TABLE 3.4 Simplex optimization

(b) Sensitivity of the dose response curve at each vertex

Vertex	^{125}I-rT_3 (μCi/μg)	% B/T	[a] P values (B_x vs B_o) rT$_3$ (nmol/L)				
			.005	.01	.02	.04	.08
A	1120	9.7	[b]+ .0477	+ .0479	+ .0880	+ .6684	.0078
B	1120	8.5	+ .8254	+ .7638	+ .4438	.0447	–
C	1120	7.9	.7427	.5655	.2176	.0210	.0001
D	1120	7.2	+ .1467	+ .4913	.1608	.0070	.0006
E	1120	6.5	.6989	.3130	.1929	.0081	.0004
F	1120	5.7	.5923	.9062	.1326	.0087	.0002
G	1120	2.6	+ .0471	.2478	.6768	.7363	.0007
E'	1830	7.1	–	.0392	.0006	.0008	
F'	1830	6.6		.6340	.0349	.0042	.0002
G'	1830	5.5		.0199	.0064	.0016	.0001
H	1830	5.5		.5342	.0644	.0005	.0001
I	1830	4.6		.1465	.0039	.0028	.0001
J	1830	4.5		.1984	.1671	.0008	.0001
K	1830	5.0		.4557	.0500	.0106	.0005

[a] The displacement of ^{125}I-rT_3 by each dose (B_x) at each vertex was tested for significance by Students unpaired t test. Significant and non-significant displacements are separated.

[b] + signifies that binding at that dose was greater than B_o at that vertex.

TABLE 3.4 Simplex optimisation

(c) Optimisation Pathway

Description of the optimisation path using the data in Table 3.5 (a) and drawn in Figure 3.22a.

The triangles tested are labelled in a clockwise manner starting at the new vertex.

Tested vertices	^{125}I-rT_3 Batch	New Vertex	Comments[a]
ACB	10	A → D	poorest sensitivity
DBC	10	B → E	highest P at .04 nmol/L
EDC	10	C → F	highest P at .04 nmol/L
FDE	10	D → G	hook
GFE	10	G → D	[b]hook
GFE	11	F → H	
HGE	11	H → F	[b]highest P at .02 nmol/L
		E → I	[c]higher P at .01 nmol/L than G
IGH	11	H → J	highest P at .02 nmol/L
JGI	11	J → H	[b]highest sensitivity
		I → K	[c]higher sensitivity than G
KGJ	11	J → E	highest sensitivity

a Reason for dropping the vertex indicated

b Generation of tested vertex

c Next best reflection

Figure 3.22 Simplex optimisation

(a) Simplex pathway (tracer concentration expressed as counts/3 min/tube)

(b) Simplex pathway (concentration of tracer and antiserum expressed as 1/K units)

$K = 4.0 \times 10^{10}$ L/mol (Figure 3.12)

Antiserum binding capacity = 160 nmol/L (Figure 3.12)

Tracer 1120 µCi/µg: 10,000 cts/3 min/tube
= 1.15 pmol/L (vertices A - G)

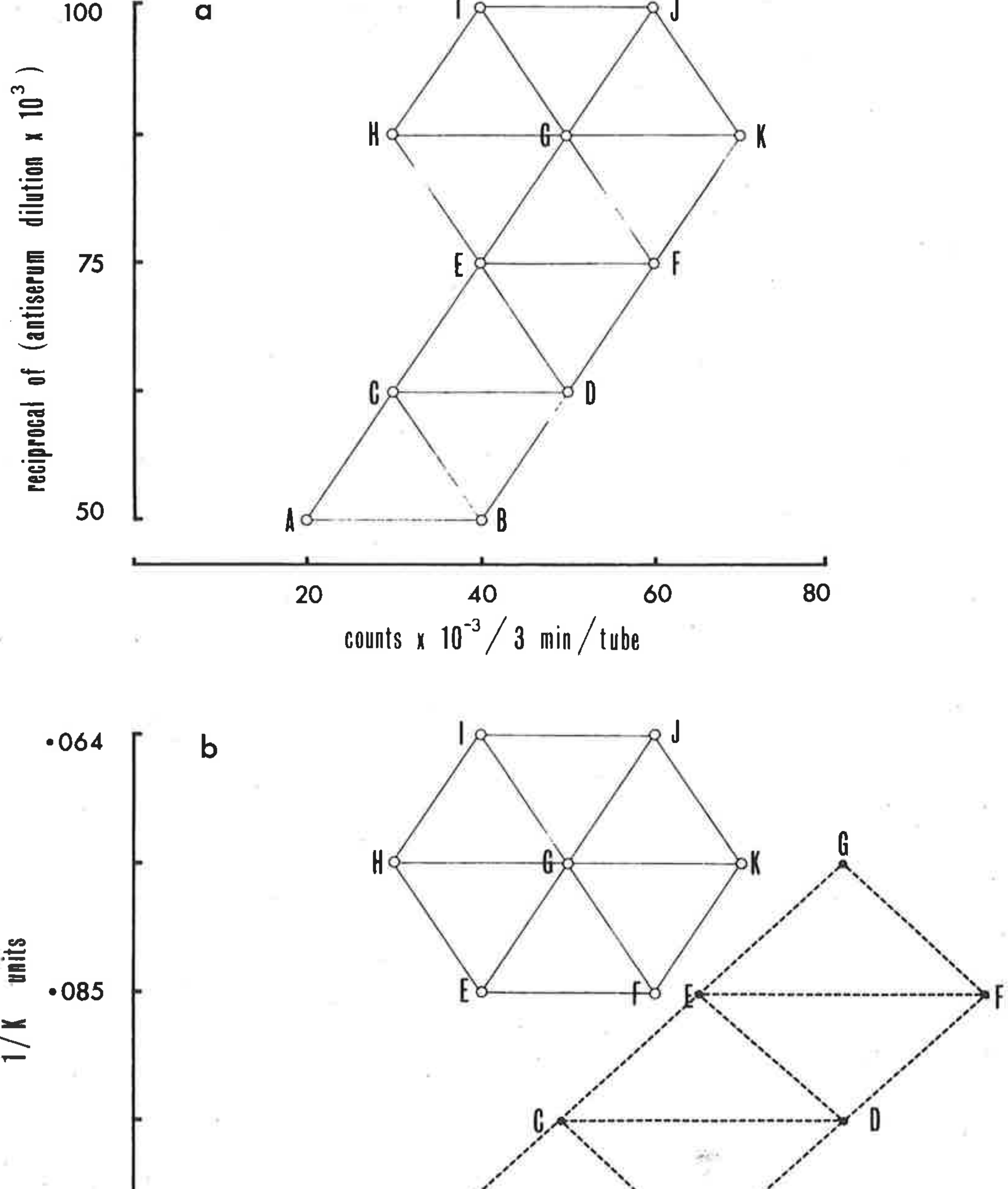
1830 µCi/µg: 10,000 cts/3 min/tube
= 0.71 pmol/L (vertices E - K)

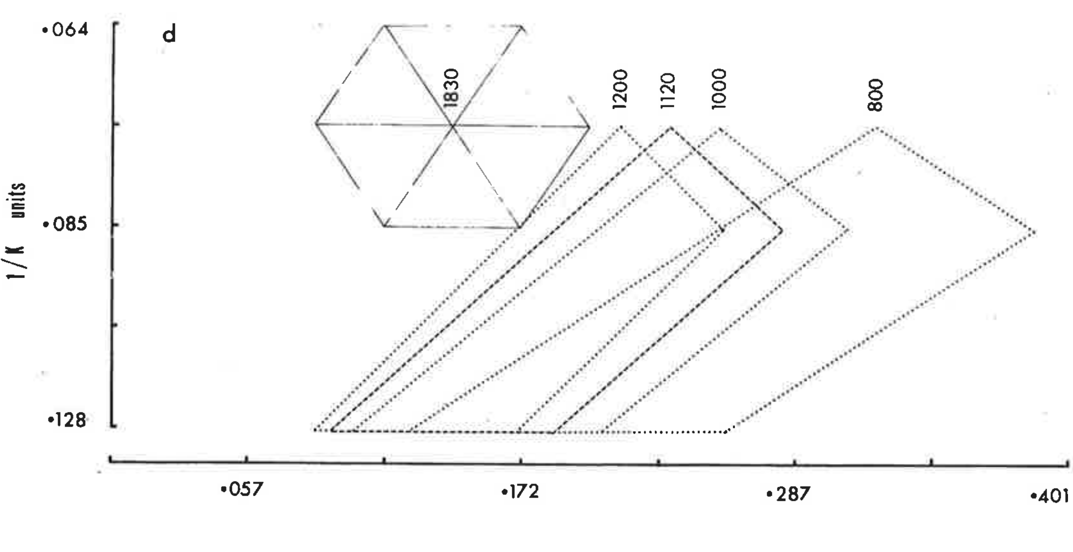
To express antiserum binding site concentration q or tracer p^* in 1/K units, divide q or p^* by K.
Abscissa represents counts/3 min/tube for the higher specific activity tracer (1830 µCi/µg) converted to 1/K units.

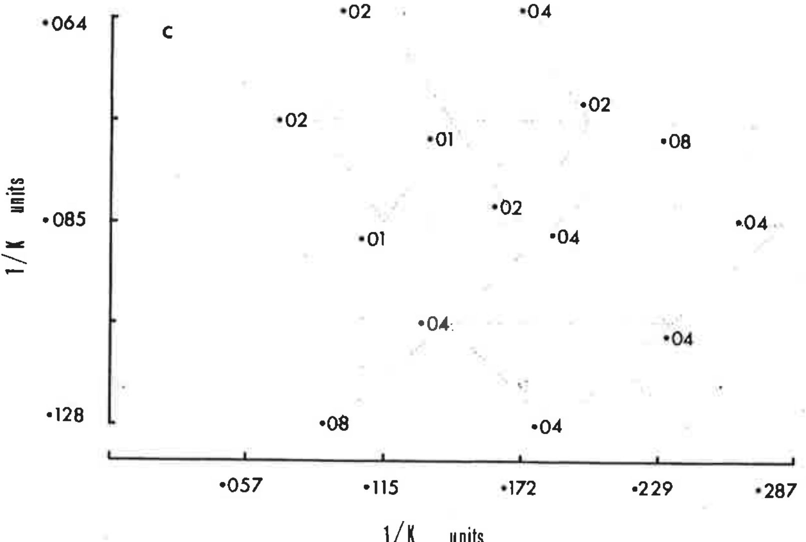
(c) Sensitivity map

(d) Effect of variation in specific activity on sensitivity

Location of vertices A - G for tracers of specific activity 800, 1000, 1120 and 1830 µCi/µg.


a
100
75
50
reciprocal of (antiserum dilution x 10^3)
I
J
H
G
K
E
F
C
D
A
B
20
40
60
80
counts x 10^{-3} / 3 min / tube
b
•064
•085
•128
1/K units
I
J
H
G
K
E
F
G
E
F
C
D
A
B
•057
•115
•172
•229
•287
1/K units


d
•064
•085
•128
1/K units
1830
1200
1120
1000
800
•057
•172
•287
•401


c
•064
•085
•128
1/K units
•02
•04
•02
•02
•01
•08
•02
•01
•04
•04
•04
•04
•08
•04
•057
•115
•172
•229
•287
1/K units

($p'/p^* = 0.35$ at E and $p'/p^* = 0.28$ at G). Thus the total ligand concentration was nearly 4 times the capacity of the antibody sites, suggesting that the antibody is saturated with ligand at all doses of rT_3 under these conditions.

3. Precision Profiles

To assess the precision of the assays at several points on the pathway, precision profiles were assessed using the p^* and q_o values at vertices A, G and E (Figure 3.23). Tracer of specific activity 1100 pCi/pg was used. The precision profile of vertex A assay was very similar to that of the empirically determined conditions, despite the higher p^*/q_o and associated lower B_o. Sensitivity was better than expected from the simplex map. The vertex E assay was less sensitive than the vertex A assay, but had a similar profile at concentrations of rT_3 seen in subjects who are either euthyroid or have elevated levels. This was of particular interest in view of the very low B_o (5.80%). The vertex G assay had poorer precision with a sensitivity of between 0.08 - 0.16 nmol/L rT_3, and was quite inadequate for routine estimation of rT_3 concentration.

4. Conclusions

The simplex method appeared to be a useful tool in the experimental optimisation of radioimmunoassay conditions. One of its greatest advantages is that it provides a rational experimental approach to optimisation which is free of the subjective judgements of the experimenter. Unfortunately, further batches of very high specific

Figure 3.23 Precision profiles of the simplex vertices A, E and G and the empirically optimised double antibody and charcoal assays

———	Empirically optimised - double antibody method
-----	Empirically optimised - charcoal method
■····■	Simplex vertex A
▲····▲	Simplex vertex E
●····●	Simplex vertex G

The charcoal assay utilized different standard concentrations (0.08, 0.16, 0.40, 0.80, 1.60, 3.20 nmol/L) to those used in the double antibody assay (Table 3.2).

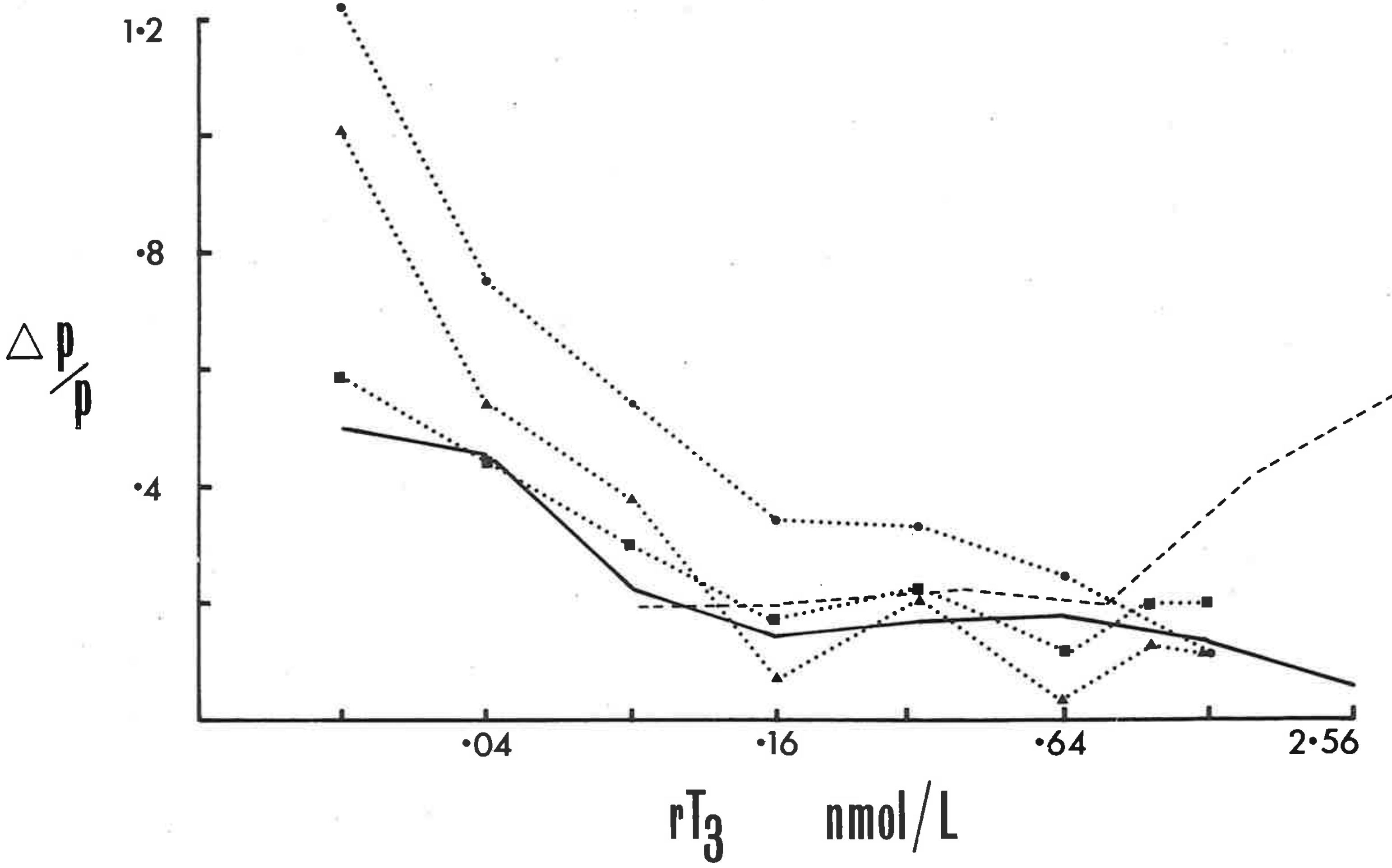
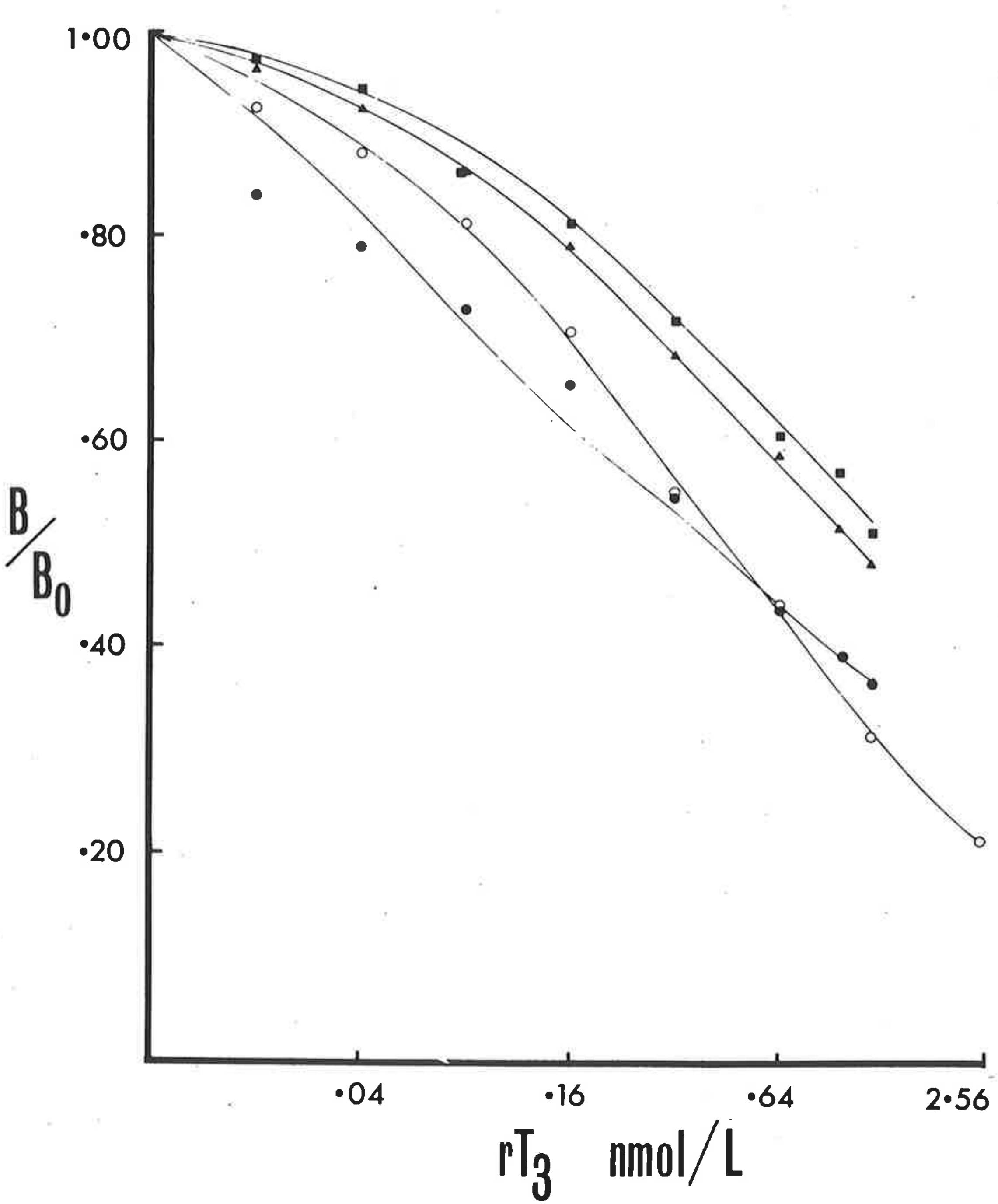

1·2
·8
·4
Δp/p
·04
·16
·64
2·56
rT3 nmol/L

Figure 3.24 Displacement curves for the simplex vertices A, E and G, and the empirically optimized assay

		% B_o/T
○——○	Empirically optimized double antibody assay	30.9
●——●	Simplex vertex A	9.7
▲——▲	Simplex vertex E	5.8
■——■	Simplex vertex G	4.7


1·00
·80
·60
·40
·20
B/B0
·04
·16
·64
2·56
rT3 nmol/L

activity were not able to be synthesized at the time of establishing the precision profiles to check the points of highest sensitivity (vertices E and G with 1830 pCi/pg tracer). However, the simplex method did produce vertices which had profiles similar to the empirical assay (A and E), despite moving in a direction opposite to that which one would take empirically.

The simplex procedure was not entirely free of subjective decisions. Thus, several of the earlier vertices were discarded by comparing P values, despite there being no evidence that the P values were significantly different. This was in a region where a number of vertices had sensitivity 0.04 nmol/L, that is, in a 'plain'. It is here that step size becomes important in the simplex method; if step size is too small, the pathway can become lost in a plain. This can be overcome by increasing the step size and carrying on until some change in the response (in this case sensitivity) is noted. A similar approach may have avoided the necessity of making rather tenuous comparisons of P values.

A particular limitation of the simplex technique is that it detects only one optimum region. To check for the possibility of other optimum regions, one must use a different starting point; this would have been of value in checking whether the empirically optimised assay could be found by the simplex technique.

The empirically derived optimum conditions were selected for use in the routine assay (Table 3.2). This

was felt to be more robust than the vertex A assay in that, although the precision profiles were similar, binding was greater in the former assay. The sensitivity under the conditions pinpointed by the simplex method were particularly prone to variations in specific activity of the tracer (Figure 3.22d). By using the empirical conditions it was unnecessary to measure specific activity routinely and it was possible to attain adequate sensitivity with lower specific activity tracer.

3.8 PROCEDURE FOR RADIOIMMUNOASSAY OF 3,3',5'-TRIIODOTHYRONINE

METHODS

1. Assay Reagents

Working Buffer - 0.25% (v/v) human serum albumin (HSA), 0.05 M phosphate buffer, pH 7.4.

Standard - L-rT_3 (Henning GmBH, Germany). Stock standard was stored at 4°C in 0.025 M NaOH-ethanol (1:1). Standards of concentration 0.02 - 2.56 nmol/L were prepared by diluting stock in 0.025 M NaOH.

T_4 - L-T_4 (Sigma, U.S.A.). Stock T_4 was stored at 4°C in 0.025 M NaOH-ethanol (1:1). Standards of concentration 31-500 nmol/L were prepared by dilution in 0.025 M NaOH. T_4 dose response curve run with each assay to enable correction for T_4 cross-reaction.

Antiserum - Rabbit 2, days 99/102 was diluted in working buffer and used at a final dilution of 1/20,000.

Tracer - ^{125}I-rT_3 (approximately 1200 pCi/pg) was stored in ethanol at room temperature. 10,000 cpm/assay tube.

Inhibitor (ANS) - 8-anilino-1-naphthalene sulphonic acid, sodium salt (ICN Pharmaceuticals, U.S.A.) was used at a concentration of 0.025% (w/v).

Thyroid Hormone Free Serum - stored at -20°C. 10% of the assay incubation volume.

Normal Rabbit Serum (NRS) - obtained regularly from in-house rabbits. 2 µL per tube.

Precipitating Antibody (GARGG) - Goat Anti-Rabbit Gamma Globulin (Calbiochem, U.S.A.) was reconstituted in 0.05 M Na_2EDTA, 0.25% HSA, 0.05 M phosphate buffer, pH 7.4, 1 unit/tube.

Incubation mix - contained ANS, $^{125}I\text{-}rT_3$ and NRS in working buffer.

2. Assay Protocol

TABLE 3.2

	Total	Blank	Zero	Standards	Test
Working Buffer	-	[a]200	100	-	100
Incubation mix	800	800	800	800	800
Standards (rT_3 or T_4)	-	-	-	100	-
Thyroid Hormone Free Serum	-	100	100	100	-
Test Serum	-	-	-	-	100
Antiserum	-	-	100	100	100
	Incubate 4°C, 24 h				
GARGG		100	100	100	100
	Incubate 4°C, 1 h Centrifuge 30 min, 4°C, 2000 g Count precipitate				

[a] All volumes are expressed as µL.

Results were calculated using the radioimmunoassay curve fitting program of Burger, Lee and Rennie (1972) adapted for use on a Wang 600 desk top calculator (Wang Computers, U.S.A.) by O'Halloran (1974)

3. Reference Sample Collection

Blood from 55 normal euthyroid male donors was collected at the Red Cross Centre (Adelaide, South Aust.). Samples were kindly donated by 54 normal euthyroid female staff members of The Queen Elizabeth Hospital (South Aust.), 30 of whom were on oral contraceptives. 10 blood samples were obtained from pregnant females at various stages of gestation and who were attending antenatal clinic (The Queen Elizabeth Hospital, South Aust.).

Sera of patients with clinical thyroid disease were selected on the basis of their thyroid function tests. 12 hypothyroid patients with low FTI, low T_4 (1 with borderline low T_4) and elevated TSH, and 11 thyrotoxic patients with elevated FTI and T_4 were included.

RESULTS AND DISCUSSION

1. Assay Reproducibility

The precision profile is presented in Figure 3.23. Precision was less than 0.20 in the euthyroid and higher concentration range and was considered quite adequate for routine analysis.

The between assay coefficient of variation was 11.8% for a serum quality control in the euthyroid range

(0.30 ± 0.03 nmol/L, uncorrected for T_4) and 10.9% for an elevated serum control (0.43 ± 0.05 nmol/L, uncorrected for T_4), run over the 20 assays used to construct the precision profile. The within assay coefficient of variation was 6.3% at 0.30 nmol/L.

2. Reference Data

Data, both uncorrected and corrected for T_4 cross-reaction are presented in Figure 3.25. The mean euthyroid concentration (0.25 ± 0.07 nmol/L) was similar to the lower concentrations reported for both extraction (Meinhold et al., 1975) and direct non-extraction methods (Table 2.1). The euthyroid range was smaller than a number of assays reported with a similar mean, agreeing with the data of Ratcliffe et al. (1976) and Premachandra (1978).

Serum rT_3 was mildly but significantly elevated in those subjects on oral contraceptives (0.33 ± 0.07 nmol/L), and was clearly elevated in pregnant subjects (0.44 ± 0.09 nmol/L), demonstrating protein binding characteristics similar to the thyroid hormones.

rT_3 concentration in hypothyroid subjects displayed marked overlap with normal euthyroid values, but was elevated in all thyrotoxic patients studied (Figure 3.25). Of particular interest was the elevated rT_3/T_4 ratio in both these groups. rT_3 has been reported to be elevated in a number of non-thyroidal systemic diseases (Chopra et al., 1975a; Chapter 6 (6.3). The non-specific nature of these elevations suggests that the elevated rT_3/T_4 seen in the patients with thyroid disease may have been a result of

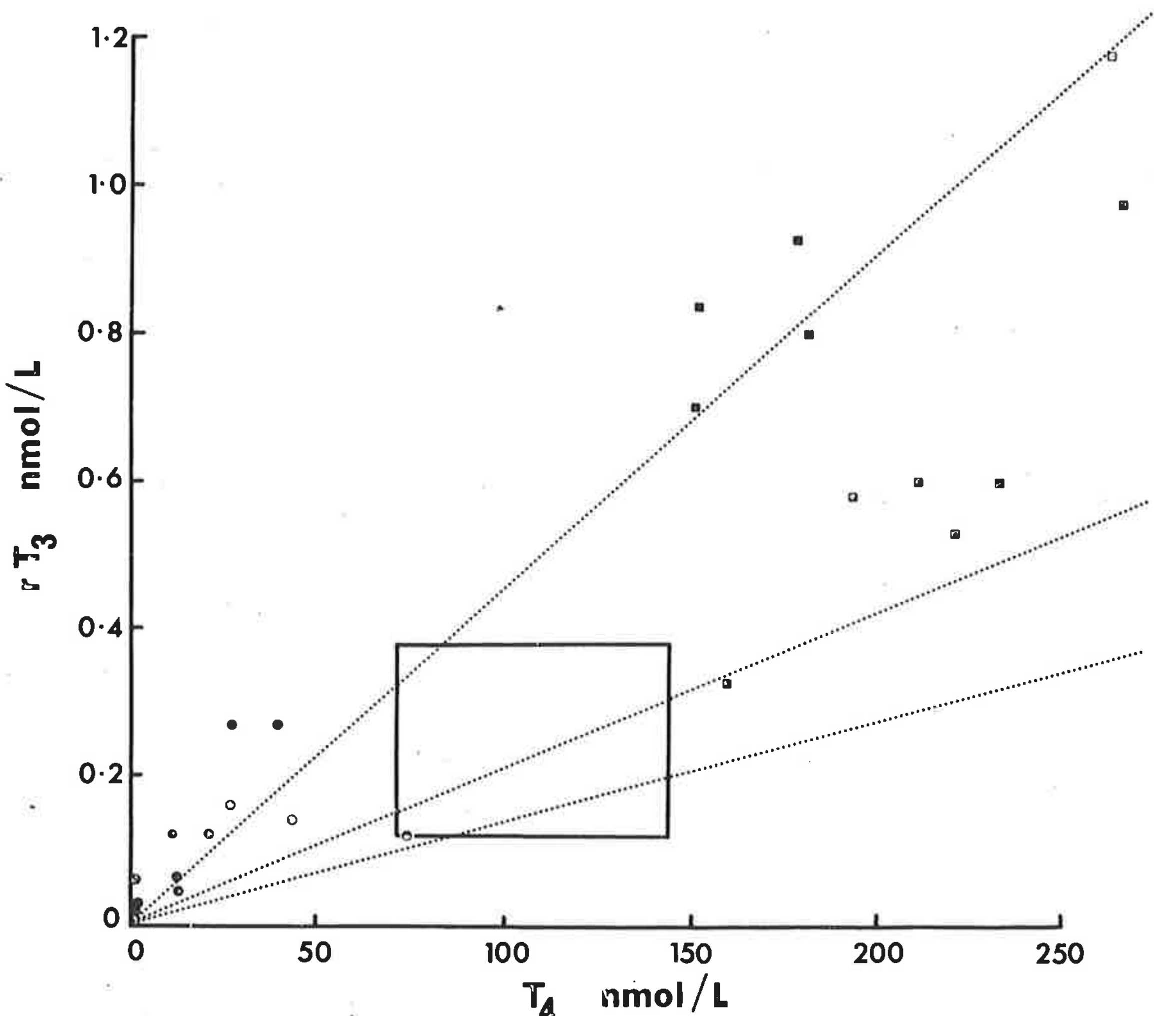
Figure 3.25 Reverse T_3 and T_4 concentration in thyroid disease

●	Hypothyroid subject
■	Thyrotoxic subject
....	represents the mean normal euthyroid T_4/rT_3 (465) and the 95% range (219 - 714).
——	represents the normal euthyroid rT_3 (0.12 - 0.38 nmol/L) and T_4 (60 - 144 nmol/L) 95% ranges.

Reference Ranges

(nmol/L rT_3 - 95% ranges)

	Uncorrected for T_4 cross-reaction	Corrected for T_4 cross-reaction
Healthy euthyroid	0.17 - 0.44	0.12 - 0.38
Oral contraceptives	0.26 - 0.57	0.19 - 0.48
Pregnancy	0.32 - 0.73	0.25 - 0.62


1·2
1·0
0·8
0·6
0·4
0·2
0
r T3 nmol/L
0
50
100
150
200
250
T4 nmol/L

systemic illness secondary to the thyroid disease.

3.9 VALIDATION OF THE DATA

The earliest rT_3 assays reported showed considerable disagreement in the estimates of mean euthyroid rT_3 concentration; the discrepancies did not appear related to the use of extraction or direct assay techniques (Table 2.1). Including recent publications, there is a six fold range in reported rT_3 concentration varying from 0.16 to 0.93 nmol/L; the data presented in this study agree with a number of assays reporting concentrations clustered in the 0.16 to 0.41 nmol/L region.

Although a variety of standards have been used, there was no clear association between the standard employed and the rT_3 concentration estimation. There has been considerable variation in reported T_4 cross-reactivity with the antisera used, with corrections being made in those assays in which cross-reactivity was greater than 0.10%. Again, however, there appeared to be no clear indication that the discrepancies in reported concentrations were due to variations in antibody specificity.

It was decided to compare the results obtained by the direct assay with those using an extraction technique; to evaluate the differences between four available rT_3 standards; and to assess whether the simple correction for T_4 cross-reaction was a valid approach to this problem.

MATERIALS AND METHODS

1. Extraction of Serum rT_3

The method of Chopra (1974) was employed. The standard, L-rT_3, was added to 1 mL aliquots of thyroid hormone free serum to give a 0.02 - 2.56 nmol/L concentration range. These samples were incubated at room temperature for 30 min and, along with the sera to be assayed, were mixed with 2 mL ethanol for 15 s and the precipitated protein pelleted at 2000 rpm for 10 min. Aliquots (300 μL) of supernatant were transferred to duplicate assay tubes and dried under nitrogen. The dried extracts were solubilized with 50 μL of 0.025 M NaOH and assayed as outlined in the protocol of Table 3.2 with the exception that thyroid hormone free serum was not present.

Recoveries were measured by supplementing the endogenous serum rT_3 concentration with 0.40, 0.80 and 1.20 nmol/L rT_3. The samples were equilibrated at room temperature for 30 min to ensure adequate exchange between the endogenous and exogenous rT_3.

2. Comparison of Standards

Two additional samples of L-rT_3 were kindly donated by the Washington Reference Laboratory (U.S.A.) and Paul Block of River Research (U.S.A.). For comparison with the Henning standard, stock solutions were prepared and stored in 0.025 M NaOH-ethanol (1:1) and diluted to give the standards 0.02 - 2.56 nmol/L on the day of use. Two serum samples were assayed as 10 replicates. The protocol in

this and the following studies was that outlined in Table 3.2.

3. Investigation of T_4 Cross-Reaction

Displacement of ^{125}I-rT_3 by 75 - 500 nmol/L T_4 was assessed in thyroid hormone free serum in the presence of 0, 0.15 and 0.45 nmol/L rT_3. These were compared to displacement of tracer by rT_3 of concentration 0.02 - 0.32 nmol/L.

In a separate experiment rT_3 was assayed in the presence of excess T_4. To reduce the variation in ^{125}I-rT_3 displacement by varying T_4 concentration between samples, rT_3 standard curves were assayed in the presence of 0, 500 and 1000 nmol/L T_4.

RESULTS AND DISCUSSION

1. Extraction Assay

There was no significant difference between the rT_3 concentration measured in the direct and extraction assays (Table 3.5). Even after correction for the low recovery in the latter assay, the results were well within the euthyroid range determined by the direct assay.

2. Standards

Both the normal and high rT_3 serum pools gave lower estimates when assayed with the Washington Reference Laboratory standard (Table 3.6). Although this was significant at only the higher rT_3 concentration, it was concluded that this standard had higher potency than the others tested.

TABLE 3.5 Comparison of extraction and direct assay

Serum pool	Concentration (nmol/L)	Percent recovery 0.40	0.80 (nmol/L added)	1.20
		Extraction Assay		
1	0.19 ± 0.04	78	71	83
2	0.19 ± 0.03	70	69	84
		Direct Assay		
1	0.21 ± 0.04	103	125	133
2	0.15 ± 0.02	100	126	109

TABLE 3.6 Comparison of rT_3 standards

Standard	rT_3 Concentration (nmol/L)	
	Serum pool	
	12	13
Little River	0.14 ± 0.02	0.43 ± 0.07
Washington Reference Laboratory	[a]0.11 ± 0.02	0.39 ± 0.07
Henning	0.16 ± 0.02	0.45 ± 0.07
Warner Lambert	0.15 ± 0.02	[b]0.53 ± 0.09

		P
a	vs Little River	0.015
	vs Henning	0.003
	vs Warner Lambert	0.004
b	vs Little River	0.034
	vs Henning	N.S.
	vs Washington Reference Laboratory	0.004

Similarly the Warner-Lambert standard was concluded to have lower potency.

Despite these differences, the results do not account for the discrepancies seen in reported rT_3 levels. In a comparison of the Washington Reference Laboratory, Henning and Jorgensen standards, Premachandra (1978) found the Reference standard to be of slightly lower potency than that of Henning's. However, the Jorgensen standard was of much lower potency, an observation which corroborates the high levels measured in all three assays using this standard.

Burrows, Cooper, Shakespear, Aickin, Fraser, Hesch and Burke (1977) reported that the Warner-Lambert standard had 0.52 the potency of Henning's and Chopra (1974) obtained high rT_3 estimates using this standard. That is, in those studies, the Warner-Lambert standard was of much lower potency than observed in this study in which the potency was 0.85 that of the Henning standard as assessed from the concentration of the high serum pool.

These differences in potency estimates highlight the problems in making an accurate determination of rT_3 concentration. Even the most frequently used standard (Henning) has been employed in assays reporting both low and high concentration of rT_3. Several publications in which the Cahnmann standard has been used may clarify the problem. Most estimates using this standard have been high and Roti, Robuschi, Bandini, Emanuele and Gnudis (1979) have recently shown this standard to give much higher estimates of rT_3 than when using the Henning standard. In contrast, Kaplan,

Schimmel and Utiger (1977) reported lower rT_3 concentrations using this standard and an antiserum with low T_4 cross-reaction. They did find however that estimates were from 2 - 10 times higher when using antisera of higher T_4 cross-reaction (about 0.10%). Similarly, Eisenstein et al. (1978) have reported different estimates of rT_3 concentration using this standard but different antisera. Of particular importance was their observation that the antiserum with slightly lower T_4 cross-reaction (0.060% at 50% displacement of tracer) actually gave nearly double the estimates of rT_3 than an antiserum of 0.080% T_4 cross-reaction. The conclusion is that the discrepancies are not simply related to differences in specificity as assessed by affinity for T_4. This is supported by the data of Kaplan et al. (1977) showing that the rT_3 estimates using some antisera were far in excess of that which could be accounted for by T_4 cross-reaction. Furthermore, even the uncorrected rT_3 levels in this study, in which an antiserum with T_4 cross-reaction nearly as high as any reported was used, were well below the higher estimates reported (some of which were made using antisera of very low T_4 affinity).

Although there are potency differences between standards, as has been pointed out by Premachandra (1978) in a review of earlier papers, the major problem would appear to be differences between antisera. There may be differences in antibody specificity for substances in serum which are iodothyronines and iodotyrosines, or which have not been characterized. The former possibility is unlikely as in a number of reports the antisera specificity have been well

characterized.

An observation pertinent to this problem has recently been reported by Grussendorf and Hufner (1980) in the assay of 3',5'-T_2. Although the antiserum used cross-reacted with T_4 (0.01%), T_3 (0.02%) and rT_3 (1.2%), calculations suggested these could account for only a small fraction of the measured 3',5'-T_2 concentrations. However, when sera were immunoextracted with antisera to T_4, T_3 and rT_3 and then assayed, there was no detectable 3',5'-T_2. This would suggest that together the iodothyronines cross-react to a greater extent than estimated by measuring displacement of tracer with only the cross-reactant present, or that there are other uncharacterized cross-reactants (which have been extracted in the immunoextraction procedure).

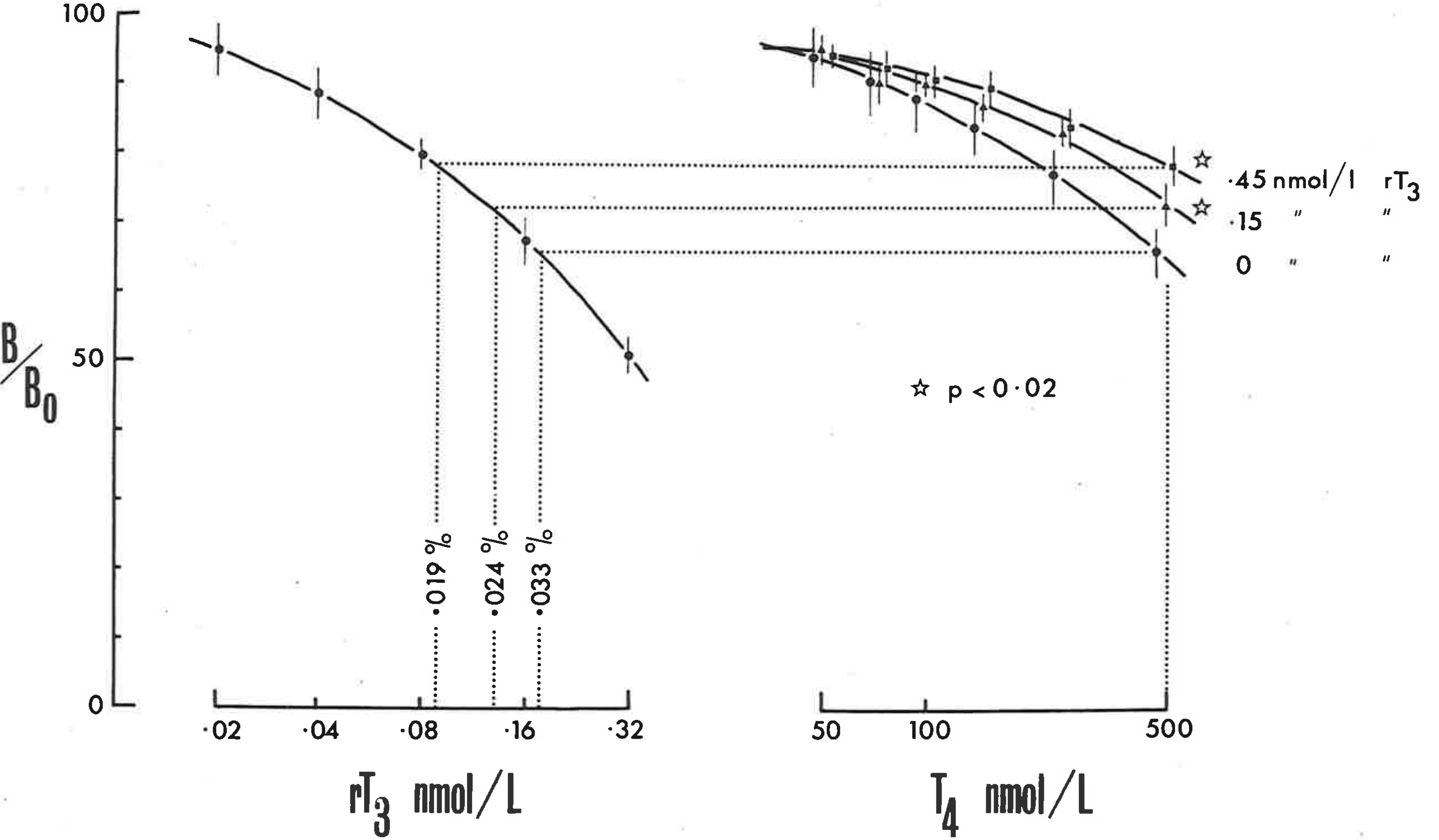
3. Antiserum Specificity

Cross-reaction by lower physiological concentrations of T_4 was not significantly altered by the presence of euthyroid (0.15 nmol/L) or elevated (0.45 nmol/L) levels of rT_3 (Figure 3.26). There was however a tendency to lower cross-reaction with increasing concentration of rT_3 present, which became significant when assessing cross-reaction by 500 nmol/L. These results suggested that correction for T_4 cross-reaction by using a T_4 dose response curve in thyroid hormone free serum in the absence of rT_3 was a valid approach. Furthermore, the potency of cross-reacting species tended to be lower in the presence of the ligand for which the antiserum had highest affinity.

Figure 3.26 Cross-reaction of T_4 at various concentrations of rT_3

T_4 Concentration (nmol/L)	Percent T_4 cross-reaction ± 1 S.D.		
	rT_3 Concentration (nmol/L)		
	0	0.15	0.45
50	.057 ± .035	.052 ± .017	.053 ± .018
75	.051 ± .026	.041 ± .012	.043 ± .015
100	.047 ± .021	.039 ± .009	.035 ± .014
150	.044 ± .017	.033 ± .007	.030 ± .010
200	.041 ± .014	.030 ± .007	.028 ± .008
500	.033 ± .006	[a].024 ± .006	[a].019 ± .005

[a] $P < .02$


100
50
0
B/B0
·02
·04
·08
·16
·32
rT3 nmol/L
50
100
500
T4 nmol/L
·019 %
·024 %
·033 %
·45 nmol/l rT3
·15 " "
0 " "
☆ p < 0·02

Assay of rT_3 in the presence of excess T_4 markedly diminished T_4 cross-reaction to very low or negligible levels (Figure 3.27). The particular advantages of this technique are that if cross-reaction is due to the presence of a second binding site, the presence of cross-reactant will tend to block these sites reducing interaction with the tracer (Feldman and Rodbard, 1972); where only one common binding site is present, the presence of excess cross-reactant smooths out the variation in endogenous cross-reactant concentration from sample to sample. However, B_o and sensitivity were markedly reduced making the assays impractical for routine use.

3.10 SUMMARY

A direct assay for the measurement of 3,3',5'-T_3 in serum has been developed using the principles developed for the radioimmunoassay of the thyroid hormones T_3 and T_4. The assay employs sodium 8-anilino-1-naphthalene sulphonate to block iodothyronine binding to the serum thyronine binding proteins.

Use of the simplex technique for optimising chemical systems led to the development of an assay with a precision profile comparable to the empirically derived assay. This assay had a B_o of 9.7% which was considerably lower than 30% B_o of the empirically developed assay. It is concluded that the simplex technique does offer a valid and objective alternative to empirical methods in optimising assay sensitivity and precision.

The double antibody technique is used to separate

Figure 3.27 Assay of rT_3 in the presence of excess T_4

(i) Percent T_4 cross-reaction ± 1 S.D.

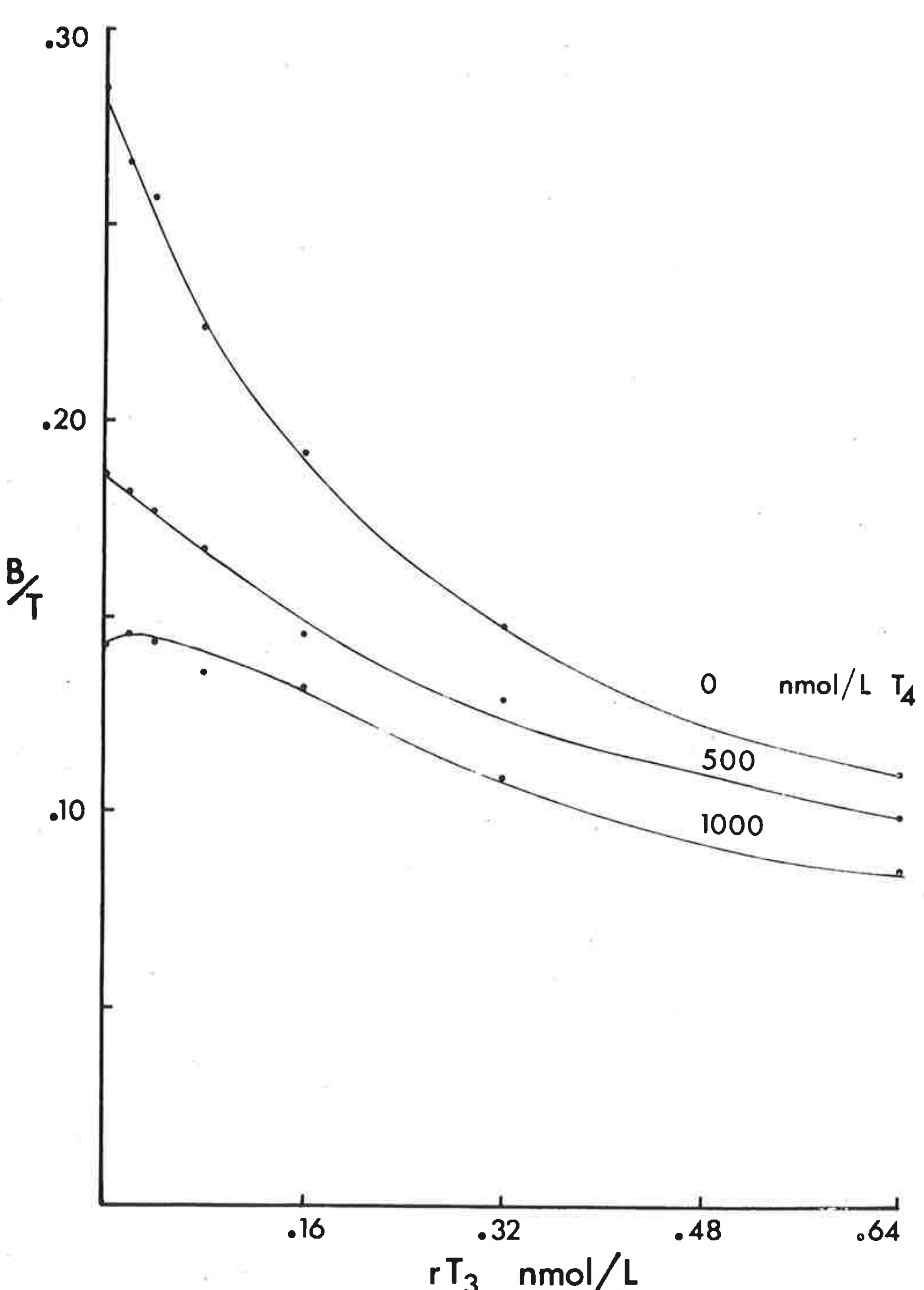
T_4 Concentration (nmol/L)	Excess T_4 concentration (nmol/L)		
	0	500	1000
100	.043 ± .007	[a].016 ± .005	< .010
150	.041 ± .005	[a].019 ± .001	< .007
200	.041 ± .005	[a].025 ± .004	< .005
500	.035 ± .006	[a].027 ± .005	[a].016 ± .005

[a] $P < .01$ (compared to cross-reaction in the absence of added T_4)

(ii) Serum pool rT_3 concentrations (nmol/L) ± 1 S.D. assayed in the presence of excess T_4

Serum pool	Exogenous T_4 concentration (nmol/L)		
	0	500	1000
Low	.17 ± .05	.17 ± .03	[b].27 ± .10
Medium	.38 ± .04	.42 ± .04	[b].52 ± .08
High	.48 ± .06	.49 ± .03	.57 ± .16

[b] $P < .001$ (compared to estimate in presence of 0 and 500 nmol/L T_4)


.30
.20
.10
B/T
0 nmol/L T_4
500
1000
.16
.32
.48
.64
rT_3 nmol/L

free and antibody bound rT_3. Use of the charcoal separation technique led to unexpected discrepancies in displacement of tracer between charcoal extracted and unreacted serum.

Cross-reaction by T_4 was significant and was corrected for by including a T_4 displacement curve with each assay. Cross-reaction by T_4 was unaffected by physiological variations in serum rT_3 concentration. The use of excess T_4 in the assay tubes to reduce the T_4 cross-reaction reduced sensitivity and was considered unsatisfactory for routine analytical purposes.

Four preparations of rT_3 standard were compared. Although these had different potencies, the differences were not of a magnitude which would account for the variation in published serum rT_3 concentrations in euthyroid individuals. It was concluded from a review of the literature that these discrepancies may be related more to the differences in antiserum used.

The measurement of serum rT_3 would appear of little use in the diagnosis of thyroid disease. The concentration of rT_3 relative to T_4 tended to increase in thyroid illness, particularly in hypothyroid patients, resulting in considerable overlap between the hypothyroid and euthyroid ranges. An assessment of the value of measuring serum rT_3 in the diagnosis of thyroid disease in patients with severe non-thyroidal illness is made in Chapter 6 (6.3).

CHAPTER 4

PRINCIPLES OF FREE THYROID HORMONE MEASUREMENT

4.1 A CHALLENGING ANALYTICAL PROBLEM

By demonstrating that serum protein bound $^{131}I\text{-}T_4$ was readily exchangeable with added unlabelled T_4 *in vitro*, Albright et al. (1955) conclusively established the reversible nature of T_4 binding to the serum thyroid hormone binding proteins, albumin and thyroxine binding protein. In keeping with Scatchard's (1949) model of the reversible binding of small molecules and serum proteins, it was concluded that unbound or free T_4 was in dynamic equilibrium with T_4 bound to the serum binding proteins.

This equilibrium was described by the law of mass action,

$$T_4 + \text{Albumin} + \text{TBP} \rightleftarrows T_4\bullet\text{Albumin} + T_4\bullet\text{TBP} \qquad (4.1)$$

where T_4 represents free T_4, Albumin and TBP represent unoccupied T_4 binding sites on albumin and thyroxine binding protein respectively, and $T_4\bullet$Albumin and $T_4\bullet$TBP represent occupied T_4 binding sites. The following relationships were derived from the law of mass action,

$$K_1 = \frac{[T_4\bullet\text{TBP}]}{[T_4][\text{TBP}]}$$

$$K_2 = \frac{[T_4\bullet\text{Albumin}]}{[T_4][\text{Albumin}]}$$

Where K_1 and K_2 are the association constants and [ ] represents the concentrations of the species in equation 4.1.

From the electrophoretic measurements of the albumin and thyroxine binding protein binding capacities, using the association constant for the binding of T_4 to bovine serum albumin as an approximation of K_2, and using an empirically calculated value for the association constant of binding of T_4 to TBP (K_1), Robbins and Rall (1957) calculated fT_4 concentrations of 30 - 90 pmol/L in nine healthy euthyroid subjects. Moreover, calculated fT_4 concentrations during pregnancy and ingestion of oral contraceptives were within this euthyroid range.

This data was consistent with the hypothesis of Recant and Riggs (1952) that the free hormone was the physiologically important moiety and provided considerable motivation for the development of suitable analytical techniques to measure fT_4 concentration.

Ingbar and Freinkel (1960) subsequently identified a third circulating T_4 binding protein, thyroxine binding prealbumin (TBPA), and demonstrated the binding of T_4 to cellular components. Assuming that free T_4 was rapidly exchanged between the extracellular and intracellular compartments, the equilibrium between free T_4 and protein bound T_4 was more accurately described by,

$$T_4 + TBG + TBPA + Albumin + B_{ic} \quad \downarrow\uparrow \quad T_4{\bullet}TBG + T_4{\bullet}TBPA + T_4{\bullet}Albumin + T_4{\bullet}B_{ic} \qquad (4.2)$$

where B_{ic} and $T_4{\bullet}B_{ic}$ represent unoccupied and occupied cellular T_4 binding sites respectively.

The measurement of circulating fT_4 concentration posed a very challenging analytical problem.

Firstly, there was the problem of measuring the free species without disturbing the equilibrium to such an extent that the measurement was inaccurate.

Secondly, new methods would have to be devised to measure the very low concentrations of fT_4. The iodine-estimation procedures commonly used to measure circulating total T_4 were too insensitive to measure fT_4 concentrations in the range 30 - 90 pmol/L.

Thirdly, there was the problem of how accurately the determination of fT_4 *in vitro* reflected the concentration *in vivo*. The measurement *in vitro* reflected the equilibrium between T_4 and the circulating binding proteins only. Furthermore, it was possible that transfer of hormone from the circulation to the intracellular compartment was so rapid in some tissues that the free hormone concentration might be very low in the blood vessels passing through that organ. In other tissues with a slower uptake (and metabolism) of the hormone, the system (equation 4.2) may be at equilibrium. In this latter case, leaving aside the question of intracellular binders, the free hormone concentration *in vitro* may reflect that *in vivo*. At the time there was no information

available to allow a resolution of these problems and it was assumed that the measurement *in vitro* was a useful approximation of the concentration *in vivo*. (This point is discussed in Chapter 5 (5.4)).

Christensen (1959) published the first semi-quantitative determination of fT_4 concentration. He proposed that if $^{131}I\text{-}T_4$ was added to serum and allowed to come to equilibrium with the endogenous T_4, then the following relationship should hold,

$$\frac{[^{131}I\text{-}T_4]}{[^{131}I\text{-}T_4 \bullet TBP]} = \frac{[T_4]}{[T_4 \bullet TBP]}$$

where $[^{131}I\text{-}T_4]$ represents the concentration of labelled free T_4 and $^{131}I\text{-}T_4 \bullet TBP$ represents the concentration of thyroxine binding sites occupied by labelled T_4.

Rearranging,

$$[T_4] = \frac{[^{131}I\text{-}T_4]}{[^{131}I\text{-}T_4 \bullet TBP]} \cdot [T_4 \bullet TBP] \qquad (4.3)$$

T_4 was known to be very strongly bound to the circulating thyroxine binding proteins, a point which Robbins and Rall's (1957) very low estimate of fT_4 concentration bore out. Thus, as a first approximation,

$$[T_4 \bullet TBP] \simeq [\text{total circulating } T_4] = [PBI]$$

and similarly,

$$[^{131}I\text{-}T_4 \bullet TBP] \simeq [\text{total circulating } ^{131}I\text{-}T_4]$$

Inserting into equation 4.3,

$$[T_4] = \frac{[^{131}I\text{-}T_4]}{[\text{total } ^{131}I\text{-}T_4]} \cdot [PBI] \qquad (4.4)$$

Christensen (1959) proposed that by using equilibrium dialysis, [$^{131}I-T_4$]/[total $^{131}I-T_4$] could be measured and the [T_4] calculated. However, Robbins and Rall's (1957) calculation of fT_4 concentration of 30 - 90 pmol/L suggested that [$^{131}I-T_4$]/[total $^{131}I-T_4$] would be in the range 0.0003 - 0.0006. That is, the percent free radiothyroxine would be approximately 0.03 - 0.06%.

Christensen considered that it was too difficult to measure such a low value of the [$^{131}I-T_4$]/[total $^{131}I-T_4$] precisely. Instead, he equilibrated $^{131}I-T_4$ with a sample of serum and then, within a dialysis cell, measured the rate at which $^{131}I-T_4$ passed from this serum through a dialysis membrane and into a sample of the same serum containing no added $^{131}I-T_4$. The rate of passage of $^{131}I-T_4$ through the membrane was assumed to be proportional to the fT_4 concentration in the dialysand. The fraction of $^{131}I-T_4$ which passed through the membrane in 24 hours was thus proportional to [$^{131}I-T_4$]/[total $^{131}I-T_4$] in the dialysand. Using this measured fraction a value for [T_4] could be calculated (equation 4.4) which, although not an accurate measure, was considered proportional to the fT_4 concentration *in vitro*.

By using the same serum sample as both dialysate and dialysand, Christensen overcame the problem of differences in osmolality between the two compartments and Gibbs-Donnan effects. Furthermore, in this system the $^{131}I-T_4$ passing the membrane was considerably greater as a percent of the [total $^{131}I-T_4$] than was the [$^{131}I-T_4$]/[$^{131}I-T_4 \bullet TBP$] in the dialysand. This permitted greater precision in calculating [T_4].

In 1962, Sterling and Hegedus published the first experimental quantitative estimation of fT_4 concentration. By also using dialysis to separate free and bound T_4, and $^{131}I\text{-}T_4$ to measure the fraction free T_4, they attempted to establish the absolute concentration of fT_4. To do this, serum containing $^{131}I\text{-}T_4$ was dialyzed against an equal volume of 0.15 M phosphate buffer, pH 7.4. After allowing the system to come to equilibrium at 37°C, samples were taken from both the dialysand and dialysate and counted to determine $[^{131}I\text{-}T_4]/[\text{total } ^{131}I\text{-}T_4]$. To overcome interference by free ^{131}I-iodide in the dialysate, $^{131}I\text{-}T_4$ was extracted from the dialysate sample on a cation exchange resin prior to counting.

Free $^{131}I\text{-}T_4$ in euthyroid subjects without thyroxine binding protein abnormalities was $0.11 \pm 0.011\%$ of the total $^{131}I\text{-}T_4$. This was similar to that predicted from Robbins and Rall's (1957) calculations and suggested that the method gave plausible results. Free T_4 concentration was calculated using the PBI (equation 4.4). The validity of the data was supported by the correlation between fT_4 concentration and clinical status (see Chapter 1, Free Thyroxine Hypothesis). This correlation highlighted the importance of developing techniques which simply and accurately measured fT_4 concentration.

4.2 ESTIMATION OF FREE THYROID HORMONE CONCENTRATION BY MEASUREMENT OF THE FREE HORMONE FRACTION

SEPARATION OF FREE AND PROTEIN BOUND HORMONE

1. Equilibrium Dialysis

Equilibrium dialysis appeared to be the method of choice for measuring the concentration of free hormone as the free and bound species were not physically separated during this procedure. None the less, it was important to know to what extent the technique altered the free hormone concentration. An analysis is presented below which determines the extent to which the equilibrium dialysis technique alters the free hormone concentration.

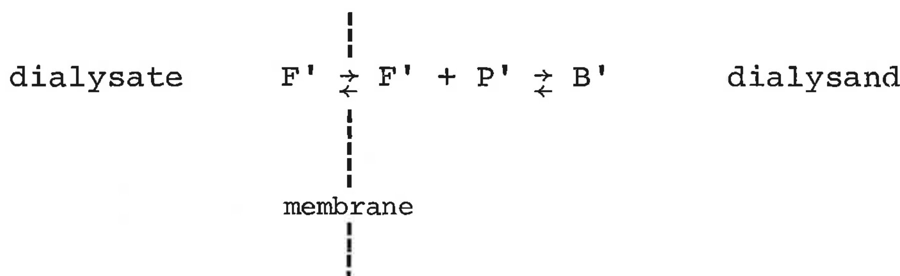
This analysis considers the general case of a ligand binding to a circulating binding protein P. Then,

$$F + P \rightleftarrows B$$

where [F] is the concentration of free hormone, [B] is the concentration of bound hormone and [P] is the concentration of unoccupied protein binding sites. During dialysis of serum, the membrane is freely permeable to the free hormone, but is impermeable to the binding protein. A new equilibrium will be established in the serum:

$$F' + P' \rightleftarrows B'$$

Note that neither the equilibrium constant, nor the total concentration of binding protein, $[P_o]$, will alter. The concentration of free hormone in the dialysate will also be [F'] and the complete system may be described as:



The solution for the concentration of free hormone, [F'], in the dialysate is derived in the appendix (equation A4).

Solving equation A4 for the concentration of fT_4, $[T_4]$, when only the binding of T_4 to TBG is considered,

$$[T_4]^2 \left[K(1+\frac{V_D}{V_S}) \right] + [T_4] \left[(1+\frac{V_D}{V_S}) + K[TBG_O] - K[TT_4] \right] - [TT_4] = 0$$

where $[TBG_O]$ is the concentration of circulating TBG and $[TT_4]$ is the concentration of total circulating T_4. Knowing the equilibrium constant K, the total concentration of binding protein $[TBG_O]$ and the total concentration of hormone $[TT_4]$, the free hormone concentration $[T_4]$ at any ratio of dialysate to serum (V_D/V_S) may be calculated.

Table 4.2 shows the effect of dialysis on the fT_4 and fT_3 concentrations and, for comparison, the effect of dialysis on a weakly bound hormone, cortisol. For strongly bound ligands such as T_4, the concentration of free ligand is not substantially affected by ratios of dialysate to dialysand volume up to 500. On the other hand, the free concentration of the more weakly bound ligand, T_3, falls significantly where the volume of dialysate is greater than 50 times that of the dialysand. The maximum V_D/V_S which does not cause substantial reduction in free cortisol concentration is even lower. That is, care must be taken to define the limit of V_D/V_S for each ligand to be measured.

Where the free hormone fraction is used to determine the concentration of free hormone, the effect of dilution is not as pronounced (Table 4.2). This can be explained

by the movement of the labelled hormone from the dialysand to the dialysate as the relative volume of dialysate is increased. The fall in absolute free hormone concentration with increasing V_D/V_S is observed as a fall in the activity of labelled hormone per unit volume in the dialysate. However, when calculating, for example the free T_4 fraction,

$$\text{free } T_4 \text{ fraction} = \frac{\text{counts/unit volume dialysate}}{\text{counts/unit volume dialysand}}$$

the activity in the dialysand is also reduced with increasing V_D/V_S, compensating for the fall in absolute free hormone concentration. For both T_3 and T_4 very high values of V_D/V_S can be tolerated without affecting the final calculation of free hormone concentration.

The appendix also outlines the solution for fT_4 taking into consideration binding to TBG, TBPA and albumin (equation A6). Although K_{TBPA} is 100 times less than that of K_{TBG}, TBPA is present in the circulation at 10 times the concentration of TBG and accounts for about a quarter of the bound circulating T_4. As shown in Table 4.2, the effects of moderate dilution are little different when considering binding to three binding proteins.

Oppenheimer and Surks (1964) considered the effects of dilution on the dialyzable fraction of T_4 and also showed no significant change up to a dilution of 1/150. Their solution for the concentration of fT_4, whilst derived from the law of mass action, involved several simplifications which restricted the analysis to only T_4 binding. The general solution offered in equation A6 allows the analysis of ligand binding with a maximum of three proteins.

TABLE 4.1 Data used for the solution of equations A4, A6, A9 and A10

Solutions for these equations are tabulated in Tables 4.2 and 4.4

(i) General

$[TBG_o]$	$[p_o]$	4.3×10^{-7}	mole/L	McDowell (1979)
$[TBPA_o]$	$[q_o]$	2.2×10^{-6}	mole/L	Oppeheimer et al. (1965)
$[alb_o]$	$[r_o]$	6.0×10^{-4}	mole/L	laboratory

(ii) T_4 binding

K_{TBG}	K_1	1.7×10^{10}	L/mole	Woeber and Ingbar (1968)
K_{TBPA}	K_2	2.3×10^{8}	L/mole	Woeber and Ingbar (1968)
K_{alb}	K_3	2.5×10^{6}	L/mole	Tritsch (1968)
$[TT_4]$	$[T]$	100×10^{-9}	mole/L	laboratory

(iii) T_3 binding

K_{TBG}	K_1	1.8×10^{9}	L/mole	Snyder et al. (1976)
K_{TBPA}	K_2	1.6×10^{7}	L/mole	Cheng et al. (1977)
K_{alb}	K_3	1.1×10^{6}	L/mole	Tabachnik and Giorgio (1964)
$[TT_3]$	$[T]$	2.0×10^{-9}	mole/L	laboratory

TABLE 4.2 Effect of serum dilution on free hormone concentration during equilibrium dialysis

(i) Free T_4:

$\frac{V_D}{V_S}$	Binding to TBG only - equation A4				Binding to TBG, TBPA and albumin - equation A6			
	[F'] (pmol/L)	[F']/[F]	D'	D'/D	[F'] (pmol/L)	[F']/[F]	D'	D'/D
0	[F] 17.82	1.000	[D] .0178	1.000	[F] 12.44	1.000	[D] .012	1.000
1	17.82	1.000	.0178	1.000	12.44	1.000	.012	1.000
5	17.80	0.999	.0178	1.000	12.43	0.999	.012	1.000
10	17.78	0.998	.0178	1.000	12.42	0.998	.012	1.000
25	17.72	0.994	.0178	1.000	12.39	0.996	.012	1.000
50	17.62	0.989	.0178	1.000	12.35	0.993	.012	1.000
250	16.86	0.946	.0176	0.990	12.01	0.965	.012	0.999
500	16.01	0.898	.0174	0.979	11.61	0.934	.012	0.995
5000	8.80	0.494	.0157	0.883	7.41	0.600	.012	0.991
							.012	0.946

Note - when V_D = 0, V_D/V_S = 0 and [F'] = [F]

TABLE 4.2 (continued)

(ii) Free T_3

$\frac{V_D}{V_S}$	Binding to TBG only - equation A4				Binding to TBG, TBPA and albumin - equation A6			
	Free T_3 [F'] (pmol/L)	[F']/[F]	D'	D'/D	free T_3 [F'] (pmol/L)	[F']/[F]	D'	D'/D
0	(F) 3.36	1.000	(D) .168	1.000	(F) 1.47	1.000	(D) .074	1.00
1	3.36	.998	.168	1.000	1.47	1.000	.074	1.00
5	3.33	.992	.168	1.000	1.47	0.997	.074	1.00
10	3.31	.984	.168	1.000	1.46	0.993	.074	1.00
25	3.23	.960	.168	1.000	1.45	0.982	.074	1.00
50	3.10	.922	.168	1.000	1.42	0.965	.074	1.00
250	2.36	.703	.167	0.995	1.24	0.844	.073	1.00
500	1.82	.543	.167	0.992	1.08	0.731	.073	1.00
5000	0.36	.106	.180	1.070	0.31	0.213	.069	0.94

TABLE 4.2 (continued)

(iii) Free cortisol - equation A4

$[CBG_o]$ = 1.35×10^{-6} mol/L laboratory

K_{CBG} = 2.5×10^{8} L/mol (Stroupe et al., 1978)

$[T]$ = 550×10^{-9} mol/L laboratory

V_D/V_S	free cortisol [F'] (nmol/L)	[F']/[F]	D'	D'/D
0	(F) 2.74	1.000	(D) .498	1.000
1	2.70	.986	.493	.990
5	2.62	.956	.488	.980
10	2.52	.921	.480	.964
25	2.28	.833	.462	.928
50	1.99	.726	.442	.887
250	1.06	.388	.372	.747
500	0.70	.255	.350	.703
5000	0.10	.038	.200	.401

2. Factors Affecting Thyroid Hormone Binding to Serum Binding Proteins

Oppenheimer, Squef, Surks and Hauer (1963) not only proposed that equilibrium dialysis was the method of choice for separating free and bound T_4 under equilibrium conditions, but also suggested dialysis of diluted serum offered particular advantages. Thus variations in the volumes of the two dialysis compartments due to osmotic and Gibbs-Donnan effects were reduced, a much smaller volume of serum could be used for analysis, and the dialyzable fraction increased linearly with dilution. As only low specific activity tracer was available, there was good reason for increasing the dialyzable fraction in order to ensure sufficient counts in the dialysate and so increase precision of dialysate counting. Furthermore, dilution of serum was found to reduce the problem of labelled iodide contamination in the dialysate as the proportion of T_4 to iodide in the dialysate increased with increasing dialyzable fraction (Oppenheimer and Surks, 1964).

Although the theoretical studies indicate that no significant alteration in free hormone concentration would be expected on diluting serum, it was found that dilution of serum by as little as 1:1 in 0.15 M phosphate buffer, pH 7.4 or in 0.16 M tris buffer, pH 7.35 caused a dramatic drop of estimated free T_4 concentration (Ingbar et al., 1965; Sterling and Brenner, 1966; Spaulding and Gregerman, 1972; Pedersen, 1974). Sterling and Brenner (1966) demonstrated that the percent dialyzable T_4 was unaffected when serum was diluted in, and dialyzed against its own ultra-

filtrate, and suggested that the use of unphysiologic buffers caused the dilution problem. Spaulding and Gregerman (1972) studied this problem in some detail and concluded that free T_4 concentration was proportional to chloride concentration. Thus, dilution in tris or phosphate buffer, by diluting the endogenous chloride, caused a fall in the dialyzable fraction. They also found it important to assess the inhibitory action of the buffer itself. Barbital buffer caused a marked increase in the dialyzable fraction, phosphate buffer caused a small increase at concentrations between 0.05 M to 0.15 M and 0.01 M Hepes appeared not to affect binding of T_4 to thyroid binding proteins at all. In fact, the dialyzable fraction was estimated to be 0.009% when serum was dialyzed against 0.01 M Hepes buffer. Although Schussler and Plager (1967) claimed that the dilution effect could be overcome by careful attention to purification of the tracer, Pedersen (1974) later showed this not to be sufficient to explain the effect, and in agreement with the foregoing studies concluded that other factors, most likely the diluting buffers, were the problem.

3. Other Separation Techniques

Although equilibrium dialysis would seem to have been the most satisfactory technique for separating free and TBP bound T_4, considerable incubation times were required to ensure equilibrium was reached. In an attempt to shorten and simplify this aspect of free T_4 analysis, a number of separating techniques were used, including ultrafiltration (Oppenheimer and Surks, 1964; Sterling and Brenner, 1966), charcoal adsorption of free (Kumagai, Jubiz and

Jessop, 1967), Sephadex G-25 adsorption of free under equilibrium conditions (Irvine, 1974), Sephadex G-25 filtration (Lee et al., 1964) and polyacrylamide gel filtration (McDonald, Robin and Siegel, 1978). In general, the filtration methods do tend to give higher estimates of free T_4 fraction, as might be expected when attempting to physically separate two species in equilibrium, the bound species having a very fast dissociation rate. In a comparison of equilibrium dialysis and polyacrylamide gel filtration, the filtration method estimate of free T_4 fraction was fifty times that of a dialysis method. Furthermore, the fraction free was linearly related to the quantity of gel used in the filtration method (McDonald et al., 1978).

THE USE OF RADIOLABELLED HORMONE TO DETERMINE THE FRACTION FREE HORMONE

Following the publications of Christensen (1959) and Sterling and Hegedus (1962) a considerable number of methods of analysis were published using a variety of techniques to separate free and bound T_4 and employing radioactively labelled T_4 to determine the fraction unbound. There was marked variation in the estimates of free T_4 fraction and fT_4 concentration and many reports attempted to investigate why variations existed between groups using the same technique and to refine the techniques accordingly. It appeared that a lot of interlaboratory and interassay variability could be explained by variable tracer quality.

1. Specific Activity

A major problem was that of tracer specific activity. It is only recently that high specific activity tracer has become available (Weeke and Orskov, 1973) and been used in the estimation of fraction free T_4 (McDonald et al., 1978). Thus most methods published have required the addition of large quantities of low specific activity ^{131}I-T_4 in order to ensure sufficient counts in the unbound fraction. The quantity of tracer added often increased endogenous T_4 concentration by 30 - 40% and would have certainly led to increases in estimated fraction free T_4. Claims that free T_4 fraction was not altered by adding these quantities of tracer (Sterling and Brenner, 1966) might possibly have been due to the insensitivity of the method inherent in using low specific activity tracer.

2. Contamination of Tracer with Iodide

A major problem with the available ^{131}I-T_4 preparations was inadequate purity. Although ^{131}I-iodide often accounted for less than 5% of the ^{131}I-T_4 activity, it could contribute at least half to the activity in the dialysate or ultrafiltrate due to its lower affinity for TBP. This problem necessitated the incorporation of procedures to separate labelled iodide and T_4 in the dialysate or ultrafiltrate which made free T_4 estimation even more time consuming and laborious. It was, in fact, by recognizing this problem and successfully extracting labelled T_4 from the dialysate that Sterling and Hegedus (1962) were able to report the first quantitative estimation of fT_4 concentration.

The clean up procedures included addition of plasma to an aliquot of dialysate and precipitation of the plasma protein bound T_4 with trichloroacetic acid (Oppenheimer et al., 1963), separation of iodide and T_4 on cation exchange resin or by paper chromatography (Sterling and Hegedus, 1962), filtration of dialysate through Sephadex G-25 with elution of iodide with buffer and subsequent elution of T_4 with serum (Liewendahl and Lamberg, 1965), addition of plasma to an aliquot of dialysate and dialysis of this mixture against buffer containing cation resin to remove iodide (Ingbar et al., 1965), addition of carrier T_4 to an aliquot of dialysate and precipitation with $MgCl_2$ (Sterling and Brenner, 1966) and selective adsorption of T_4 in polyacrylamide gel filtrate onto talc (McDonald et al., 1978).

A further strategy to reduce the effect of contaminating ^{131}I-iodide was the dialysis of diluted serum against a large volume of dialysate (Oppenheimer and Surks, 1964). Being only weakly bound to the serum proteins, iodide existed mainly in the free form. Thus, by diluting serum before dialysis little further increase in fraction free iodide would be expected while fraction free T_4 would rise. That is, by increasing serum dilution and V_D/V_S, the ratio of [^{131}I-T_4]/[^{131}I-iodide] in the dialysate could be raised.

3. Tracer Contamination Other than Iodide

In addition to iodide, T_3 and rT_3 were found to be contaminants of commercial ^{131}I-T_4 in amounts similar to contaminating iodide (Volpert, Martinez and Oppenheimer,

1967). Due to their lesser affinities for TBP, these tri-iodothyronines led to substantial errors in estimation of free T_4 fraction. Their recommendations of preliminary purification of $^{131}I-T_4$ were supported by Schussler and Plager (1967) who found estimates of fraction free T_4 to be considerably reduced when using purified tracer. Volpert et al. (1967) did find, however, that $^{125}I-T_4$ preparations were less contaminated with iodide, T_3 and rT_3, and suggested purification of the $^{125}I-T_4$ to be unnecessary. More recently Jiang and Tue (1977) have highlighted the problems of batch variation and ageing in commercial $^{125}I-T_4$ supplies. They did find estimates of free T_4 fraction to be lower when using freshly purified $^{125}I-T_4$, and that the estimate increased with ageing of the tracer if no further purification was carried out. Batch variation appeared to be independent of the ageing effect.

Although those purifying the tracer before use have in general reported lower fraction free T_4, the additional procedures of dialysate clean up and tracer prepurification have not in themselves resulted in estimates of free T_4 as low as those obtained by the more recent direct methods to be discussed.

MEASUREMENT OF FREE TRIIODOTHYRONINE CONCENTRATION

Measurement of free T_3 concentration has involved problems similar to those in measuring fT_4 concentration. The earliest report gave an estimate of the percent free T_3 only as no assay was available for total T_3 (Ingbar et al., 1965). The mean percent free T_3 in 12 healthy euthyroid

subjects was 0.48% and was considerably higher than the percent free T_4. Nauman et al. (1967) reported an assay for total circulating T_3 in which T_3 and T_4 in a serum extract were separated by gel filtration and the T_3 concentration determined by iodine analysis. By measuring the dialyzable fraction of ^{131}I-T_3 added to serum samples they were able to calculate a mean fT_3 concentration of 23.4 pmol/L in euthyroid subjects.

Although there was subsequent reasonable agreement on the measured percent free T_3, there was considerable variation in reported free T_3 concentrations. The differences were thought to be most likely due to difficulties in measuring circulating total T_3 either by iodine analysis, or more recently, by radioimmunoassay. Despite the apparent lower variation in estimation of percent free T_3, Pedersen (1974) stressed the requirement for standardized conditions as T_3 binding to TBP was found to be dependent on pH, temperature and chloride ion concentration just as was T_4 binding to TBP.

In summary, the gamut of methods developed reflects attempts to simplify the procedure and to overcome the variations in reported levels. Despite the continued variation in reported results, several groups found differing techniques of separation and dialysate or ultrafiltrate clean up to give similar results when performed by the same group (Schussler and Plager, 1967; Bird and Abiodun, 1973).

4.3 DIRECT MEASUREMENT OF FREE THYROID HORMONE CONCENTRATION *IN VITRO*

EQUILIBRIUM DIALYSIS-RADIOIMMUNOASSAY

Ellis and Ekins (1975) reported the first direct measurement *in vitro* of serum free T_4 and free T_3. This involved the equilibrium dialysis of diluted serum against 0.01 M Hepes, 0.11 M NaCl, pH 7.4, and measurement of T_3 and T_4 in aliquots of the dialysate by ultrasensitive radioimmunoassay. Euthyroid T_4 concentration ranged from 9.7 to 21.9 pmol/L and T_3 concentration from 4.5 to 8.4 pmol/L. The use of radioimmunoassay overcame the problems inherent in the indirect methodologies of tracer impurities and the associated laborious techniques required to purify both the tracer and the T_3 or T_4 in the dialysate or ultrafiltrate. Furthermore, the new method was claimed to measure directly and specifically the physiological entity and to be less subject to variability as only one serum parameter, namely free thyroid hormone concentration, was being measured. Although the method was a two step procedure, it was straightforward and potentially applicable to the area of routine assessment of thyroid status.

Using the same technique, Yeo et al. (1977) reported a mean fT_4 concentration of 10.4 pmol/L in euthyroid subjects, and Jiang and Tue (1977), by dialyzing against 0.15 M phosphate, pH 7.4, reported a mean level of 9.8 pmol/L. That is, the direct method has yielded the lowest and most consistent estimations of fT_4 concentration, the levels measured representing a percent free T_4 of 0.010%.

These estimates are unlikely to be artifactually low as Jiang and Tue (1977), by taking particular care over purity and age of the ^{125}I-T_4, showed the indirect and direct methodologies to both give a percent free T_4 of 0.010%.

Ellis and Ekins (1975) have confirmed that the concentration of free T_4 is only slightly affected by dilution of the serum while, as expected free T_3 concentration did decrease with dilution, reflecting the lower affinity of T_3 for the serum binding proteins.

Petersen, Giese, Larsen and Karger (1977) have recently developed a very sensitive gas liquid chromatography system for the measurement of T_3 and T_4 in dialysates. This elaborate method involves the dialysis of serum against 0.15 M phosphate, pH 7.4, purification of the dialysates on cation exchange columns, evaporation of the eluant and derivatization of the residue, and quantitation of the hepta-fluorobutyryl methyl ester derivatives by electron capture detection - gas liquid chromatography (ECD - GLC). Free T_4 concentration was measured to be 24.4 pmol/L being over double the concentration estimated by the radioimmunoassay procedures, and mean fT_3 concentration was 6.5 pmol/L, similar to the radioimmunoassay measurements.

KINETIC MEASUREMENT OF FREE T_4 CONCENTRATION

1. Principles

Ekins (1979a) has outlined a new approach to measuring fT_4 concentration *in vitro*. This approach can be termed the kinetic method of measuring fT_4 concentration. If a

binding reagent is added to serum, then the binding of a ligand L, to the reagent R, is described as:

$$R + L \underset{k_2}{\overset{k_1}{\rightleftarrows}} RL$$

where RL is the reagent bound ligand and k_1 and k_2 are the association and dissociation rate constants respectively. The rate of formation of RL is described by the equation:

$$\frac{d[RL]}{dt} = k_1[R][L] - k_2[RL]$$

where $k_1[R][L]$ is the rate of binding of ligand to the reagent, and $k_2[RL]$ is the rate of dissociation of ligand from the reagent. [R] is the concentration of unoccupied reagent sites, [L] the concentration of unbound ligand, and [RL] the concentration of bound ligand. The assumption is made that the forward reaction is slower than the dissociation reaction, that is that binding to the ligand is the rate limiting step. This is reasonable in view of the fact that the association reaction is bimolecular and so likely to be much slower than the unimolecular dissociation reaction. In this case then,

$$\frac{d[RL]}{dt} \simeq k_1[R][L]$$

At the initiation of binding to reagent, all reagent sites will be unoccupied. That is, $[R] = [R_o]$, and

$$\frac{d[RL]}{dt} \simeq k_1[R_o][L]$$

That is, the rate of binding of ligand will be proportional to the free ligand concentration [L].

Thus the binding of T_4 to a specific binder such as antibody in a given period of time can be used to measure the concentration of fT_4. Alternatively, the rate of binding of labelled T_4 to antibody or binding protein will allow the calculation of the fraction T_4 bound and, knowing the total T_4 concentration, fT_4 concentration may be calculated. To ensure an accurate measurement of free hormone the concentration of R should be such that free hormone concentration is not altered by excessive stripping of hormone from the serum binding proteins. Ekins recommended that less than 5% of the total hormone be bound to R during incubation.

In the case of T_4, where greater than 99% of the hormone is bound to the serum binding proteins, the amount of T_4 bound to R at equilibrium will be approximately proportional to fT_4 as long as the amount of hormone bound to R is small, for example less than 5% of total hormone. If, under these conditions, only the binding of ligand to R is considered, then as the reaction proceeds, the concentration of unoccupied sites [R] diminishes and the forward reaction slows down. Finally with the attainment of equilibrium

$$\frac{d[RL]}{dt} = k_1[R][L] - k_2[RL] = 0$$

That is, there is no net formation of RL. At this point, the reaction may be described by the law of mass action,

$$K = \frac{[RL]}{[R][L]}$$

$$\text{As} \quad [R] = [R_o] - [RL]$$

$$K[L] = \frac{[RL]}{[R]} = \frac{[RL]}{[R_o] - [RL]}$$

$$\frac{1}{K[L]} = \frac{[R_o] - [RL]}{[RL]} = \frac{[R_o]}{[RL]} - 1$$

that is, $$\frac{1}{K[L]} + 1 = \frac{[R_o]}{[RL]}$$

or, $$\frac{1 + K[L]}{K[L]} = \frac{[R_o]}{[RL]}$$

and $$\frac{[RL]}{[R_o]} = \frac{K[L]}{1 + K[L]} \qquad (4.5)$$

where $[RL]/[R_o]$ is the fractional occupancy or the ratio of occupied reagent binding site concentration [RL] to the concentration of total reagent binding sites $[R_o]$.

Providing the system has not altered the free ligand concentration and that less than 5% of bound hormone is bound to R then equation 4.5 states that the fractional occupancy, or the amount of ligand bound to the reagent, will be dependent on the free ligand concentration.

2. Determination of the Fractional Occupancy of the Binding Reagent

After adding the binding reagent and allowing the reaction to proceed either for a given time, or to equilibrium, the fractional occupancy of the binding reagent (proportional to the free ligand concentration) may be determined in several ways (Ekins, 1979a).

(a) Back titration - in this method, fractional occupancy is determined by separating the reagent from the serum binding proteins and unbound ligand and then incubating the reagent with tagged ligand. The tracer bound is inversely proportional to the concentration of occupied reagent binding sites or fractional reagent binding site occupancy. This in turn is proportional to the fT_4 concentration.

Clinical Assays (Travenol, U.S.A.) supply a kit which measures free T_4 by this method. 50 µL of serum is added to reaction tubes containing 1 mL of buffer, and anti-T_4 adsorbed to the tubes. The binding reaction is allowed to proceed for 20 min at 37°C after which time the buffer is completely aspirated from the tubes. At this time, T_4 bound to the adsorbed antibody is proportional to the fT_4 concentration of the serum. Buffer containing $^{125}I-T_4$ is then added to the tubes, the tubes are incubated for 60 min at 37°C, the buffer is then aspirated and the tubes counted. The counts in the tube will be inversely related to the fractional occupancy or fT_4 concentration. A standard curve is constructed using standards with known fT_4 concentration.

(b) Sandwich technique - once again the binding reagent is added to buffer containing the test serum and incubation allowed to proceed for a given time, or to equilibrium if this does not strip too much ligand from the serum binding protein. Test serum binding protein and unreacted ligand are then removed. The concentration of reagent bound ligand is determined directly by incubation with a labelled antibody

to the ligand. Unreacted labelled antibody is removed and the resulting labelled antibody-ligand-reagent sandwich quantitated to give a measure of fractional reagent occupancy. Again, this is related to the fT_4 concentration by using standards.

(c) Direct tracer technique - in this method, labelled ligand is added to the incubation mixture with the binding reagent and serum sample. After a given time the reagent is separated and the fraction of tracer bound to the reagent is determined (fractional occupancy). The quantity of hormone bound to the reagent is determined by multiplying the fraction tracer bound by the total ligand concentration of the serum sample. Standards are used to relate the amount of ligand bound to the free ligand concentration.

The Corning Free-T_4-^{125}I Radioimmunoassay Test System (Corning Medical, U.S.A.) is based on this principle. ^{125}I-T_4 and 25 μL of serum are incubated together for 20 min at room temperature to ensure equilibration of ^{125}I-T_4 binding to the serum protein. The binding reagent, anti-T_4 bound to glass beads, is added and incubated for 30 min at room temperature after which the antibody is spun down and the bound ^{125}I-T_4 determined. The counts bound are expressed as a fraction of the counts added to the tube. Binding of the tracer in the system can be determined as:

$$*T_4 + T_4 + TBP + R \rightleftarrows *T_4 \bullet TBP + T_4 \bullet TBP + *T_4 \bullet R + T_4 \bullet R$$

where ($*T_4 \bullet R + T_4 \bullet R$) represents the occupied reagent binding sites.

The occupancy of the binding reagent (antibody bound to glass bead) by the T_4 in the sample is then determined by multiplying the fraction of counts bound to the reagent by the total T_4 of the serum sample. The concentration of T_4 bound to reagent is then related to the fT_4 concentration by using standards with known fT_4 concentration.

Table 4.3 illustrates how the Corning test system responds when measuring fT_4 in sera from subjects with a variety of different thyroid states.

(d) Tracer displacement technique - the Damon Diagnostics kit (Damon Diagnostics, U.S.A.) employs this principle. The kit uses a binding reagent (anti-T_4) encapsulated in a semipermeable nylon membrane (Halpern and Bordens, 1979). This reagent is saturated with ^{125}I-T_4. That is, occupancy by ^{125}I-T_4 is 100%. On addition of serum to the incubation solution, T_4 diffuses into the gel displacing ^{125}I-T_4 from the antibody into the extra-gel space (incubation solution). In a given time, the displacement of tracer into the incubation solution is proportional to the T_4 diffusing into the gel which in turn is proportional to the fT_4 concentration.

4.4 DERIVED INDICES OF FREE THYROXINE CONCENTRATION

MEASUREMENT OF SERUM UNOCCUPIED T_4 BINDING SITE CONCENTRATION

To overcome the problems associated with the measurement of fT_4 concentration, attempts were made to develop assays which provided values proportional to the free

TABLE 4.3 Rationale of the Corning kinetic method for the measurement of fT_4

Group	$[T_4]$	$[TBG_o]$	$[TT_4]$	Rate of T_4 binding to antibody	Tracer specific activity	$^{125}I-T_4$ bound to antibody (A/T)	T_4 bound to antibody (A/T x T_4)
Pregnancy Oral contraceptives Idiopathic elevated TBG	N	↑	↑	N	↓	↓	N
Severe illness Androgens Idiopathic low TBG	N	↓	↓	N	↑	↑	N
Serum dilution	N	↓	↓	N	↑	↑	N
Salicylates	N	N	↓	N	↑	↑	N
Hypothyroid	↓	N	↓	↓	↑	N	↓
Thyrotoxic	↑	N	↑	↑	↓	N	↑

hormone concentration.

By considering only the binding of T_4 to thyroxine binding protein, Osorio, Jackson, Gartside and Goolden (1962) derived the following expression for $[T_4]$ from the law of mass action,

$$[T_4] = \frac{[T_4 \bullet TBP]}{K[TBP]}$$

that is, $$K[T_4] = \frac{[\text{occupied } T_4 \text{ binding sites}]}{[\text{unoccupied } T_4 \text{ binding sites}]} \quad (4.6)$$

where K is the association constant for binding between T_4 and TBP.

The concentration of unoccupied T_4 binding sites was determined by incubating $^{131}I\text{-}T_3$ with serum and subsequently separating the unbound $^{131}I\text{-}T_3$ by binding to red blood cells. It was reasoned that because T_3 had lower affinity for TBP than T_4, $^{131}I\text{-}T_3$ would bind to the unoccupied binding sites without displacing significant quantities of bound T_4. Red blood cells provided a large capacity source of low affinity binding sites which could bind $^{131}I\text{-}T_3$ not bound to TBP. Osorio, Jackson, Gartside and Goolden (1961) demonstrated that red blood cell uptake of $^{131}I\text{-}T_3$ was indeed inversely proportional to the concentration of unoccupied TBP binding sites. Using the red blood cell uptake to calculate the concentration of unoccupied T_4 binding sites and PBI as an approximation of the concentration of occupied T_4 binding sites they were able to calculate $K[T_4]$.

The semiquantitative factor, $K[T_4]$, correlated well

with the clinical thyroid status in normals, in euthyroid pregnant women, hypothyroid and thyrotoxic subjects and in subjects on the antiepileptic drug, phenytoin. They concluded, however, that the test was too laborious for routine use.

FREE THYROXINE INDEX

A similar approach was made by Clark and Horn (1965). They used ion exchange resin to bind excess $^{131}I\text{-}T_3$ and simplified the calculations of Osorio et al. (1962) to provide a simple and rapid assay.

By using simply the counts taken up by the resin and the PBI they calculated the Free Thyroxine Index (FTI). That is,

$$(\text{counts } ^{131}I\text{-}T_3 \text{ bound to resin}) \propto \frac{1}{[\text{unoccupied } T_4 \text{ binding sites}]}$$

Inserting into equation 4.6,

$$K[T_4] \propto \text{PBI x (counts } ^{131}I\text{-}T_3 \text{ bound to resin)}$$

and,

$$\text{FTI} = \text{PBI x (counts } ^{131}I\text{-}T_3 \text{ bound to resin)} \quad (4.7)$$

The FTI was found to differentiate between euthyroid, thyrotoxic and hypothyroid groups, and was normal in euthyroid groups with altered TBP. The FTI was more amenable to most diagnostic laboratories than the more elaborate free T_4 concentration analyses and has been widely adopted with either ion exchange resin or Sephadex G-25 being used to bind free $^{125}I\text{-}T_3$.

The counts bound to the resin are usually normalized by comparison to a normal control serum sample and called the T_3 uptake, or T_3U.

$$T_3U = \frac{\text{(counts bound to resin in test sample)}}{\text{(counts bound to resin in control sample)}}$$

Thus from equation 4.7,

$$FTI = [TT_4] \times T_3U \qquad (4.8)$$

where $[TT_4]$ represents the total circulating T_4 concentration.

It should be stressed that the FTI bears only an indirect relation to the fT_4 concentration. Although both T_4 and T_3 are bound principally to TBG, there is significant lower affinity binding to TBPA and albumin. Furthermore, when considering the latter two binding proteins, the differences in binding affinities for T_3 and T_4 are less pronounced. That is, some competition for binding may take place between the ^{125}I-T_3 and bound T_4. Despite this limitation the FTI has been found to correlate well with fT_4 concentration (Wellby and O'Halloran, 1966; Anderson, 1968; Stein and Price, 1972).

The form of the T_3U used in equation 4.8 is not universally accepted and is sometimes expressed in terms of the counts remaining in the serum after removal of the exchange resin or Sephadex G-25. In this case the T_3U represents the ^{125}I-T_3 bound to the serum binding proteins and is directly proportional to the concentration of unoccupied binding sites. That is,

$$T_3U = \frac{\text{(counts bound to serum proteins in test sample)}}{\text{(counts bound to serum proteins in control sample)}}$$

and $$FTI = [TT_4] \times \frac{1}{T_3U}$$

Furthermore, T_3U may be expressed simply as the percent ^{125}I-T_3 bound to either the serum protein or resin in the test sample.

Labelled T_4 has been used to determine the concentration of unoccupied binding sites but has the disadvantage that most of the added tracer is bound to the serum (Liewendahl and Helenius, 1975).

With the recent publication of data on TBG concentration in various physiological states, it is possible to calculate the concentration of unoccupied TBG binding sites. Data published by McDowell (1979) was used to estimate the concentration of unoccupied TBG binding sites in euthyroid subjects with abnormal TBG concentration. The equation used to determine the concentration of unoccupied binding sites [TBG],

$$[TBG]^2 K + [TBG]\left[K[TT_4] - [TBG_o]K + 1\right] - [TBG_o] = 0$$

is derived in the appendix (equation A9). K is the association constant for binding between T_4 and TBG, $[TBG_o]$ the total circulating TBG concentration, and $[TT_4]$ the total circulating T_4 concentration. Table 4.4(a) and Figure 4.1 show the percent changes in unoccupied binding sites [TBG], are similar to the percent changes in the total TBG concen-

TABLE 4.4 (a) Concentration of unoccupied TBG binding sites, [TBG], in euthyroid subjects with altered TBG concentration, $[TBG_o]$.

[TBG] was calculated using the equation

$$[TBG]^2 K + [TBG]\left[K[TT_4] - K[TBG_o] + 1\right] - [TBG_o] = 0$$

derived in the appendix (equation A9)

$K = 1.7 \times 10^{10}$ (Woeber and Ingbar, 1968)

Molecular weight of TBG = 50,000 daltons

The data for $[TBG_o]$, $[T_4]$ and T_3U are taken from McDowell (1979)

Group	$[TBG_o]$ mg/L	$[TBG_o]$ mol/L	% change	$[TT_4]$ nmol/L	% change	calc. [TBG] mol/L	% change	(T_3U)	% change
Normal	21.6	4.3×10^{-7}	-	95	-	3.4×10^{-7}	-	1.00	-
Sick euthyroids	16.8	3.4×10^{-7}	-22	53	-44	2.9×10^{-7}	-14	0.80	-11
Oral contracep.	30.5	6.1×10^{-7}	+41	133	+41	4.8×10^{-7}	+42	1.15	+15
Pregnancy	35.3	7.1×10^{-7}	+63	141	+49	5.7×10^{-7}	+70	1.22	+22

The T_3U was calculated from ^{125}I-T_3 binding to serum proteins, not resin. That is,

$$T_3U = \frac{\text{(counts bound to test serum proteins)}}{\text{(counts bound to reference serum proteins)}}$$

In this form, the T_3U is directly related to the concentration of unoccupied binding sites

The percent changes are plotted in Figure 4.1.

TABLE 4.4 (b) $[TT_4]/[TBG_o]$ in euthyroid subjects with altered TBG concentration, $[TBG_o]$

$[TT_4]/[TBG_o]$ was calculated using the equation -

$$\frac{[TT_4]}{[TBG_o]} = \frac{K[T_4]}{1 + K[T_4]} + \frac{[T_4]}{[TBG_o]}$$

derived in the appendix (equation A10)

$[TT_4]$, $[TBG_o]$ and FTI are taken from McDowell (1979).
$[T_4]$ the healthy euthyroid mean was taken from data presented in this thesis (measured by E/D-RIA).

Group	$[TBG_o]$ (mol/L)	$[TT_4]$ (nmol/L)	$\frac{[TT_4]}{[TBG_o]}$	$[T_4]$ pmol/L	Calculated $[TT_4]/[TBG_o]$ (equ. A10)	FTI
Normal	4.3×10^{-7}	95	.221	10.2	.148	.89
Sick euthyroid	3.4×10^{-7}	53	.156	10.2	.148	.59
Oral contraceptives	6.1×10^{-7}	133	.218	10.2	.148	1.08
Pregnancy	7.1×10^{-7}	141	.199	10.2	.148	1.09
Hypothyroid	5.4×10^{-7}	44	.081	3.7	.059	.37
Thyrotoxic	4.2×10^{-7}	186	.443	19.2	.246	2.22

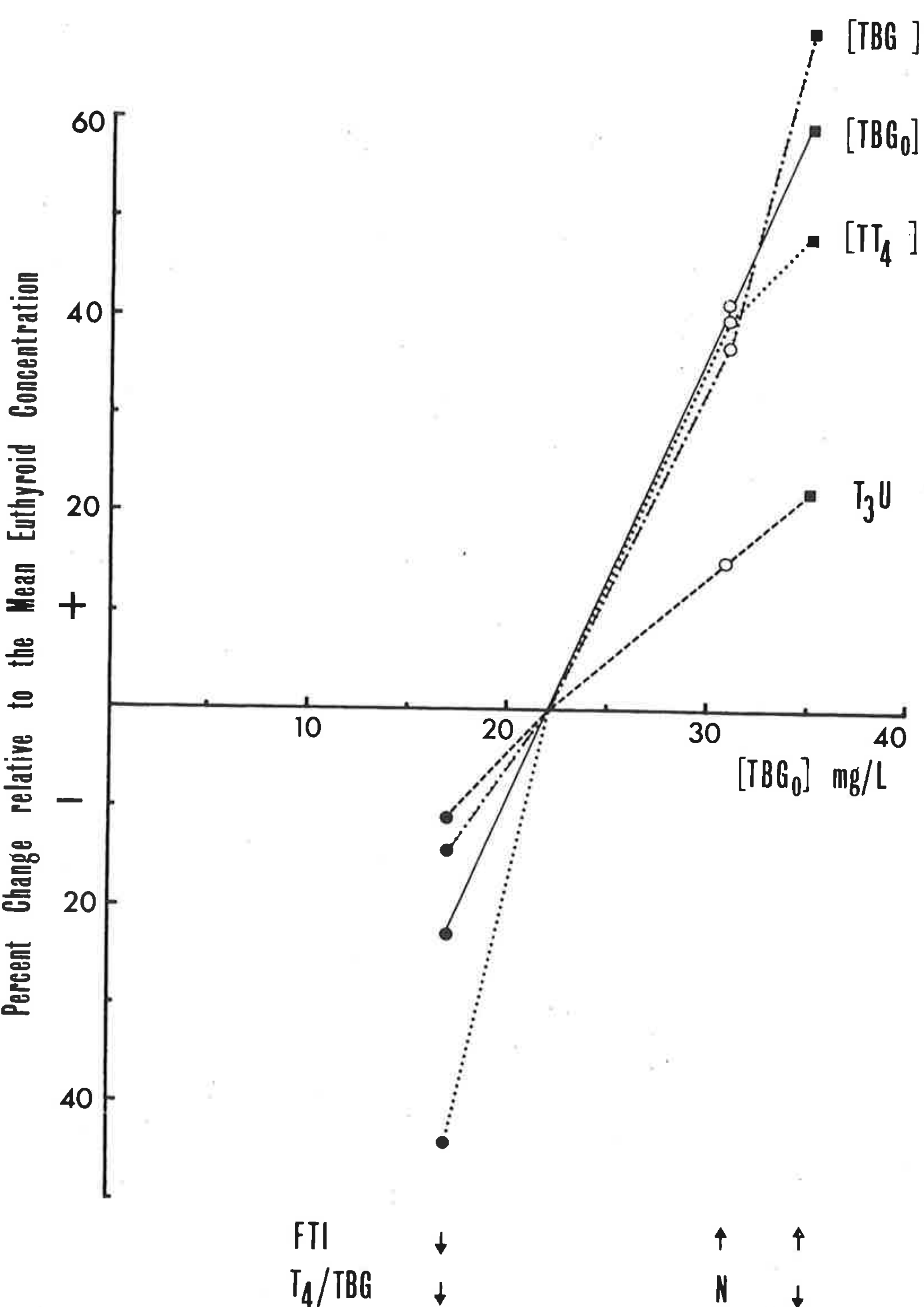
Figure 4.1 Changes in $[TT_4]$, $[TBG_o]$, $[TBG]$ and T_3U in acute illness, during pregnancy and during ingestion of oral contraceptives (adapted from McDowell (1979))

Unoccupied binding site concentration $[TBG]$ was calculated using equation A9 in the appendix.

T_3U represents uptake by the serum protein, not the resin. That is, FTI = $[TT_4]/T_3U$.

Symbol	Meaning
●	Acute illness
○	Oral contraceptives
■	Pregnant
———	$[TBG_o]$
-----	T_3U
·······	$[TT_4]$
—·—	$[TBG]$

Also indicated at the bottom of the diagram are the changes in FTI and T_4/TBG relative to the healthy euthyroid group with normal binding protein concentration expected from this data.



[TBG]
[TBG0]
[TT4]
T3U
60
40
20
+
-
20
40
10
20
30
40
[TBG0] mg/L
Percent Change relative to the Mean Euthyroid Concentration
FTI
T4/TBG
N

tration $[TBG_o]$. Thus, if changes in T_4 are due only to changes in TBG concentration, $[TBG_o]$, then any method which measures unoccupied binding sites and gives results varying linearly with variations in the concentration of these sites should correct the changes in T_4 to give a normal FTI (this is discussed in more detail in Chapter 5 (5.4).

T_4/TBG

The recent availability of a radioimmunoassay for TBG (Levy, Marshall and Velayo, 1970) has allowed the calculation of an FTI using the measured total T_4 and TBG concentrations (Burr, Ramsden, Evans, Hogan and Hoffenberg, 1977). Considering the binding between T_4 and TBG:

$$T_4 + TBG \rightleftarrows T_4 \bullet TBG$$

then at equilibrium,

$$K = \frac{[T_4 \bullet TBG]}{[T_4][TBG]}$$

where the concentration of unoccupied binding sites,

$$[TBG] = [TBG_o] - [T_4 \bullet TBG]$$

Solving for $[TT_4]/[TBG_o]$,

$$\frac{[TT_4]}{[TBG_o]} = \frac{K[T_4]}{K[T_4] + 1} + \frac{[T_4]}{[TBG_o]}$$

(equation A10, appendix).

$[TT_4]/[TBG_o]$ is seen to be a function of not only the fT_4 concentration, but also the concentration of circu-

lating TBG. However, as the solutions for this equation show (Table 4.4(b)),

$$\frac{[T_4]}{[TBG_o]} << \frac{K[T_4]}{K[T_4]+1}$$

that is, $[TT_4]/[TBG_o]$ can be considered a function only of fT_4. Roosdorp and Joustra (1979) have recently shown that when binding to TBPA and albumin is considered as well, $[TT_4]/[TBG_o]$ does vary with varying $[TBG_o]$ independent of changes in fT_4. Furthermore, where changes in T_4 are due mainly to changes in TBPA (sick euthyroid subjects), $[TT_4]/[TBG_o]$ does not necessarily reflect fT_4 concentration (Table 4.4(b)).

Use of the FTI and T_4/TBG is discussed in detail in Chapter 5 (5.4).

DUAL COMPETITIVE PROTEIN BINDING ASSAYS - THE EFFECTIVE THYROXINE RATIO AND NORMALIZED THYROXINE TESTS

Although initial assessment showed the FTI to be a simple, cheap and effective guide to fT_4 concentration, it had the disadvantage of being a two step procedure. Mincey, Thorson and Brown (1971) introduced a single step, or 'one tube', estimate of a parameter indirectly proportional to the fT_4 concentration. This was termed the Effective Thyroxine Ratio, or ETR, and the procedure is best described as a competitive protein binding assay for T_4 carried out in the presence of a small volume of the test sample serum. The assay described by Mincey et al. (1971) involved the ethanol extraction of endogenous T_4 from the test serum

sample. This extract, together with a very small volume of the test serum, was added to the $^{125}I-T_4$ - binding globulin reagent and mixed with an anion exchange resin. The counts remaining in solution after a given incubation time were counted and the ETR calculated as:

$$\text{ETR} = \frac{(\text{counts remaining in solution in reference sample})}{(\text{counts remaining in solution in test sample})}$$

$$= \frac{\begin{pmatrix}\text{counts bound to} \\ \text{globulin reagent}\end{pmatrix} + \begin{pmatrix}\text{unoccupied reference serum} \\ \text{binding protein}\end{pmatrix}}{\begin{pmatrix}\text{counts bound to} \\ \text{globulin reagent}\end{pmatrix} + \begin{pmatrix}\text{unoccupied test serum} \\ \text{binding protein}\end{pmatrix}}$$

Reference to Table 4.5 shows how the ETR remains in the narrow range around 1 where T_4 is normal, elevated due to increased TBG, or low due to decreased TBG, but is low in hypothyroidism and high in thyrotoxic patients. Where T_4 is abnormal, the specific activity of the $^{125}I-T_4$ after addition of the ethanol extract varies accordingly compared to the specific activity in the normal reference serum assay vial. Furthermore, the concentration of binding sites in the assay vial is the sum of the globulin reagent sites and the unoccupied sites added with the serum sample. This concentration will be higher than in the reference sample vial on addition of elevated TBG and hypothyroid samples and lower than the reference sample vial on addition of low TBG or thyrotoxic samples. Mincey et al. (1971) optimized the procedure by experimentally determining that volume of serum which must be added to the test vial so that variations in specific activity of $^{125}I-T_4$ balanced the changes in binding of T_4 and tracer to the globulin reagent and

introduced unoccupied TBG sites in samples where abnormal T_4 values were due to abnormal TBG concentration. On the other hand both specific activity and binding site concentration are increased in hypothyroid samples leading to low ETR, whereas specific activity and binding site concentration are decreased in thyrotoxic serum thus giving an elevated ETR.

A variety of dual competitive protein binding assays have since been reported. The normalized T_4 assay of Ashkar and Bezjian (1972) is based on similar principles. A Sephadex G-25 column has been used to both extract endogenous T_4 from the serum and to carry out the CPBA in the presence of test sample serum (Abreau, Azizi, Vagenakis, Ingbar and Braverman, 1973; Howorth and McKerron, 1974).

The disadvantage of this type of assay is that the results provide no information on abnormalities in TBG concentration. However, many regard these abnormalities clinically irrelevant. It is of interest that Wong, Cradduck and Wood (1974) developed a system where each sample was assayed in two tubes. Extracted T_4 and competitive protein binding assay reagents were added to both tubes and test serum to only one. Thus one gave an estimate of T_4 concentration and the other, after comparison to a reference tube, gave a result corrected for any variations in binding protein concentration.

TABLE 4.5

				FTI		
	$[TBG_o]$	$[TT_4]$	fT_4	^aT_3U		
				b	c	FTI
Pregnancy Oral contraceptives Idiopathic TBG	↑	↑	N	↓	↑	N
Severe illness Androgens Idiopathic TBG	↓	↓	N	↑	↓	N
Salicylates	N	↓	N	↑	↓	N
Hypothyroid	N	↓	↓	↓	↑	↓
Hyperthyroid	N	↑	↑	↑	↓	↑

a T_3U - see text of Section 4.4

b $$T_3U = \frac{\text{resin counts-test}}{\text{resin counts-control}}$$

c $$T_3U = \frac{\text{supernatant counts-test}}{\text{supernatant counts-control}}$$

TABLE 4.5 (continued)

^{d}ETR			
$^{125}I-T_4$ specific activity	Concentration of binding sites	Counts not bound to resin	ETR
↓	↑	N	N
↑	N	N	N
↑	↓	⋮↓	↑⋮
↑	↑	↑	↓
↓	↓	↓	↑

^{d} ETR - See text of Section 4.4

⋮↓ ↑⋮ results expected to be borderline low or high respectively.

CHAPTER 5

THE ANALYSIS OF FREE THYROID HORMONE CONCENTRATION

5.1 INTRODUCTION

Chapter 1 outlined the development of the free thyroid hormone hypothesis. This hypothesis states the circulating free form of the thyroid hormone determines the availability of the thyroid hormones to the intracellular compartment and the subsequent biological actions of these hormones. The conclusion was that, in view of available theoretical considerations and experimental evidence, the measurement of fT_3 and fT_4 concentrations was the most reliable test of thyroid function.

This part of the thesis was undertaken with the aim of establishing an analytical procedure to accurately measure the free thyroid hormones. This procedure was to be selected on the criterion that, on theoretical grounds, the method most accurately measured the free hormone concentration in serum. Chapter 4 outlined in detail the methodologies which have been developed to measure free thyroid hormone concentration. Of the techniques available, the equilibrium dialysis-radioimmunoassay (E/D-RIA) procedure of Ellis and Ekins (1975) appeared to afford the most accurate measurement of free hormone concentration. The establishment of this technique is described in detail.

Ellis and Ekins (1975) used a computer optimisation technique (Ekins and Newman, 1968) to determine those concentrations of tracer and antiserum which afforded assays of the sensitivity required. This chapter describes several

simpler models of ligand binding derived from the law of mass action. The use of these models for optimising assay sensitivity is evaluated and compared to empirically established assays.

The chapter then goes on to record the results of the further aims of this project. Firstly there was a need to establish the limitations of the existing routine indirect estimates of free T_4 concentration (the free thyroxine index) by comparison with the equilibrium dialysis technique. Secondly, a recently available commercial kit claiming to directly measure fT_4 concentration by a kinetic approach was evaluated by comparison with the dialysis technique.

The results of several clinical studies using the equilibrium dialysis method are described in Chapter 6.

5.2 MATERIALS AND METHODS

APPARATUS AND MATERIALS

The dialysis cells were of the same construction used by Ellis and Ekins (1975) (Figure 5.1). 30 cells were turned from 2.54 cm teflon rod (Menzel Plastics, S. Aust.). Using these cells, dialysis was carried out at 37°C in a water bath with a speed adjustable reciprocating tray (Paton Industries, South Aust.). Dialysis membrane was Visking tubing of 43 mm flat width (Union Carbide, U.S.A.).

The reagents and apparatus used for the synthesis of ^{125}I- derivatives and in the production of antibodies are described in Chapter 3. The iodothyronines, Na-L-3,3'-T_2,

Na-L-T_3 and Na-L-T_4 used for iodination and as standards were supplied by Sigma (U.S.A.). Commercial supplies of high specific activity ^{125}I-T_3 and ^{125}I-T_4 have recently become available and when used, were obtained from The Radiochemical Centre (U.K.).

All buffer reagents and solvents used were A.R. grade and were available from most chemical suppliers. 1/10,000 (w/v) sodium azide (BDH, U.K.) was used as a bacteriostat in the buffers, which were stored at 4°C to further reduce bacterial growth. The gelatine used in the assay buffer was supplied by BDH (U.K.). The anti-gamma-globulin used as the second antibody in the double antibody separation technique was a donkey anti-sheep, goat gamma-globulin (Wellcome, U.K.). In the charcoal separation technique, activated Norit A charcoal (BDH, U.K.) was used.

METHODS

The methodology used to measure free thyroid hormones in serum was similar to the published method of Ellis and Ekins (1975). The procedure involved the over-night dialysis of a small volume of serum and subsequent measurement of the iodothyronine of interest in the dialysate by serum-free radioimmunoassay.

The method used for the preparation of iodinated iodothyronines, analysis of the products of radioiodination by thin layer chromatography, determination of specific activity, conjugation of iodothyronines to bovine serum albumin, production of antisera, the determination of precision profiles, and radioimmunoassay curve fitting have

been fully described in Chapter 3. Described in this section are those points pertinent to the application of these procedures in the development of the E/D-RIA technique.

1. Dialysis

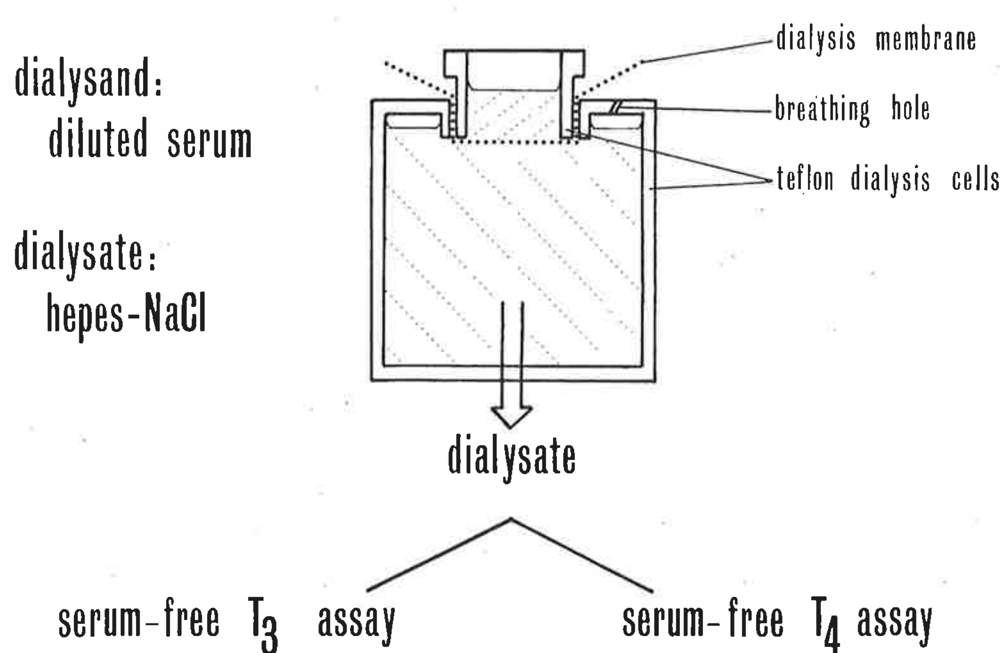
5 mL 0.01 M Hepes, 0.11 M NaCl, 1.5 mM sodium azide, pH 7.4., was added to the dialysate compartment of each cell (Figure 5.1). Dialysis tubing, which had been stored in the dialysate buffer at 4°C, was slit along the fold of one side of the flat tubing and cut into squares measuring about 85 mm x 85 mm. All handling of tubing was done using sterile gloves. A piece of membrane was placed over the dialysate compartment of each cell and the sample compartment pushed into the dialysate compartment such that the membrane formed a seal between the two units. 500 μL of dialysate buffer and 100 μL of serum were added to each sample compartment. The cells were placed on the tray of a reciprocating tray water bath, with the cells in about 15 mm of water at 37°C. After gently shaking overnight, the dialysand and membrane were discarded and 1 mL aliquots of the dialysate were pipetted into assay tubes. These were either assayed that day or stored at 4°C for not more than three days prior to assay.

2. Preparation of Radioiodinated Iodothyronines

The sensitivities required in the radioimmunoassays for measuring iodothyronines in dialysate necessitate the use of high specific activity tracer. The method of iodination is based on the chloramine T technique of Weeke and Orskov (1973) in which near theoretically maximum specific

Figure 5.1 Analysis of free thyroid hormones by equilibrium dialysis - radioimmunoassay

Free Thyroid Hormone Analysis



activities can be obtained. Synthesis of radioiodinated T_3 or T_4 involved the chloramine T oxidative radioiodination of 3,5-T_2 with formation of monolabelled T_3 and bilabelled T_4, or radioiodination of 3,3',5-T_3 with formation of mono-labelled T_4.

A 12.5 μL aliquot of 0.5 M phosphate buffer, pH 7.4, was added to 1 - 2 m Ci of ^{125}I-iodide in the vial in which the iodide was received. Either 10 μL 3,5-T_2 (2.1 nmol) or 5 μL 3,3',5-T_3 (1.6 nmol) was then pipetted into the vial. The reaction was started by adding 12.5 μL chloramine T (89 nmol) and allowed to proceed for 15 - 20 s at room temperature with shaking. Sodium metabisulphite (658 nmol/ 25 μL) was added to stop the reaction and eluting buffer added to fill the vial. The reaction mixture was then eluted through Sephadex G-25 with 0.025 M NaOH or through LH-20 with ethyl acetate/methanol/NH_4OH/water (400:100:4:36) respectively, to remove unreacted ^{125}I-iodide and to fractionate the labelled products. The aqueous fractions were neutralized and diluted to 50% with ethanol for storage at 4°C. The ethyl acetate fractions were dried down and stored in ethanol/water (1:1) at room temperature.

When necessary, identification of the ^{125}I-iodothyronine products in the reaction mixture was made using thin layer chromatography. The routine method for checking the adequacy of the iodination reaction was to check the binding of the product to the relevant antiserum under the normal conditions of assay. It was found important also to check the binding to charcoal in the absence of anti-serum to assess contamination of the tracer with free ^{125}I-

iodide (this is simply the 'blank' tube described in the assay protocol section). If binding to the antiserum was not as good as required, the specific activity of the tracer was checked. If possible the tracer concentration in the assay incubate was adjusted to improve binding, being careful not to reduce sensitivity to an impractical level. If the specific activity was too low, the iodination was repeated.

3. Production of Antisera

High titre sheep antisera to T_3 and T_4 were kindly supplied by Ms. Jan Gooden and Ms. Lucyna Guthrie (Department of Clinical Chemistry, The Queen Elizabeth Hospital, South Aust.).

Bovine serum albumin conjugates of T_3 or T_4 (10 mg) were dissolved in 5 mL Freunds complete adjuvant and 2.5 mL of the thoroughly mixed emulsion injected into the shorn back of a sheep by the multiple site intradermal injection technique (Vaitukaitis et al., 1971). About 25 intradermal injections along each side of the spine were made using a 26 guage needle. The remaining 2.5 mL of emulsion was injected intramuscularly into both sides of the rump. Booster injections of 5 mg of conjugate were given intradermally at 6 weeks and 12 weeks post primary immunization. Antiserum titre was assessed weekly, and 100 mL of blood drawn when the titre became acceptable. The serum was separated and stored as 1 mL aliquots at a dilution of 1/100 in 0.1% BSA, 0.025 M barbital buffer, pH 8.6.

Sheep antiserum to T_3 #478 and antiserum to T_4 #492 were used to establish the serum-free thyroid hormone assays.

4. Standards

Sodium-L-T_3 (100 μg) was stored in 100 mL of 0.025 M NaOH/ethanol (1:1) at 4°C (1.55 μmol/L). The stock solution was diluted 1/1250 with 0.025 M NaOH to make up the highest standard, 1.24 nmol/L. This standard solution was serially diluted 1:2 in 0.025 M NaOH providing the 0.62, 0.31, 0.16, 0.08 and 0.04 nmol/L standards. As only 50 μL standard solution was added to the assay incubates as compared to 1 mL of dialysate, these standards were equivalent to 62, 31, 15.5, 7.8, 3.9 and 1.9 pmol/L dialysate T_3 concentration respectively. Standards were prepared in batches sufficient for 20 assays and stored as 250 μL aliquots at -20°C.

Sodium-L-T_4 was stored as an 800 μmol/L stock in 0.025 M NaOH/ethanol (1:1) at 4°C. The stock solution was diluted 1/160,000 in 0.025 M NaOH providing the highest standard of 5.0 nmol/L. This standard was serially diluted 1:2 with 0.025 M NaOH to furnish the 2.5, 1.25, 0.63, 0.31 and 0.16 nmol/L standards. These were equivalent to 250, 125, 62.5, 31.3, 15.6 and 7.8 pmol/L dialysate T_4 concentration respectively. The standards were stored as 250 μL aliquots at -20°C.

5. Separation Techniques

Charcoal separation of free and antibody bound hormone was routinely used in the radioimmunoassay. The procedure used to optimise the charcoal concentration was that of Binoux and Odell (1973). Five grams activated charcoal was suspended in 250 mL 0.01 M Hepes, 0.011 M Nacl, 1.5 mM

sodium azide, pH 7.4 and stored at 4°C.

In those studies where the second antibody technique was used, donkey anti-sheep, goat gammaglobulin (DASGG) was the precipitating agent. Normal sheep serum was used as the carrier protein, and was added to the tubes prior to the first incubation (0.25 μL per tube for the fT_4 assay and 1 μL per tube for the fT_3 assay). After the first incubation 8 μL of DASGG was added to each tube in the fT_4 assay or 12 μL DASGG per tube in the fT_3 assay and the tubes incubated for 1 h at 4°C. The tubes were centrifuged at 2000 rpm for 45 min, the supernatant tipped off and the precipitate counted.

6. Optimisation of Assay Sensitivity

Assays of sufficient sensitivity were established using empirical techniques to select the best combination of tracer and antibody concentration. Charcoal was used to separate free and antibody bound hormone during these studies. The general approach was to perform an antiserum dilution curve using a concentration of tracer similar to the sensitivity required.

Displacement of tracer by unlabelled hormone was assessed at those dilutions of antiserum which bound between 40 - 80% of the tracer. The dilution giving the best sensitivity was selected and the suitability of the tracer concentration used checked by measuring the binding at a variety of tracer concentrations. A precision profile was then experimentally determined.

A second approach to optimising assay sensitivity

was by making use of models describing the equilibrium binding of ligand to antibody. Two models of binding were derived from the law of mass action, one describing the binding of a ligand to one binding site population and the other describing ligand binding to two species of binding site. The equations derived (see Appendix) allowed the determination of the concentration of bound ligand, pq, at any concentration of ligand, p_o. Using equations to describe the slope of the binding curve at any ligand concentration, precision profiles were constructed assuming a constant percent coefficient of variation in measuring the percent tracer bound (2.5% and 5% were analyzed). From these profiles, the sensitivity of assays using a variety of antiserum dilutions was assessed and compared to the experimental data.

7. Radioimmunoassay Protocol

The assays were carried out in 5 mL disposable polystyrene serology tubes (Disposable Products, South Aust.). Working solutions of tracer and antiserum were made up just prior to assay. Tracer was diluted in 1% gelatine, 0.01 M Hepes, 0.11 M NaCl, 1.5 mM sodium azide, pH 7.4 to a concentration of 4000 cpm 100 µL. Antiserum was diluted in 0.1% gelatine, 0.01 M Hepes, 0.11 M NaCl, 1.5 mM sodium azide, pH 7.4 to ensure the final dilution desired. At the time of writing, anti-T_4 (#492) was used at a final dilution of $1/(2 \times 10^6)$ and anti-T_3 (#478) used at a final dilution of $1/(6 \times 10^6)$.

The volumes of reagents added to the assay tubes

are outlined in Table 5.1. The tubes were covered with Parafilm (3M Company, U.S.A.) and incubated for 24 h at 4°C. Toward the end of incubation, the charcoal mixture was mixed for 5 min at room temperature. One serology tube cap per tube was placed upside down on the bench and 250 µL of the 2% charcoal solution added to each cap. The caps were placed on the tubes and the racks mixed end-over-end for 1 min after which the tubes were centrifuged at 4°C for 15 min commencing 5 min after the start of mixing. The supernatants were poured into clean tubes and counted to 4000 counts per tube. Dialysate concentration of fT_3 or fT_4 were calculated from the raw data using Rodbard's curve fitting program for radioimmunoassays Chapter 3 (3.8).

8. Other Assays

The T_3 Sephadex uptake (T_3SU) was used as a semi-quantitative estimation of the unoccupied serum protein binding site concentration (Liewendahl and Helenius, 1975). Patient or reference serum (50 µL) was added to 5.2 mL of Sephadex G-25 suspension in 0.067 M phosphate buffer, pH 7.5, containing $^{125}I\text{-}T_3$. After rotating for 30 min the particles were allowed to settle and a 1 mL aliquot of supernatant counted. The T_3SU was calculated as,

$$T_3SU = \frac{(\text{counts/mL reference supernatant})}{(\text{counts/mL patient supernatant})}$$

Expressed in this form, the T_3SU is inversely proportional to the concentration of unoccupied binding sites.

The FTI was then calculated as,

$$FTI = [TT_4] \times T_3SU$$

TABLE 5.1 Protocol for Radioimmunoassay of T_3 and T_4 in Dialysates

All volumes are expressed as microlitres (μL)

Reagents	Total	Blank	Zero	Standard	Dialysate
Hepes/NaCl	1300	1050	1000	1000	
Dialysate					1000
0.025 M NaOH		50	50		50
Standard				50	
Tracer	100	100	100	100	100
Antiserum			50	50	50

Incubate 4°C, 24 h

250 μL 2% (w/v) charcoal, 5 min

Centrifuge 2000 rpm, 4°C, 15 min

Serum thyroxine concentration was assayed using an established T_4 radioimmunoassay. The diluent buffer was 0.1% BSA, 0.025 M barbital buffer, pH 8.6. Each tube contained in a 1 mL volume of incubate, 10 μL serum, 300 μg of the thyroid hormone binding protein inhibitor, 8-anilino-1-naphthalene sulphonic acid (Sigma, U.S.A.), 50,000 cpm ^{125}I-T_4 (The Radiochemical Centre, U.K.; 600 μCi/μg) anti-T_4 at a final dilution of 1/7,000 (sheep batch #357), 0.05 μL normal sheep serum, 1/85 final dilution of donkey anti-sheep goat gammaglobulin (Wellcome, Aust.) and 25 μL of either thyroid hormone free serum in the standard tubes, or 25 μL of test serum in the sample tubes. The tubes were incubated for 2 h at 37°C, centrifuged at 4°C, 2000 rpm for 30 min, the supernatant aspirated, and the precipitates counted.

The concentration of serum triiodothyronine was measured with an established laboratory radioimmunoassay. The diluent buffer was the same as used in the T_4 assay. Each tube contained in 1 mL of incubate, 50 μL serum, 65 μg 8-anilino-1-naphthalene sulphonic acid, 10,000 cpm ^{125}I-T_3 (Abbot Laboratories, U.S.A.; 500 μCi/μg), and T_3 antiserum at a final dilution of 1/200,000. Polyethylene glycol (BDH, U.K.) was added to a final concentration of 20% (w/v), the tubes incubated overnight at 4°C, centrifuged, and the precipitate counted.

Thyroid stimulating hormone was also assayed with an established radioimmunoassay. The diluent buffer was 0.05 M phosphate, 0.15 M NaCl, pH 7.8. The incubation volume was 1 mL and contained 100 μL serum and antiserum (National Pituitary Agency, U.S.A.) at a final dilution of 1/1,000,000.

The standard was the 68/38 reference preparation (National Institute for Medical Research, U.K.). After a 24 h incubation, 10,000 cpm ^{125}I-TSH/tube was added and incubated for a further 24 h. Donkey anti-rabbit gamma globulin (Wellcome, Aust.) was then added to precipitate the antibody bound tracer over a 24 h period. All incubations were performed at room temperature.

9. The Corning Free T_4-^{125}I Radioimmunoassay Test System

Free T_4 concentration in some samples was assayed not only with the equilibrium dialysis-radioimmunoassay technique, but also with the Corning Free T_4-^{125}I Radioimmunoassay Test System (Corning Medical, U.S.A.). The principle of this method has been described in Chapter 4 (4.3).

Two methods have been employed to calculate the fT_4 concentration using this kit. The original method recommended by the manufacturer was the relative rate technique. In this procedure, two sets of tubes were established for each standard and sample to be assayed. To one series (A series) was added ^{125}I-T_4, while to the second series (B series) was added ^{125}I-T_4 and the thyronine binding protein inhibitor, merthiolate. Anti-T_4 bound to glass beads was added to all tubes. After a 30 min incubation at room temperature the antibody was separated by centrifugation and the ratio of counts in the first series of tubes to the counts in the second series of tubes was calculated for each standard and a standard curve constructed. Similarly this ratio was calculated for each test sample and the fT_4 concentration read from the graph.

The second method, called the modified method, has been recently recommended by the manufacturer (Corning, 1979). The assay procedure is the same as that described above, but the calculation of fT_4 concentration now requires the estimation of total T_4 concentration in each sample. The second series of tubes, containing the thyronine binding protein inhibitor, provides a conventional radioimmunoassay for the total T_4 concentration. Using the A series tubes, without binding protein inhibitor, the fraction of $^{125}I-T_4$ in each sample which has bound to antibody in the incubation period (A/T) is calculated. Knowing the total T_4 concentration, the amount of T_4 bound to the antibody during the incubation ($A/T \times T_4$) is calculated for each standard and a standard curve constructed. By calculating ($A/T \times T_4$) for the test samples, the fT_4 concentration can be determined from the standard curve.

10. Sample Collection

Fifteen to twenty mL of blood was collected by venepuncture from all volunteers. Samples from normal euthyroid male subjects were collected during blood donation (Red Cross Blood Transfusion Centre, South Aust.) and samples were collected from normal euthyroid female subjects only where it could be ascertained that TBG was not elevated due to pregnancy or ingestion of oral contraceptives. Volunteers among the female staff at The Queen Elizabeth Hospital, South Aust., who were on oral contraceptives kindly donated samples for inclusion in the elevated TBG group. Samples with elevated TBG were also collected from pregnant women attending the ante-natal clinic of The Queen Elizabeth

Hospital. Cord blood samples collected at parturition were obtained from the blood bank of The Queen Elizabeth Hospital. Samples from patients on either anticonvulsant or salicylate therapy were supplied by Mr. Tim Wilkie of the Commonwealth Health Laboratories, Toowoomba, Queensland.

Samples collected from patients with abnormal thyroid function were assayed only where thyroid disease had been established by both clinical criteria and biochemical tests.

The separated sera were stored at -20°C until assayed.

5.3 ESTABLISHMENT OF THE FREE THYROID HORMONE ASSAYS

SYNTHESIS OF $^{125}I-T_3$ and $^{125}I-T_4$

1. Elution of the Iodination Reaction Products

The major products of the radioiodination of both 3,5-T_2 and T_3 were $^{125}I-T_3$, $^{125}I-T_4$ and unreacted ^{125}I-iodide. These products were well separated on Sephadex G-25 at high pH (Figure 5.2), as was originally demonstrated by Blasi and deMasi (1967). Despite the good separation on Sephadex G-25, the Sephadex LH-20 filtration system of Williams et al. (1969) was considered more convenient as the fractions could be simply and rapidly dried down for storage in ethanol/water (1:1) or for thin layer chromatography analysis.

The elution pattern on Sephadex LH-20 was quite different to that on Sephadex G-25, demonstrating altered adsorption characteristics. $^{125}I-T_3$ eluted before and was well resolved from ^{125}I-iodide (Figure 5.2b). In contrast,

$^{125}I-T_4$ elution was much more variable and the ratio of the elution volume of T_4 to that of iodide (V_e/V_t) ranged between 1.1 - 1.8 (Figure 5.3a - e). On one occasion a peak of co-eluting ^{125}I-iodide and $^{125}I-T_4$ eluted between and was well resolved from an ^{125}I-iodide peak and an $^{125}I-T_4$ peak (Figure 5.3a and Figure 5.4). However, separation of $^{125}I-T_4$ from ^{125}I-iodide was assured when freshly prepared eluting solvent was used (Figure 5.3b and e). The formation of $^{125}I-T_3$ during the radioiodination of T_3 (Figure 5.3) suggested that significant exchange took place during the iodination reaction. No exchange was apparent in the absence of chloramine T, confirming that this reaction occurs by a mechanism similar to that of iodine substitution (Mayberry, 1972).

2. Structural Integrity of the Tracer Preparations

The $^{125}I-T_3$ (Figure 5.5) and $^{125}I-T_4$ preparations exhibited self-displacement parallel to displacement of tracer by unlabelled T_3 or T_4 respectively verifying that the tracer was adequately purified after iodination. The specific activity of the mono-radioiodinated preparations ranged between 1200 - 1600 pCi/pg. Although the [^{125}I-3'5']-T_4 formed during radioiodination of 3,5-T_2 had very high specific activity, its lesser stability compared to that of the [^{125}I-3']-T_4 formed during radioiodination of T_3 made it unsuitable for routine use in the assay.

3. Storage

Thin layer chromatography of a number of samples of $^{125}I-T_3$ stored in ethanol/water (3:1, v/v) and of varying

Figure 5.2 Elution of reaction mixture post radio-iodination of 3,5-T_2

Elution profiles and thin layer chromatograms

(a) Elution of iodination reaction mixture through a 24 x 0.5 cm Sephadex G-25 (fine) column.
Eluting buffer was 0.025 M NaOH
Fraction size was 1 mL

			Ve/Vt
fraction	5	^{125}I-Iodide	1
"	45	^{125}I-T_3	9
"	105	^{125}I-T_4	21

(b) Elution of iodination reaction mixture through a 22 x 1.1 cm Sephadex LH-20 column.
Eluting solvent was ethylacetate/methanol/NH_4OH/H_2O (400:100:36:4)
Fraction size was 2 mL

			Ve/Vt
fraction	24	^{125}I-T_3	0.6
"	42	^{125}I-Iodide	1
"	51	^{125}I-T_4	1.2

(The thin layer chromatogram shows contaminating ^{125}I-T_3 in fraction 51; 'T_3 Am' is ^{125}I-T_3 obtained from The Radiochemical Centre, U.K., and used here as a marker.)

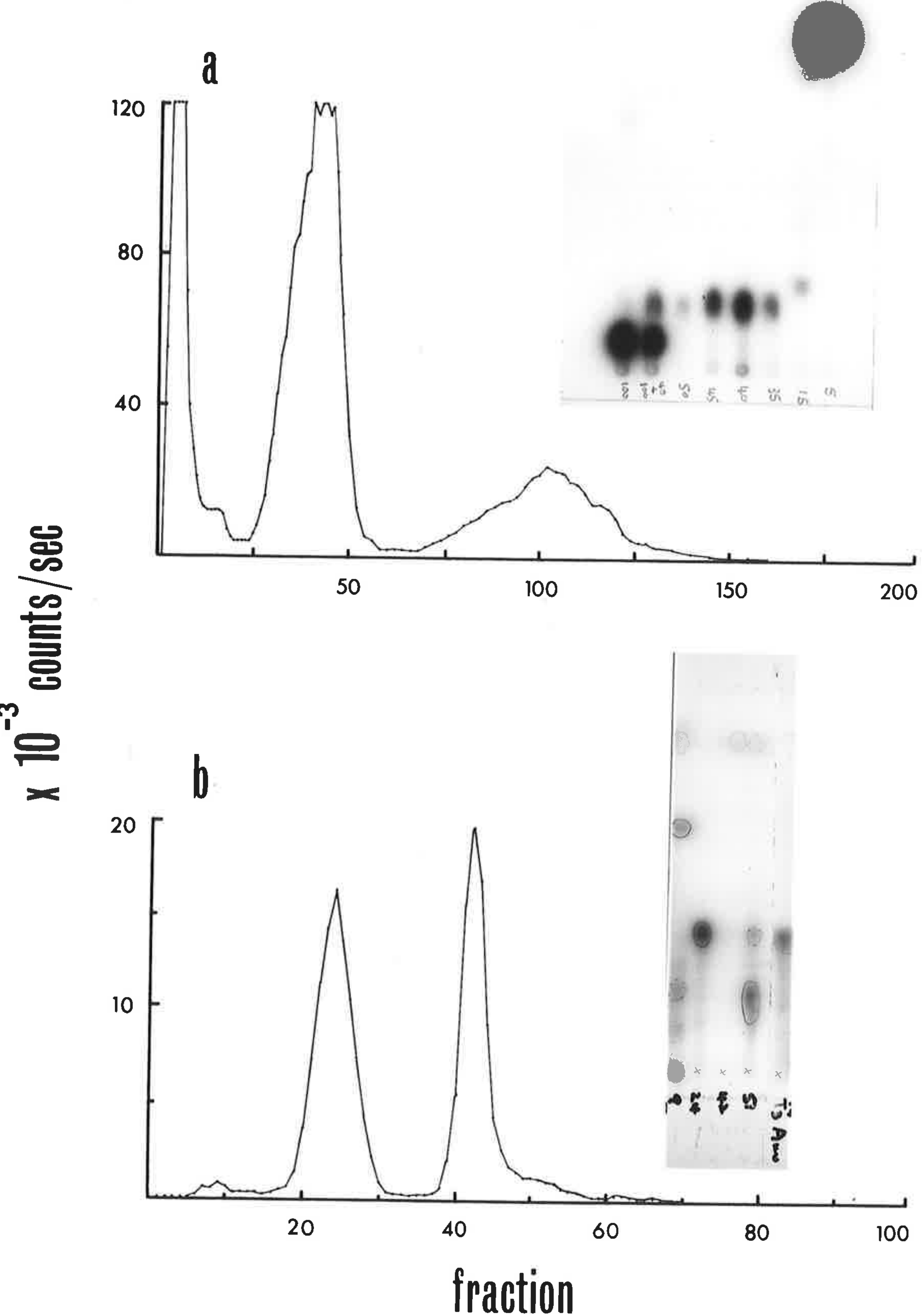
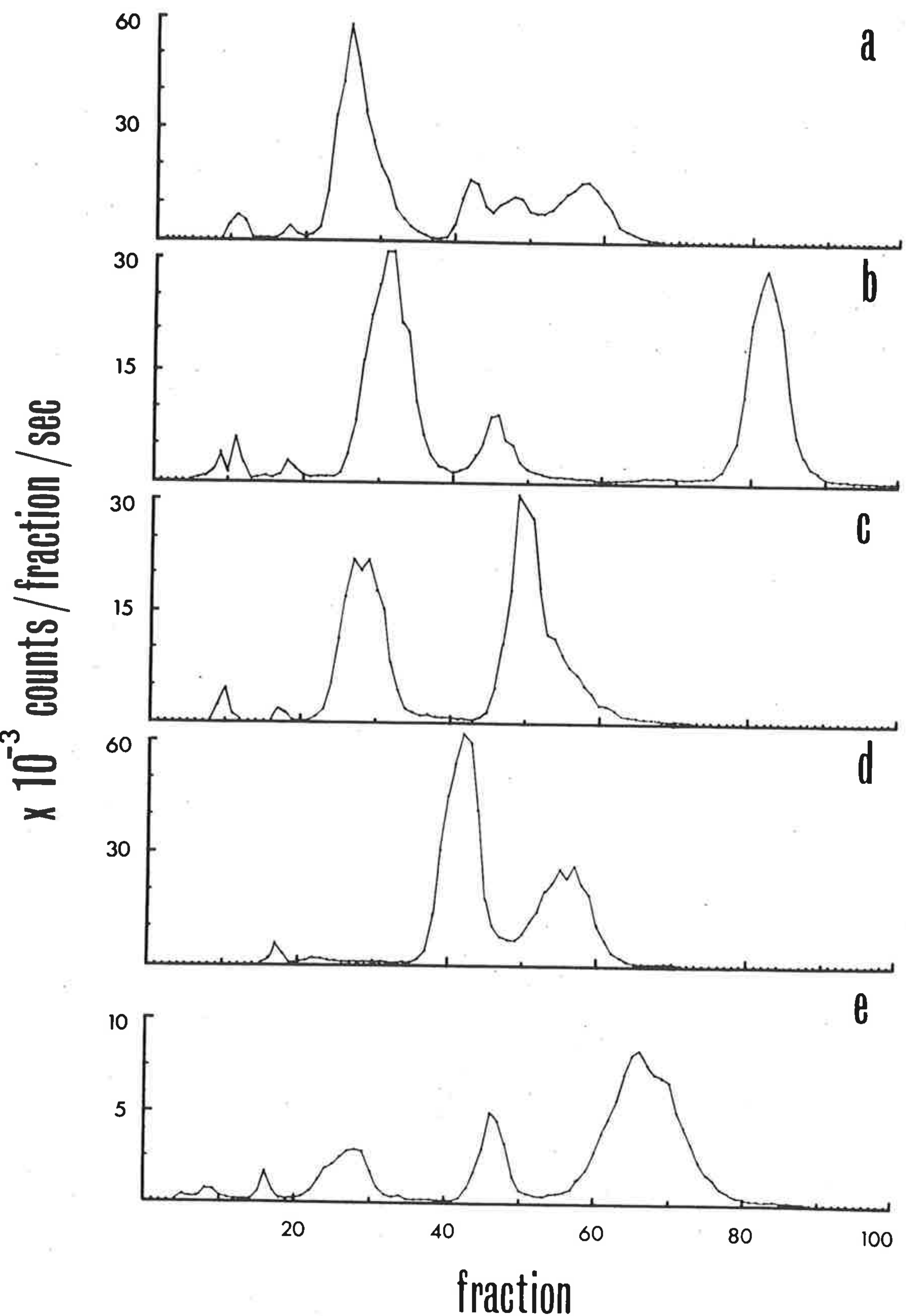

a
120
80
40
50
100
150
200
x 10-3 counts/sec
b
20
10
20
40
60
80
100
fraction

Figure 5.3 Elution of reaction mixture post radio-iodination of T_3

Elution of reaction mixture through a 22 x 1.1 cm Sephadex LH-20 column. Eluting solvent was ethyl acetate/methanol/NH_4OH/H_2O (400:100:36:4).
Fraction size was 2 mL.
Represented are 5 different reaction mixture elution profiles.
Thin layer chromatograms of fractions in profiles (a), (d) and (e) are shown in Figure 5.4.

	Fraction	Identity	Ve/Vt
(a)	27	$^{125}I\text{-}T_3$	0.6
	43	$^{125}I\text{-}I^-$	1
	49	$^{125}I\text{-}T_4 + ^{125}I\text{-}I^-$	
	58	$^{125}I\text{-}T_4$	1.4
(b)	32	$^{125}I\text{-}T_3$	0.7
	46	$^{125}I\text{-}I^-$	1
	82	$^{125}I\text{-}T_4$	1.8
(c)	28	$^{125}I\text{-}T_3$	0.6
	48	$^{125}I\text{-}I^-$	1
	56	$^{125}I\text{-}T_4$	1.1
(d)	42	$^{125}I\text{-}I^-$	1
	57	$^{125}I\text{-}T_4$	1.4
(e)	28	$^{125}I\text{-}T_3$	0.6
	45	$^{125}I\text{-}I^-$	1
	66	$^{125}I\text{-}T_4$	1.5



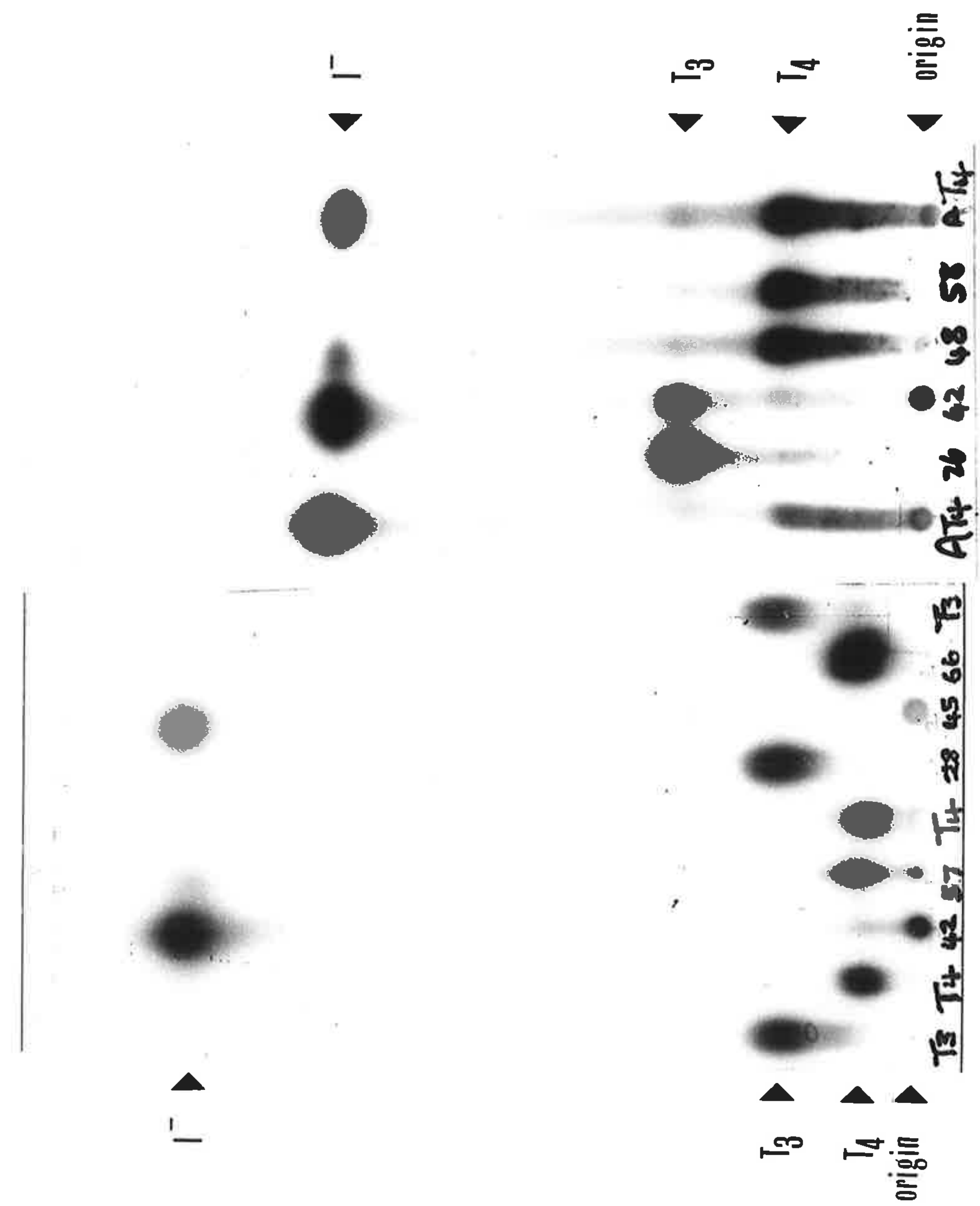
60
30
a
30
15
b
30
15
c
60
30
d
10
5
e
x 10^{-3} counts/fraction/sec
20
40
60
80
100
fraction

Figure 5.4 Thin layer chromatography of fractions eluted from the T_3 iodination reaction mixture

Chromatograms of fractions shown in profiles in Figure 5.3.

Shown are fractions 42 and 57 of profile (d); fractions 28, 45 and 66 of profile (e); and fractions 26, 42, 48 and 58 of profile (a).

$^{125}I-T_3$ and $^{125}I-T_4$ markers are included.



I⁻
T3
T4
origin

Figure 5.5 Self-displacement of $^{125}I-T_3$

$^{125}I-T_3$ from The Radiochemical Centre, U.K.:

□——□ Self-displacement

■······■ Displacement by unlabelled T_3

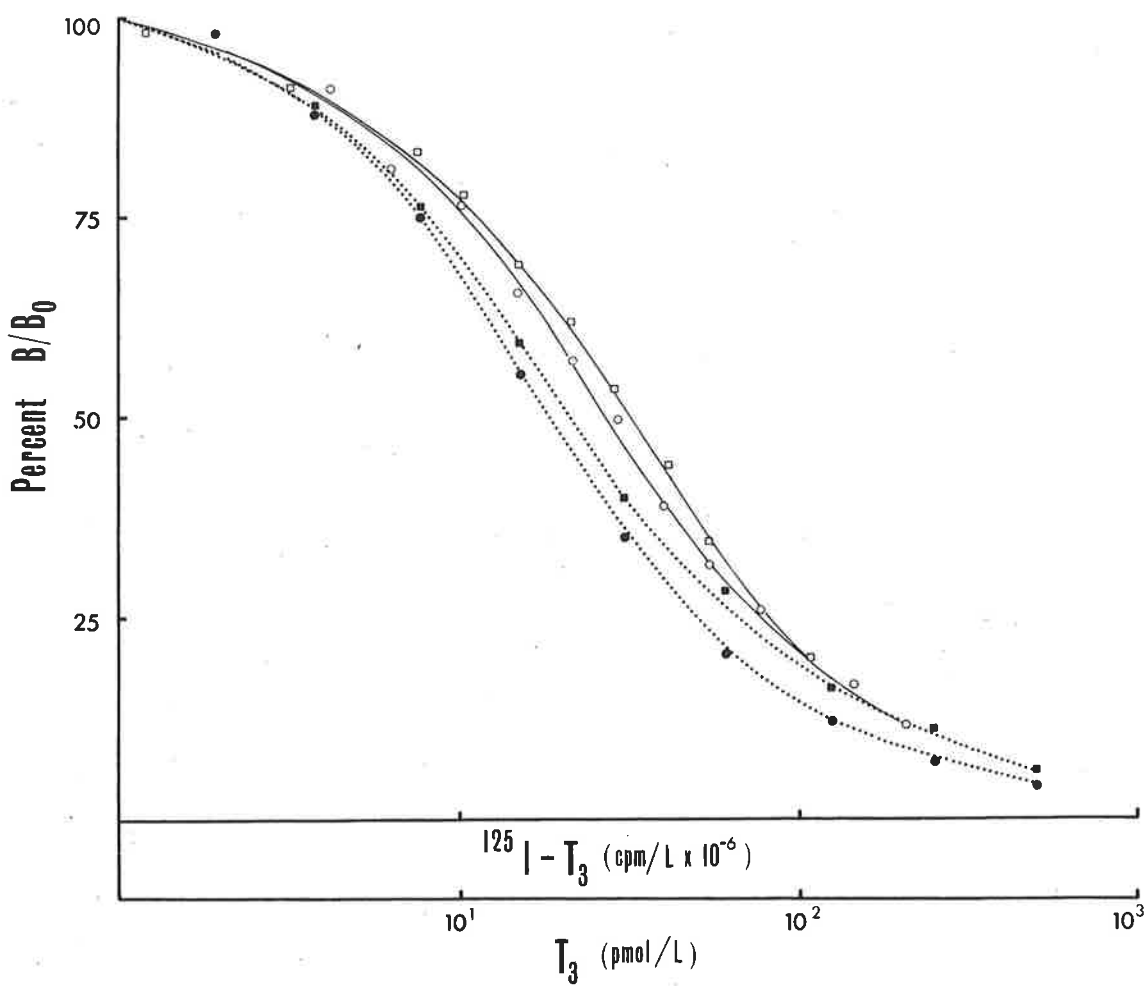
Laboratory preparation of $^{125}I-T_3$:

○——○ Self-displacement

●······● Displacement by unlabelled T_3.

The average specific activity of the Radiochemical Centre preparation was 1389 pCi/pg and that of the laboratory preparation 1376 pCi/pg.

Both $^{125}I-T_3$ (cpm/L x 10^{-6}) and T_3 (pmol/L) are represented on the same scale.



100
75
50
25
Percent B/B0
125 I - T3 (cpm/L x 10-6)
10¹
10²
10³
T3 (pmol/L)

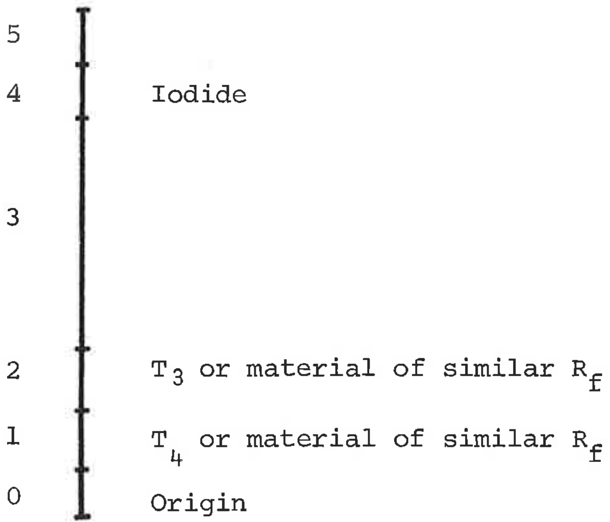
age, showed the tracer to be particularly stable in this solvent (Table 5.2). Charcoal binding of iodinated material did not increase substantially with age, even in samples up to 6 months old, suggesting limited accumulation of ^{125}I-iodide with age. This was confirmed by thin layer chromatography of the tracer samples. However, the ^{125}I-T_3 content did decrease with age and was followed by a concomitant fall in tracer binding to antiserum. Although little ^{125}I-iodide was evident, there was an accumulation of material with a similar R_f to T_4. Although it was not identified, it would seem unlikely that this material was ^{125}I-T_4. Kochupilla and Yalow (1978) have demonstrated similar excellent stability of ^{125}I-T_3 in 100% ethanol, ammoniated methanol, and 50% polyethylene glycol.

^{125}I-T_4 underwent a different pattern of changes during storage (Table 5.2). The percent of radioactivity with R_f similar to T_4 diminished with age, whereas the percent of radioactive material with R_f between that of T_3 and iodide and with R_f similar to iodide increased. Binding of tracer to antiserum and charcoal confirmed this pattern of deiodination.

^{125}I-T_4 preparations showed more rapid aging than ^{125}I-T_3, as has been confirmed by both Kochupilla and Yalow (1978) and Kjeld et al. (1975). The commercial ^{125}I-T_4 preparations (The Radiochemical Centre, U.K.) showed consistently greater adsorption to charcoal than the laboratory prepared tracer. It was concluded that either the commercial preparations were less highly purified or that ^{125}I-iodide was accumulating during shipment.

TABLE 5.2 Storage of ^{125}I-T_3 and ^{125}I-T_4 in Ethanol/Water (3:1)

Aging of stored tracer was studied by thin layer chromatographic analysis of different batches of tracer which were of varying age (expressed as days post iodination). The chromatograms were divided into the following regions after autoradiographic localization of the chromatographed species.



Each region was scraped from the chromatographic plate, counted, and expressed as a percent of the sum of all the regions.

Stored tracer was also assessed for binding to charcoal (% blank), binding to antiserum (B_o/T), and assay sensitivity in the case of ^{125}I-T_3 (expressed as % $B_{7.8}/B_o$ or the relative displacement of tracer in the presence of 7.8 pmol/L standard).

TABLE 5.2 (continued)

Batch	Age (days)	Chromatographic region 0	1	2	3	4	5	% Blank	B_o/T	$B_{7.8}/B_o$
		^{125}I-T_3 (Amersham)								
75	185	5.2	30.6	53.5		7.4	5.3	9.6	.331	71
76	171	5.3	19.2	62.0	9.0	3.5	1.1	9.4	.498	71
77	157	3.5	14.4	66.9	9.8	4.7	0.7	11.3	.548	75
80	115	3.1	11.4	69.8	9.8	4.7	1.2	13.8	.627	78
82	87	2.5	5.7	56.1	11.3	23.4	0.9	39.9	.452	80
84	59	3.1	8.8	75.0	9.2	3.3	0.7	9.0	.662	84
86	31	2.2	6.2	79.9	8.5	2.2	0.9	16.2	.401	73
88	3	1.5	5.8	85.0	5.5	1.7	0.5	8.8	.710	80
		^{125}I-T_4 (Amersham)								
104	84	5.1	57.7	4.2	10.1	22.0	0.8			
104	21							29.9	.400	
104	15							27.7	.546	
108	42	5.1	69.2	3.5	7.5	14.3	0.5			
108	14							20.3	.466	
112	0	2.3	79.5	2.6	4.8	9.4	1.4			

Both $^{125}I-T_4$ and $^{125}I-T_3$ were less stable in the ethyl acetate eluting solvent than in aqueous ethanol. Fractions were thus dried down and stored in ethanol/water (1:1).

ANTISERUM CHARACTERISTICS

1. Cross-Reaction

None of the iodothyronines or iodotyrosines studied showed levels of cross-reaction in the serum-free T_3 assay which would cause interference when assaying the dialysates (Table 5.3). Specificity requirements for an antiserum in the fT_3 assay are quite different to those in the serum T_3 assay. Thus serum T_4 concentration is approximately 50 times that of T_3 whereas the ratio of the serum fT_4 and fT_3 concentrations ($[fT_4]/[fT_3]$) is between 1 - 2. Serum concentrations of 3,5-T_2 and 3,3'-T_2 are 20 - 40 times less than that of T_3 whereas, in view of the lower affinity of these diiodothyronines for the thyronine binding proteins, it is likely that $[fT_2]/[fT_3]$ is considerably less than 20 - 40.

In the serum-free T_4 assay, only rT_3 cross-reacted at a level which might cause interference in the assay (Table 5.3). Direct measurements of rT_3 in the dialysates by radioimmunoassay showed the free rT_3 (frT_3) concentration to be as high as 3pmol/L. Using the dialyzable fraction of rT_3, Chopra et al. (1975a) showed frT_3 to vary between 0.9 - 2.5 pmol/L in normal adults. At 7.5% cross-reaction, 3pmol/L frT_3 would be equivalent to 0.23 pmol/L fT_4. This was considered insignificant for the purposes of assaying fT_4.

TABLE 5.3 Cross-reaction of iodothyronines and iodotyrosines in the serum-free T_3 and T_4 assays

$$\text{percent cross-reaction} = \frac{\text{moles of cross-reactant giving 50\% displacement of tracer}}{\text{moles of standard hormone giving 50\% displacement of tracer}} \times 100$$

	Serum-free T_3 assay antiserum # 478	Serum-free T_4 assay antiserum # 492
T_4	0.39	100
T_3	100	0.22
3,3'-T_2	1.67	0.08
3,5-T_2	1.50	0.015
rT_3	0.008	7.54
MIT	0.01	0.002
DIT	0.001	0.025

2. Concentration and Affinity of Antibody Binding Sites

Analysis of the displacement of ^{125}I-T_3 from the T_3 antiserum #478 revealed two species of binding sites (Figure 5.6). One species had a very high affinity site (K_1 1.72 x 10^{11} L/mol, q_o 4.24 x 10^{-5} mol/L), whilst the other was of lower affinity and had nearly three times the binding capacity (K_2 4.73 x 10^9 L/mol, r_o 1.17 x 10^{-4} mol/L).

A Scatchard plot of the displacement of ^{125}I-T_4 from the T_4 antiserum #492 revealed one binding species with association constant of 4.98 x 10^{10} L/mol and concentration 2.3 x 10^{-5} mol/L (Figure 5.12).

OPTIMISATION OF ASSAY SENSITIVITY AND PRECISION

1. Empirical Procedures

(i) Free T_3 Assay - For the purposes of titrating the T_3 antiserum, a tracer concentration was selected which was similar to the sensitivity required, namely 1 to 2 pmol/L. A study of the binding of ^{125}I-T_3 at antiserum dilutions between 1/(1 x 10^6) and 1/(64 x 10^7) suggested that dilutions in the range 1/(2 x 10^6) to 1/(8 x 10^6) would be most suitable for establishing an assay (Figure 5.7).

Displacement studies at the dilutions 1/(2 x 10^6), 1/(4 x 10^6), 1/(6 x 10^6) and 1/(8 x 10^6) showed greatest displacement by each dose of unlabelled T_3 added at the latter two dilutions (Figure 5.8). While there was little difference in the relative displacement of tracer by unlabelled T_3 at these two antiserum dilutions, binding of ^{125}I-T_3 at an antiserum dilution of 1/(6 x 10^6) was nearly

Figure 5.6 Characterization of T_3 antiserum # 478

Scatchard Analysis of displacement of $^{125}I\text{-}T_3$ from antiserum # 478 by unlabelled T_3.

p* 1.71 fmole/tube

Antiserum dilution $1/(8 \times 10^6)$

B - concentration of labelled + unlabelled T_3 bound to antiserum (pmol/L)

K_1 1.72×10^{11} L/mol

q_o 4.24×10^{-5} mol/L

K_2 4.73×10^{9} L/mol

r_o 1.17×10^{-4} mol/L

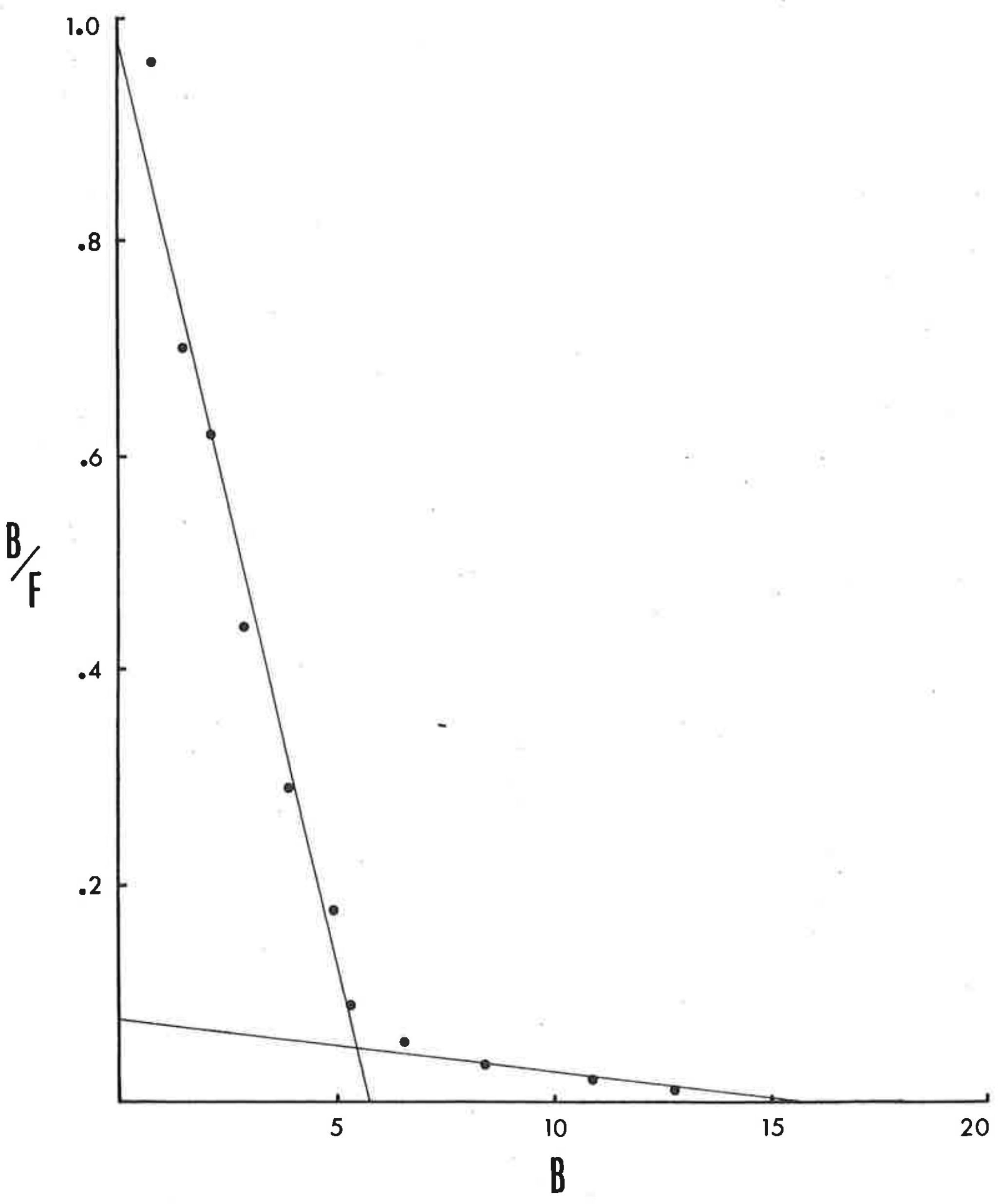

1.0
.8
.6
.4
.2
B/F
5
10
15
20
B

Figure 5.7 Optimisation of serum-free T_3 assay reagent concentrations

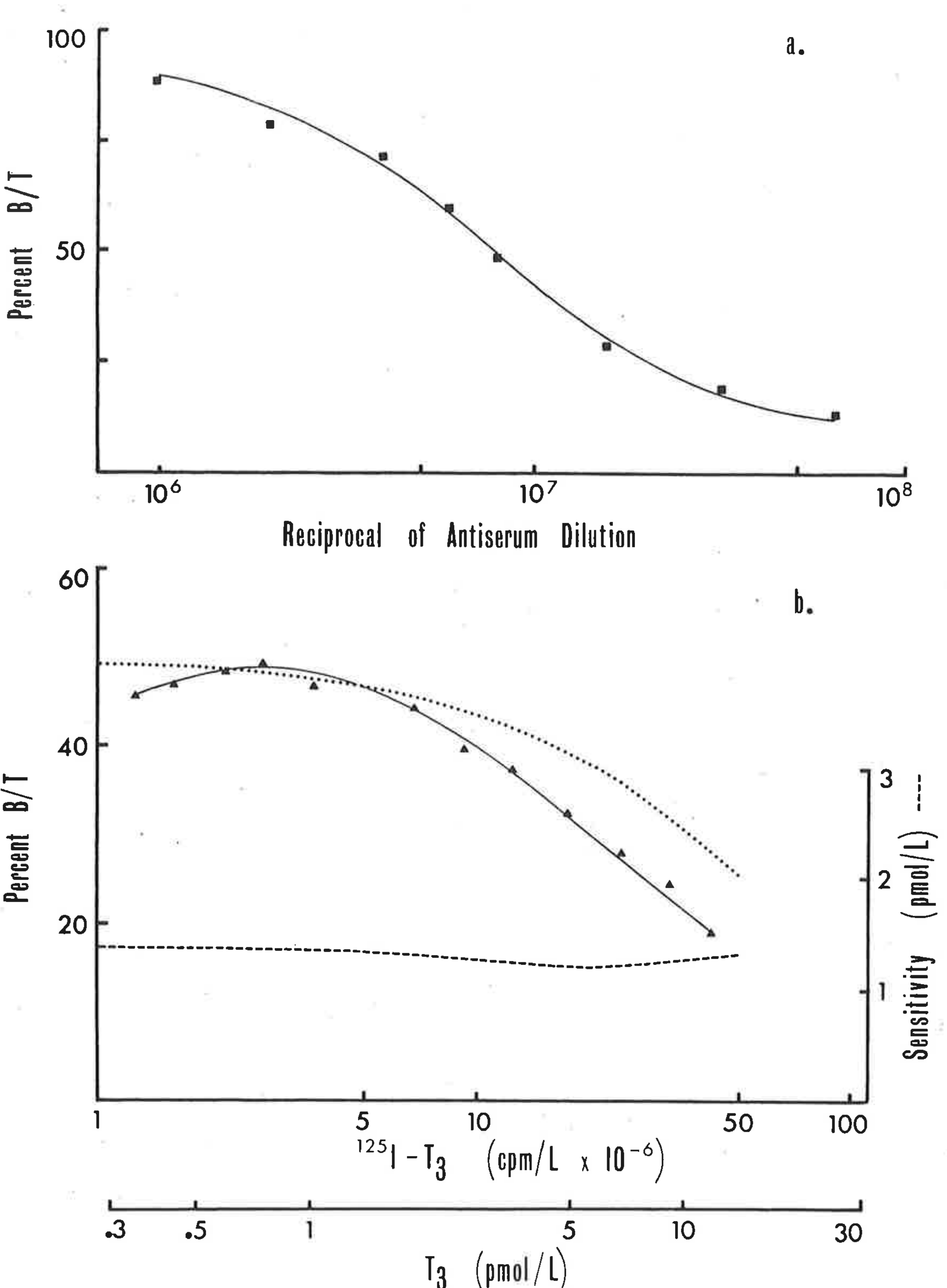
(a) Titration of T_3 antiserum # 478

(b) Binding of ^{125}I-T_3 to T_3 antiserum # 478 at varying concentrations of added tracer in the absence of unlabelled T_3. Antiserum was at a final dilution of $1/6 \times 10^6$.

▲——▲ experimental data

······· theoretical data (one binding species model - equation A11)

----- theoretical calculation of sensitivity at various concentrations of ^{125}I-T_3 (one binding species model - equation A14)



a.
100
50
Percent B/T
10^6
10^7
10^8
Reciprocal of Antiserum Dilution
b.
60
40
20
Percent B/T
3
2
1
Sensitivity (pmol/L) ---
1
5
10
50
100
125I - T3 (cpm/L x 10^-6)
.3
.5
1
5
10
30
T3 (pmol/L)

Figure 5.8 Optimisation of serum-free T_3 assay reagent concentrations

One binding species model - Appendix equation A11.

p*	1.71 fmole/tube
K	1.72×10^{11} L/mol
q_o	4.6×10^{-5} mol/L

(a) Displacement of ^{125}I-T_3 by added unlabelled T_3 at various antiserum dilutions

●—● experimental data

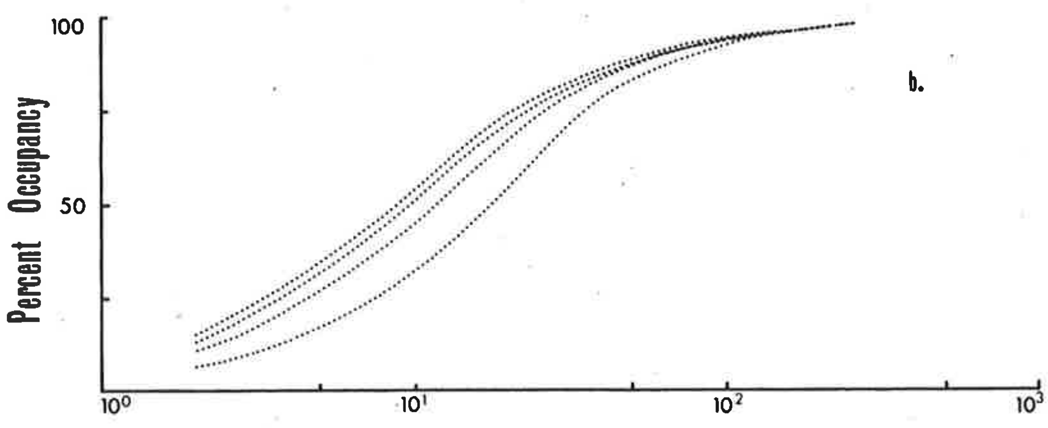
······· theoretical data

Reading from left to right antiserum dilutions are $1/(8 \times 10^6)$, $1/(6 \times 10^6)$, $1/(4 \times 10^6)$, $1/(2 \times 10^6)$.

Antiserum	B_o/T		Sensitivity (pmol/L)	
dilution	expt.	theor.	expt.	theor.
$1/(2 \times 10^6)$	.7366	.7836	5.2	4.8
$1/(4 \times 10^6)$	.7003	.6374	0.8	3.7
$1/(6 \times 10^6)$	.6012	.5349	0.5	3.0
$1/(8 \times 10^6)$	.4909	.4582	0.3	1.9

(b) Occupancy of binding sites calculated using equation A12.

Reading from left to right antiserum dilutions are $1/(2 \times 10^6)$, $1/(4 \times 10^6)$, $1/(6 \times 10^6)$, $1/(8 \times 10^6)$.


100
50
Percent Occupancy
b.
10^0
10^1
10^2
10^3

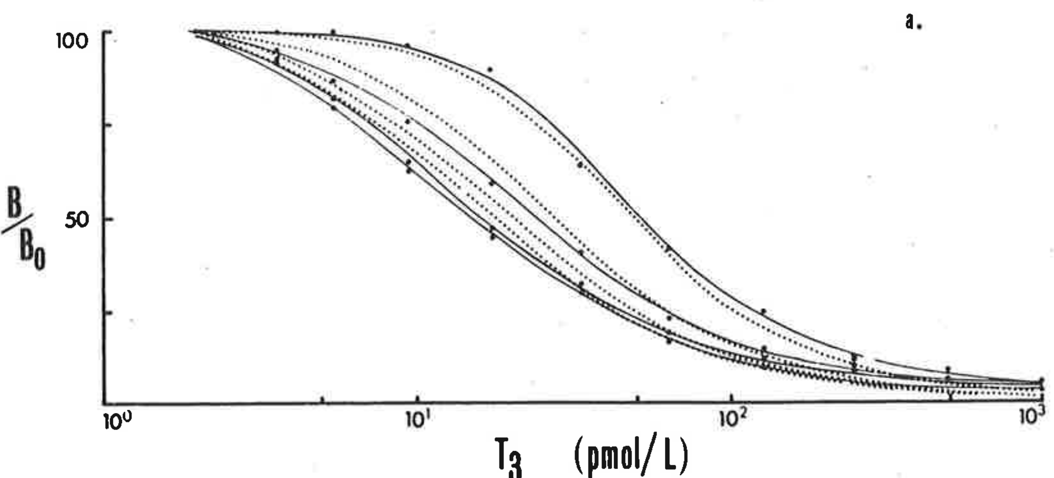

a.
100
50
B/B0
10^0
10^1
10^2
10^3
T3 (pmol/L)

Figure 5.9 Optimisation of serum-free T_3 assay reagent concentrations

Two binding species model - Appendix equation A15

p^* 1.71 fmol/tube

K_1 1.72×10^{11} L/mol

K_2 4.73×10^{9} L/mol

q_o 4.24×10^{-5} mol/L

r_o 1.17×10^{-4} mol/L

(a) Displacement of $^{125}I\text{-}T_3$ by added unlabelled T_3 at various antiserum dilutions

●——● experimental data

······· theoretical data

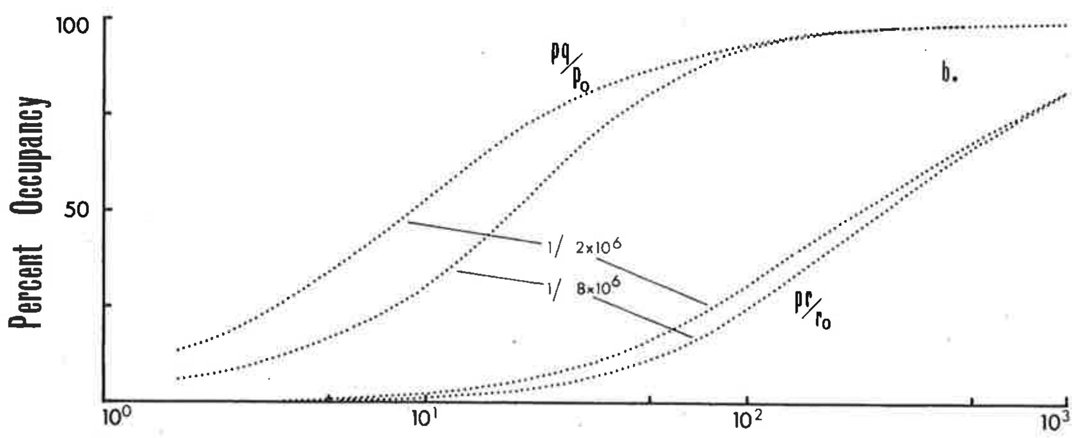
Reading from left to right antiserum dilutions are $1/(8 \times 10^6)$, $1/(6 \times 10^6)$, $1/(4 \times 10^6)$, $1/(2 \times 10^6)$.

Antiserum dilution	B_o/T expt.	B_o/T theor.	Sensitivity (pmol/L)
$1/(2 \times 10^6)$	.7366	.8117	5.2
$1/(4 \times 10^6)$	.7003	.6784	0.8
$1/(6 \times 10^6)$	.6012	.5790	0.5
$1/(8 \times 10^6)$	.4909	.5008	0.3

(b) Occupancy of binding sites calculated using equation A16 and A17.

high affinity species (pq/q_o)

low affinity species (pr/r_o)


100
50
Percent Occupancy
pq/p0
b.
1/ 2x10^6
1/ 8x10^6
pr/r0
10^0
10^1
10^2
10^3

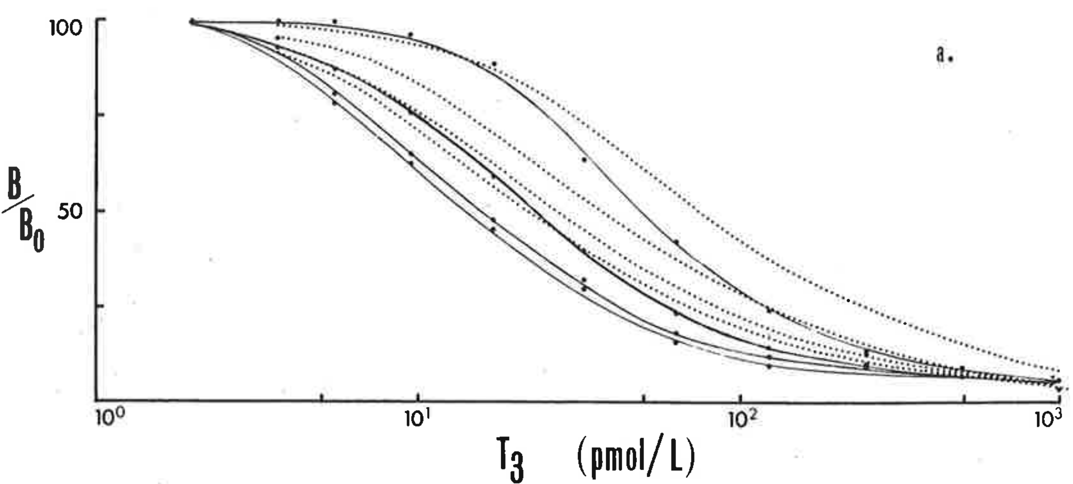

100
50
B/B0
a.
10^0
10^1
10^2
10^3
T3 (pmol/L)

Figure 5.10 Serum-free T_3 assay precision profile

a. Experimentally determined precision profile.

○——○	antiserum # 395; charcoal
△——△	antiserum # 395; double antibody
□——□	antiserum # 478; charcoal

All antisera at dilution of $1/(6 \times 10^6)$.

b. Theoretically determined precision profile (calculations outlined in the appendix Section A4).

———	(calculated using 2.5% C.V. in measurement of concentration of tracer bound. Reading from left to right antiserum dilutions are $1/(8 \times 10^6)$, $1/(6 \times 10^6)$, $1/(4 \times 10^6)$, $1/(2 \times 10^6)$.
-----	calculated using 5.0% C.V. in measurement of concentration of tracer bound. Antiserum dilution $1/(6 \times 10^6)$.

Experimental data on the coefficient of variation in measuring the concentration of tracer bound is tabulated on the page following the figure.

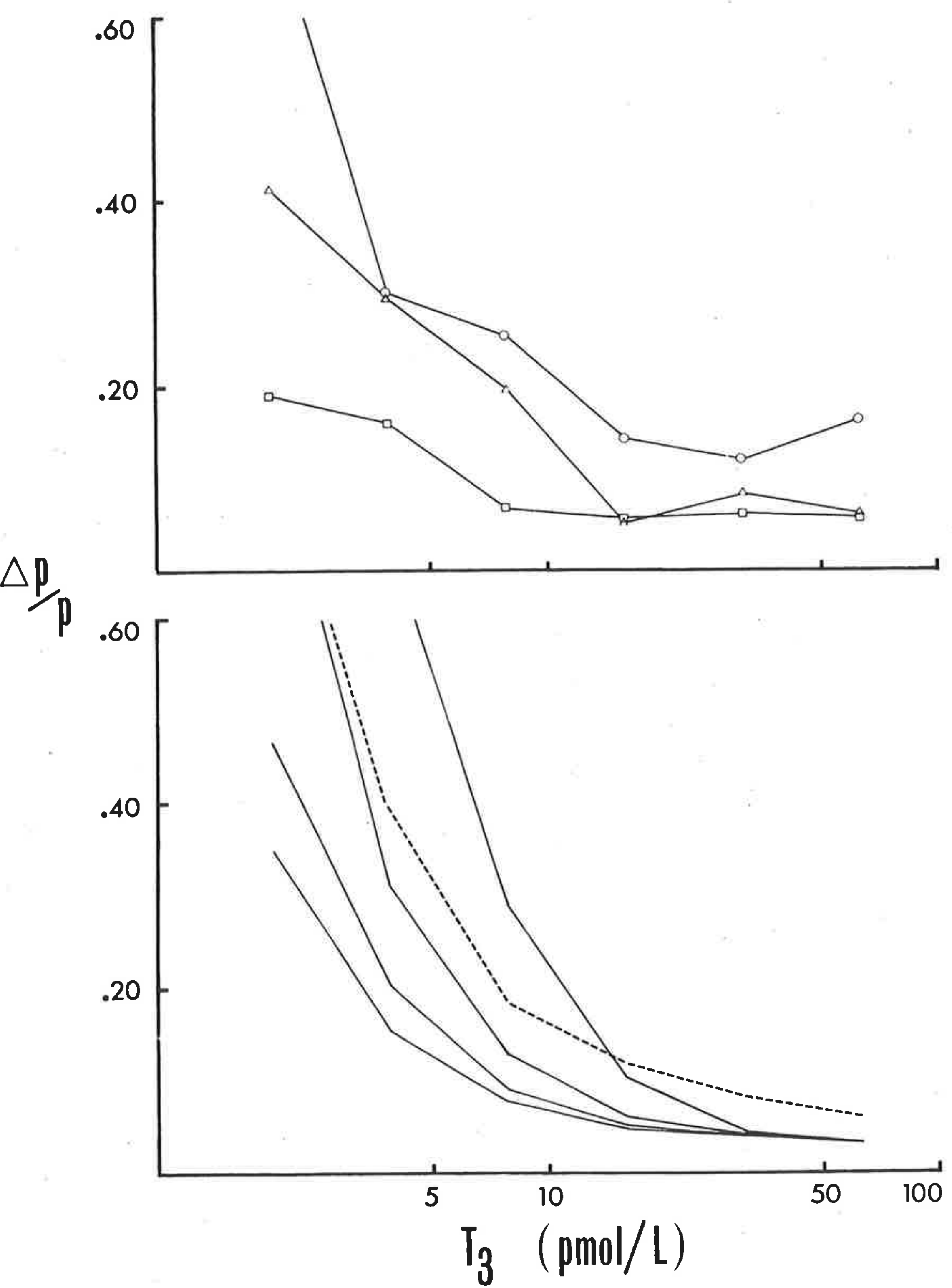

.60
.40
.20
$\Delta p/p$
.60
.40
.20
5
10
50
100
T_3 (pmol/L)

Figure 5.10 (continued) Tabulation of experimentally determined percent coefficients of variation in measuring the concentration of tracer bound

	Charcoal # 395 $1/(6 \times 10^6)$	Double antibody # 395 $1/(6 \times 10^6)$	Charcoal # 478 $1/(6 \times 10^6)$
B_o/T	.2618	.2608	.7499
B_o (cpm)	14,000	14,045	9,088
T_3 (pmol/L)			
0	3.45	3.90	3.45
2.0	2.74	5.06	1.93
3.9	3.95	3.88	3.31
7.8	5.12	6.86	2.45
15.6	2.22	6.08	3.28
31.3	10.6	5.53	5.51
62.5	10.4	6.53	5.66

Data were determined by calculating the standard deviation of the counts bound at each concentration of unlabelled T_3.

Figure 5.11 95% confidence limits of estimates of T_3 concentration

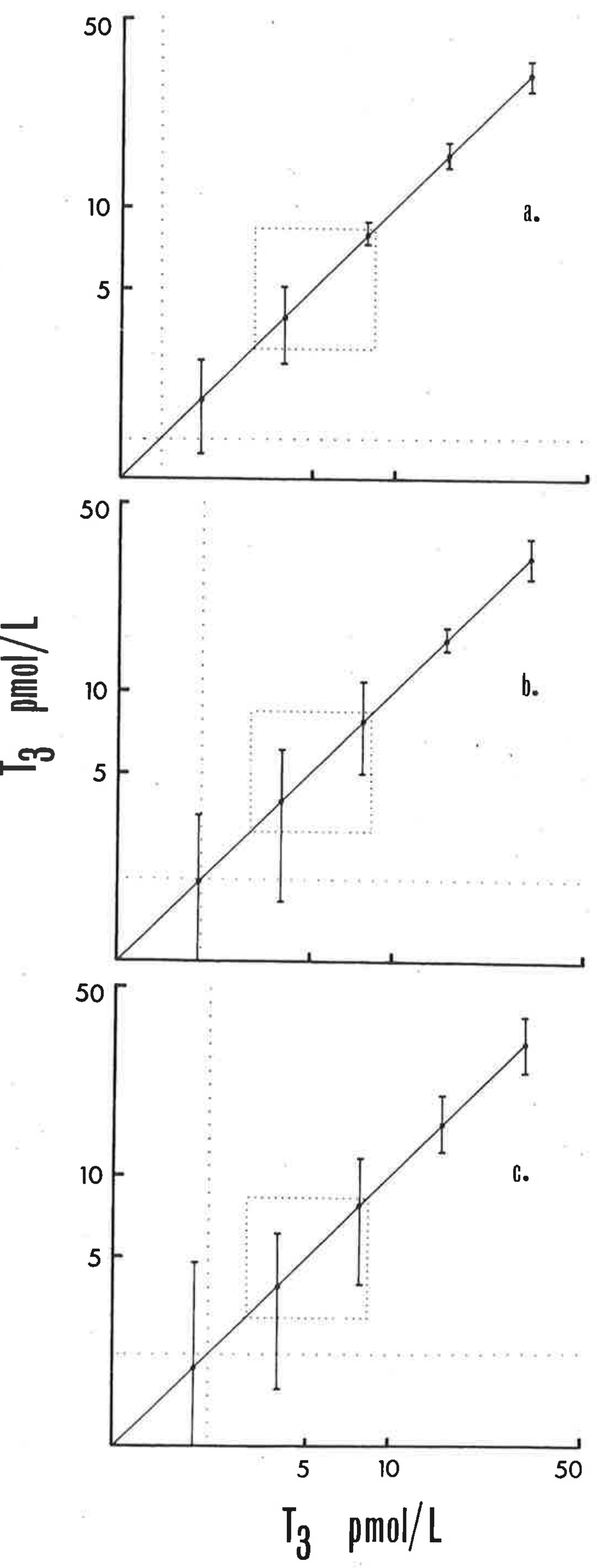
Precision profiles are shown in Figure 5.10a

a. Charcoal assay; antiserum # 478, $1/(6 \times 10^6)$ dilution.

b. Double antibody assay; antiserum # 395, $1/(6 \times 10^6)$ dilution.

c. Charcoal assay; antiserum # 395, $1/(6 \times 10^6)$ dilution.

Marked on each diagram is the fT_3 reference range (square) and the experimentally determined sensitivity (calculated from the standard deviation of B_o/T).


50
10
5
a.
50
10
5
b.
50
10
5
c.
5
10
50
T_3 pmol/L
T_3 pmol/L

20% greater than that at antiserum dilution $1/(8 \times 10^6)$, and the dilution $1/(6 \times 10^6)$ was thus chosen for further assessment.

The percent binding of $^{125}I\text{-}T_3$ was checked at concentrations of tracer ranging from 0.40 - 10 pmol/L (Figure 5.7). The percent $^{125}I\text{-}T_3$ bound rose to a maximum at 0.90 pmol/L and then fell steadily at higher concentrations of tracer. It was concluded that a concentration of tracer between 0.50 - 1.50 pmol/L was suitable, the higher concentrations having the advantage of providing higher counts per tube. In practise, 5000 - 6000 cpm per tube was usually employed which, depending on specific activity, ensured 1.5 - 1.7 fmoles tracer per tube.

The precision profile of the fT_3 assay using antiserum #478 is shown in Figure 5.10. The profile was calculated from duplicate data pooled from 9 assays. Using the standard deviation of the percent binding of tracer in the absence of standard (percent Bo/T), the sensitivity was calculated as 1.4 pmol/L. The precision profile shows the sensitivity to be less than 2.0 pmol/L as the 2.0 pmol/L standard had a 2 S.D. range of 1.2 - 2.7 pmol/L, well above zero. Furthermore, the profile shows the assay is capable of assaying fT_3 concentration below the normal range of 2.8 - 8.4 pmol/L.

(ii) Free T_4 Assay - To establish the most suitable concentration of tracer and antibody a comprehensive initial study was undertaken. The antiserum was titrated at four different tracer concentrations ranging from 1 to 6.2 pmol/L (Figure

5.13). Binding of $^{125}I-T_4$ to antiserum in the presence of 15.6 pmol/L unlabelled T_4 at these antibody and tracer concentrations was also measured. Binding in the presence of 15.6 pmol/L T_4 was expressed as a percent of binding in the absence of unlabelled T_4 and used as a guide to the sensitivity which could be expected at a particular tracer and antiserum concentration (Figure 5.13).

Greatest displacement of tracer by 15.6 pmol T_4 was observed at antiserum dilutions of $1/(2 \times 10^6)$ and $1/(4 \times 10^6)$ (Figure 5.13). An antiserum dilution of $1/(2 \times 10^6)$ was selected for further assessment as the displacement study suggested it was unlikely that better sensitivity would be attained at higher dilutions.

$^{125}I-T_4$ binding increased at each antiserum dilution as the tracer concentration approached 4 fmoles/tube (equivalent to 13,800 cpm/tube at a specific activity of 1600 pCi/pg) (Figure 5.15a). Displacement of $^{125}I-T_4$ by 15.6 pmole/L of unlabelled T_4 was maximum at this tracer concentration. At higher tracer concentrations, binding began to diminish due to self-displacement, and displacement by 15.6 pmol/L unlabelled T_4 decreased (Figure 5.13b). The precision profile was calculated from duplicate data pooled from four assays (Figure 5.16). Using the standard deviation of the zero binding (Bo/T), sensitivity was calculated to be 0.6 pmol/L, well below the normal range (3.5 - 13.6 pmol/L). This may be an underestimate of the sensitivity, as the precision of the lowest standard was 3.9 ± 2.5 (2 S.D.) pmol/L. The sensitivity would be more likely to be somewhere closer to 2.5 pmol/L (the 2 x S.D. of

the 3.9 pmol/L standard).

(iii) Summary - These studies demonstrate that serum free assays which have adequate sensitivity to measure free thyroid hormone concentration can be established by straightforward empirical means. While high specific activity tracer is now readily available commercially, high affinity antisera are not generally obtainable and must usually be produced in the laboratory. It is particularly interesting that the concentrations of antibody and tracer found to give assays of the required sensitivity are quite different to those recommended by Ekins et al. (1968). Ekins suggested, as a guide to establishing assays of high sensitivity, tracer concentration of 4/K and antibody concentration 3/K.

Considering only the high affinity T_3 binding site, the recommended concentration of tracer is 10 - 20 times that used in both the fT_3 and fT_4 assays. Similarly, the recommended antibody concentration is 3 - 5 times greater than that used in these assays. While the concentration of reagents suggested by Ekins to obtain optimum sensitivity applied only to assays with one binding species, the discrepancy between the concentration of reagent used and those recommended were as marked in the serum-free T_4 assay as those in the serum-free T_3 assay.

2. Models of Ligand Binding

Equations have been derived from the law of mass action describing the binding of hormone to one species of antibody under both equilibrium conditions (Ekins et al., 1968; Yalow and Berson, 1968) and non-

Figure 5.12 Scatchard analysis of T_4 antisera

●——● # 492 - bleed 1

$K = 4.98 \times 10^{10}$ L/mol

$q_o = 2.26 \times 10^{-5}$ mol/L

○---○ # 492 - bleed 2

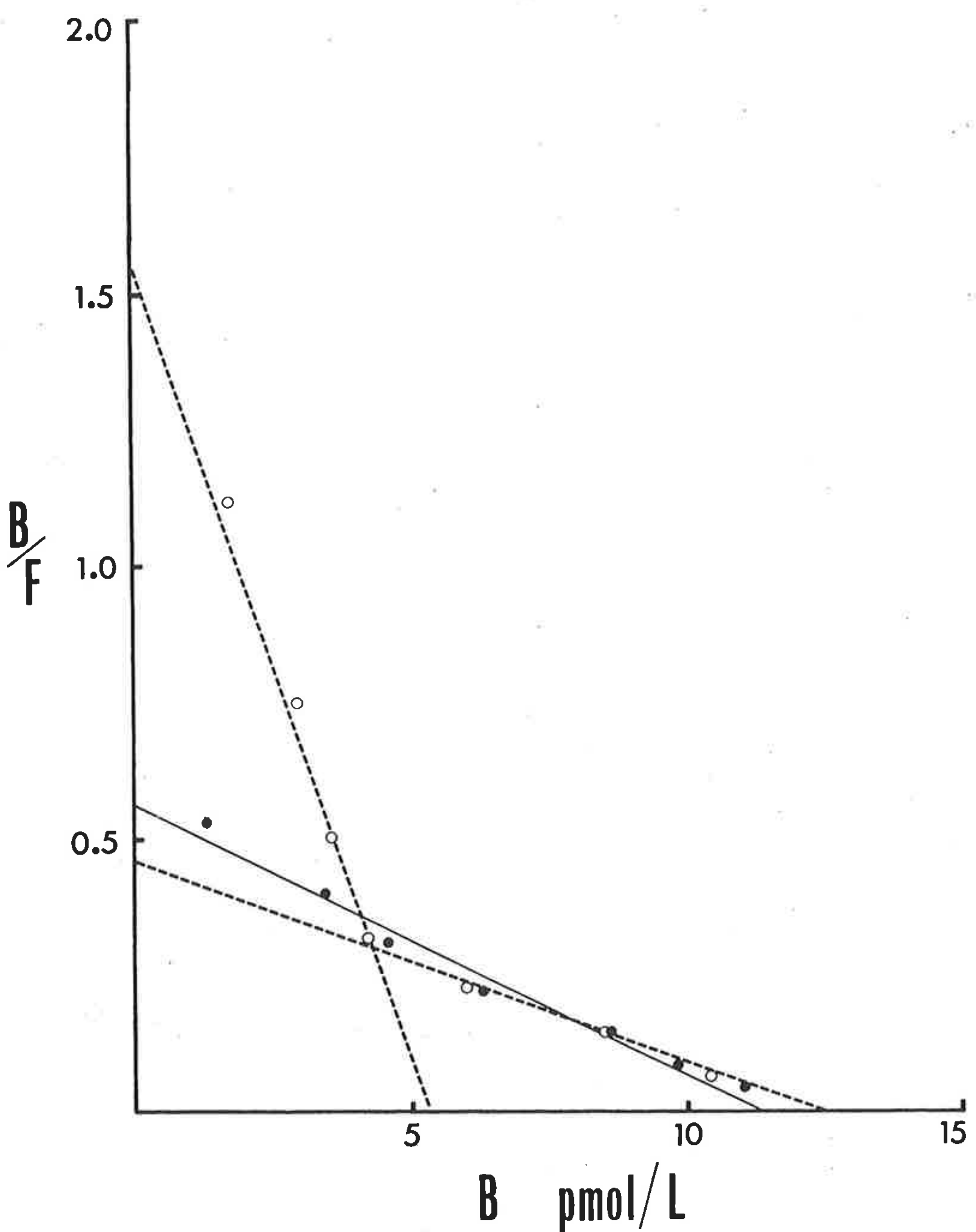
$K_1 = 2.87 \times 10^{11}$ L/mol

$q_o = 1.08 \times 10^{-5}$ mol/L

$K_2 = 3.66 \times 10^{11}$ L/mol

$r_o = 2.46 \times 10^{-5}$ mol/L

Antisera dilution $1/(2 \times 10^6)$



2.0
1.5
1.0
0.5
B/F
5
10
15
B pmol/L

Figure 5.13 Characterization of T_4 antiserum # 492

(a) Binding of $^{125}I-T_4$ to T_4 antiserum at various concentrations of tracer and antiserum dilutions.

(b) Relative displacement of $^{125}I-T_4$ by 15.6 pmol/L unlabelled T_4 at the same tracer concentrations and antiserum dilutions as in (a).

Symbol	Count	Concentration
●—●	3367 cpm/tube	(1.02 pmol/L)
○—○	9823 cpm/tube	(2.85 pmol/L)
■—■	14568 cpm/tube	(4.22 pmol/L)
□—□	21498 cpm/tube	(6.23 pmol/L)

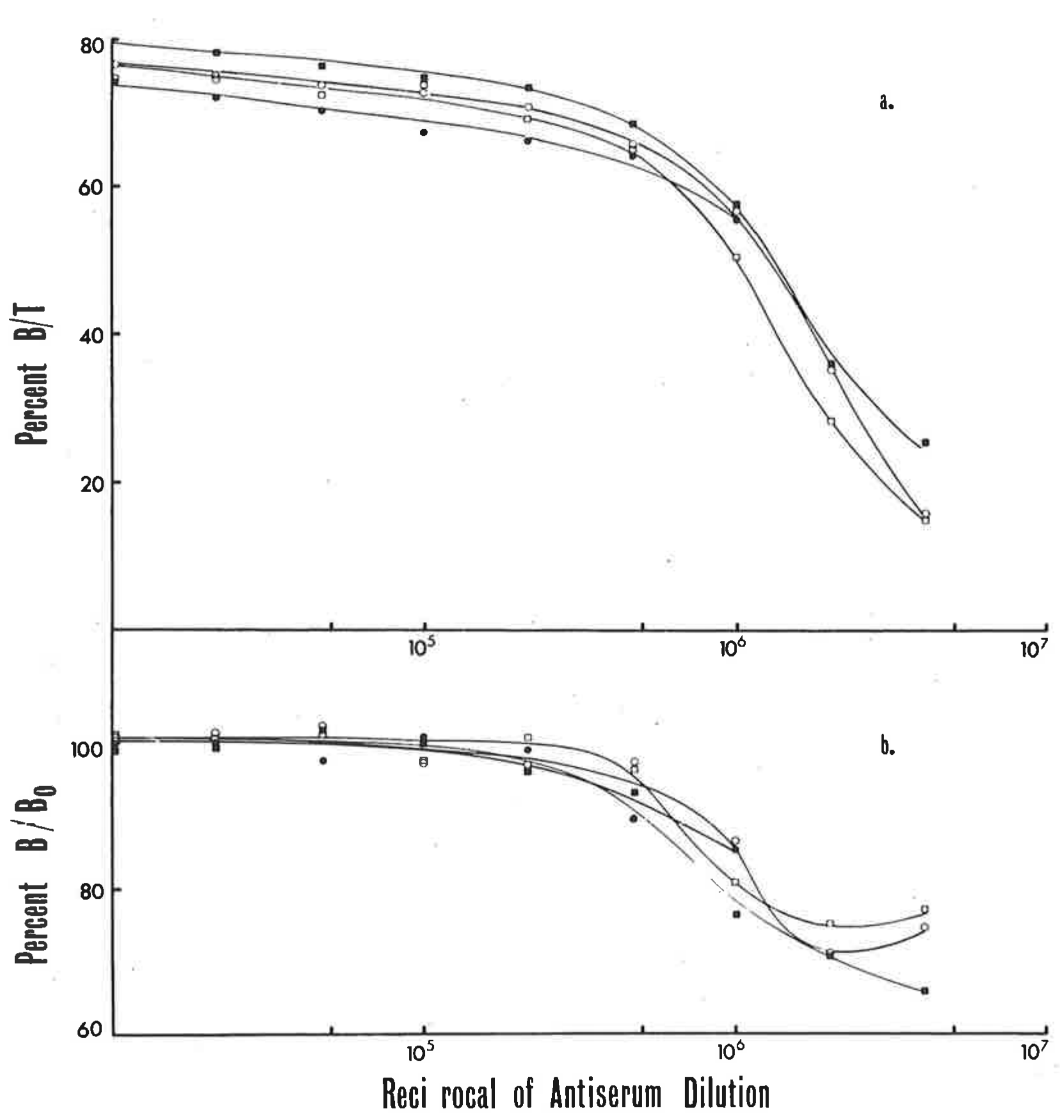

80
60
40
20
Percent B/T
a.
10^5
10^6
10^7
100
80
60
Percent B / B_0
b.
10^5
10^6
10^7
Reci rocal of Antiserum Dilution

Figure 5.14 Optimisation of serum-free T_4 assay reagent concentration

One binding species model - Appendix equation All.

p*	4.0 pmol/L
K	4.98×10^{10} L/mol
q_o	2.3×10^{-5} mol/L

Displacement of $^{125}I-T_4$ by added T_4 at various antiserum dilutions.

●——● experimental data ($1/(2 \times 10^6)$ dilution of T_4 antiserum # 492 bleed 1).

······· theoretical data
reading from left to right antiserum dilutions are $1/(4 \times 10^6)$, $1/(2 \times 10^6)$, $1/(1 \times 10^6)$, $1/(464 \times 10^3)$.

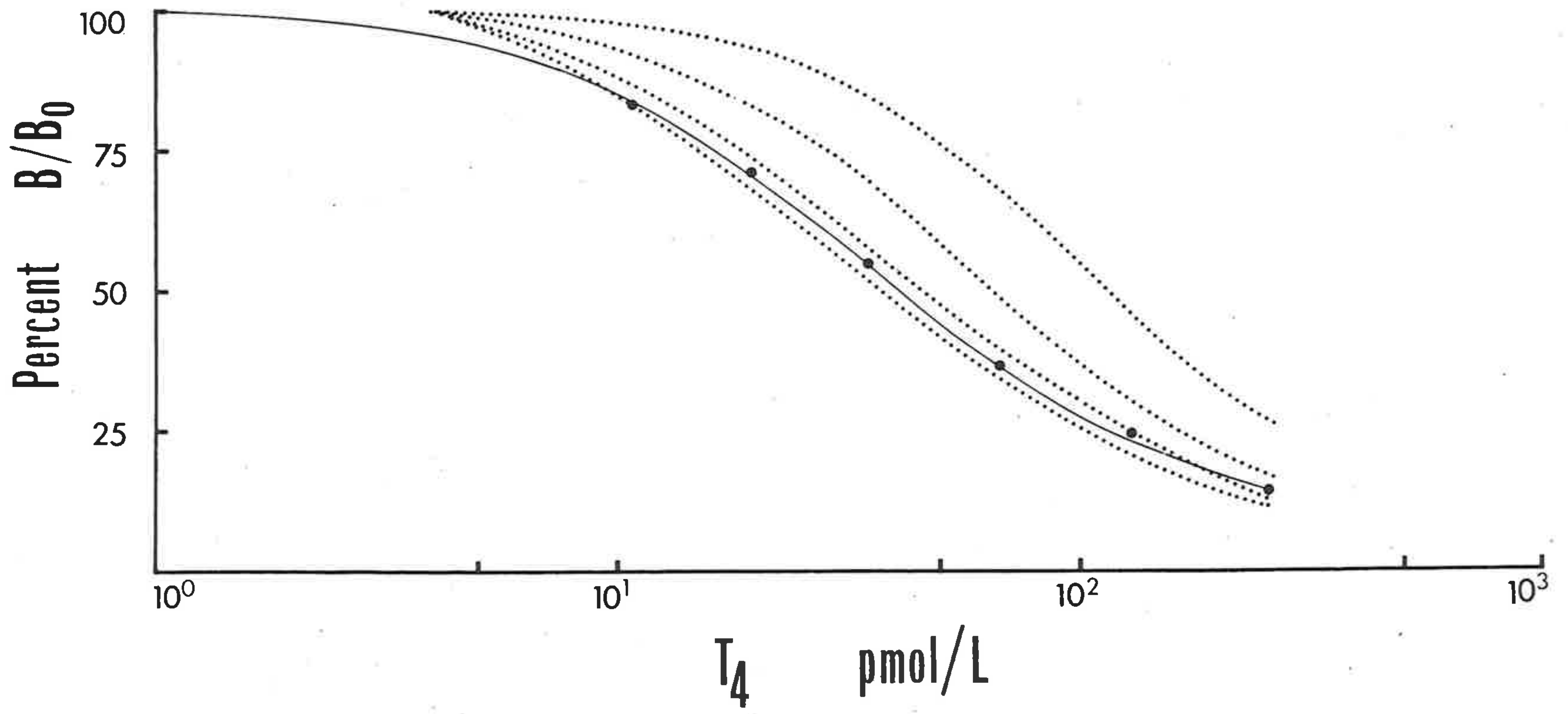

100
75
50
25
Percent B/B0
10^0
10^1
10^2
10^3
T4 pmol/L

Figure 5.15 Optimisation of serum-free T_4 assay reagent concentration

One binding species model

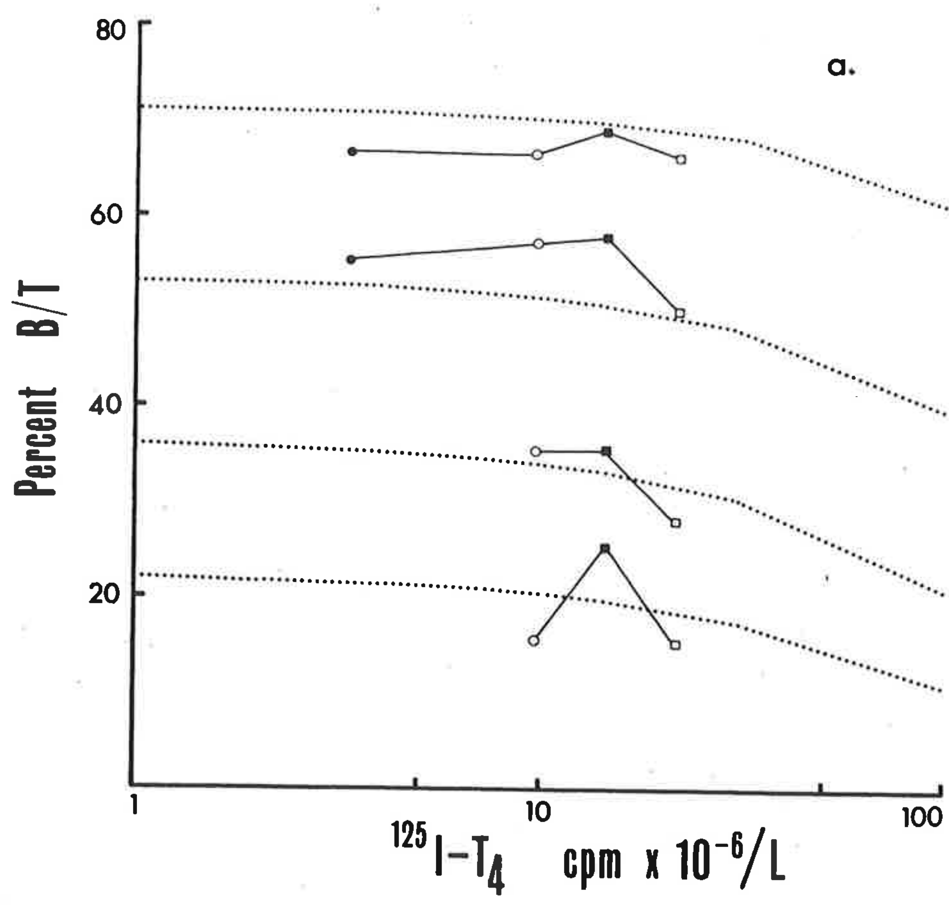
p*	4.0 pmol/L
K	4.98×10^{10} L/mol
q_o	2.3×10^{-5} mol/L

a. Self-displacement of $^{125}I-T_4$ from various dilutions of T_4 antiserum # 492 - bleed 1 (read $1/(464 \times 10^3)$, $1/(1 \times 10^6)$, $1/(2 \times 10^6)$, $1/(4 \times 10^6)$ from top to bottom).

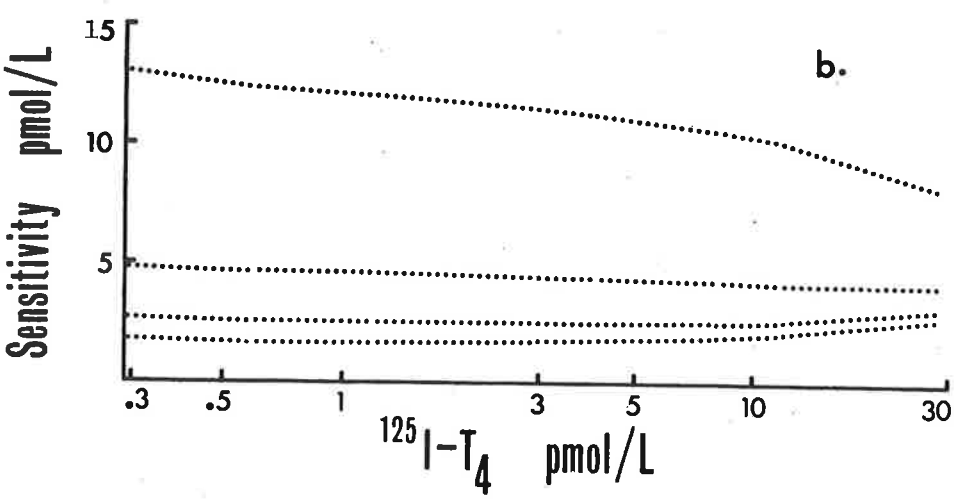
——— experimental data
(tracer concentrations same as in Figure 5.14).

······· theoretical data
(Appendix equation A11).

b. Theoretical calculation of sensitivity at various concentrations of $^{125}I-T_4$ (one binding species model - Appendix equation A14. The antiserum dilutions are in the same order as in (a).



80
60
40
20
Percent B/T
a.
1
10
100
^{125}I-T$_4$ cpm x 10^{-6}/L



15
10
5
Sensitivity pmol/L
b.
.3
.5
1
3
5
10
30
^{125}I-T$_4$ pmol/L

Figure 5.16 Serum-free T_4 assay precision profile

a. Experimentally determined precision profile.

○——○ antiserum # 492, bleed 1; charcoal separation

△——△ antiserum # 492, bleed 1; double antibody separation

□——□ antiserum # 492, bleed 2; charcoal separation

Antisera at dilution of $1/(2 \times 10^6)$.

Experimental data on the coefficient of variation in measuring the concentration of tracer bound is tabulated on the page following the figure.

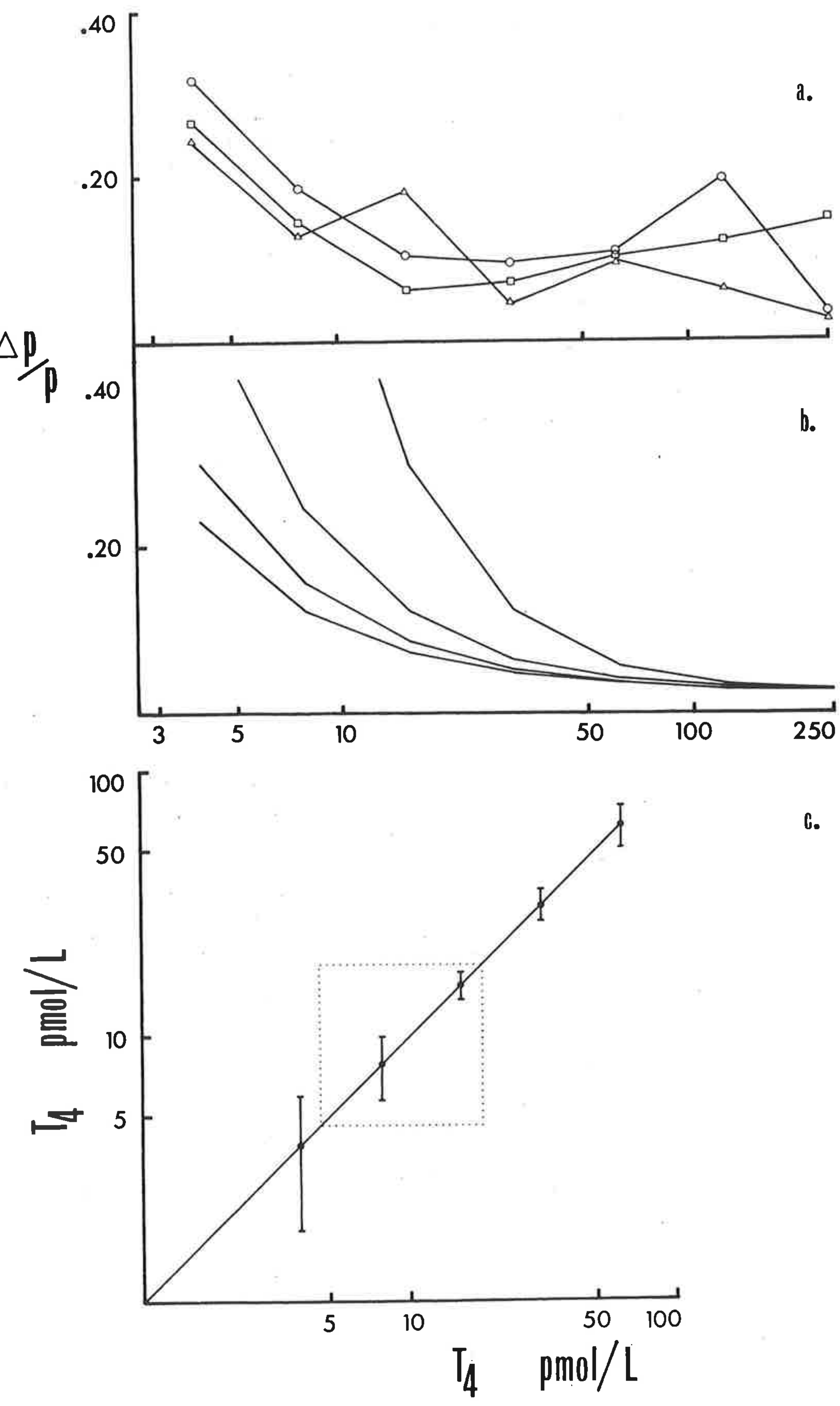
b. Theoretically determined precision profile.

—— calculated using 2.5% C.V. in measurement of concentration of tracer bound.

Antiserum dilutions are $1/(464 \times 10^3)$, $1/(1 \times 10^6)$, $1/(2 \times 10^6)$, $1/(4 \times 10^6)$ reading from right to left.

c. 95% confidence limits of estimation of T_4 concentration.

Antiserum #492, bleed 1; charcoal separation $1/(2 \times 10^6)$ dilution.



.40
.20
a.
Δp/p
.40
b.
.20
3
5
10
50
100
250
100
50
c.
T_4 pmol/L
10
5
5
10
50
100
T_4 pmol/L

Figure 5.16 (continued) Tabulation of experimentally determined percent coefficients of variation in measuring the concentration of tracer bound

	Charcoal # 492 bleed 1	Double antibody # 492 bleed 1	Charcoal #492 bleed 1
B_o/T	.2925	.2462	.5081
T_4 (pmol/L)			
0	0.2	1.3	1.1
3.9			4.8
7.8	2.7	1.9	4.5
15.6	3.0	5.4	3.1
31.3	4.9	2.3	4.2
62.5	7.2	7.0	6.3
125	11.3	2.6	11.0
250	22.9	10.8	13.7

Data were determined by calculating the standard deviation of the counts bound at each concentration of unlabelled T_4.

equilibrium conditions (Vassent, 1974). By incorporating expressions for experimental error, Ekins and Newman (1968) were able to optimise sensitivity, and to optimise precision at any given concentration of added unlabelled hormone.

It was decided to evaluate this approach to optimizing sensitivity and precision by comparing the data derived with the experimental data obtained during the empirical development of these assays. Furthermore, it was of interest to know whether this approach actually simplified the amount of experimental work required to optimize the assay. The general solution of Wosilait and Nagy (1976) for solving the concentration of ligand bound at equilibrium in the presence of any given number of binding site species was used. It was possible to derive equations providing solutions for the concentration of ligand bound in the presence of one binding species (fT_4 assay; Appendix equation A11) or two binding site species (fT_3 assay; Appendix equation A15) at any given concentration of added unlabelled hormone. To enable the optimisation of sensitivity and precision it was necessary to derive an equation for the slope of the displacement curve. This was formulated for only the single binding site model (Appendix, equation A13). By assuming that the coefficient of variation of the concentration of hormone bound was constant, a precision profile could be constructed for any combination of tracer and antibody concentration. A 2.5% coefficient of variation was used as a minimum estimate of experimental error.

(i) Serum-Free T_3 Assay - Figure 5.8 illustrates the displacement of $^{125}I-T_3$ from the T_3 antiserum as predicted by the one binding site model. As was expected and illustrated by the experimental data, Bo/T decreased and the slope of the displacement curve increased with increasing antiserum dilution. The relationship between slope and antiserum dilution suggested that any sensitivity could be attained by simply diluting the antiserum. As was borne out by the experimental data in which there was little difference in the sensitivity and slope at $1/(6 \times 10^6)$ and $1/(8 \times 10^6)$ antiserum dilution, this does not occur in practise. The limiting factor is the experimental error or the standard deviation of the measured concentration of tracer bound.

The precision profiles at the four antiserum dilutions used in the displacement studies are depicted in Figure 5.10. The decision to assess the precision profile by assuming a constant experimental error (coefficient of variation) in the measurement of the concentration of tracer bound at each unlabelled hormone dose was justified by the experimental data (legend, Figure 5.10; antiserum #395 was used during optimization of the separation technique). The experimental error was constant except at the highest doses, where displacement was greatest. While the use of a coefficient of variation of 2.5% appeared to be a minimum estimate of the experimental error, precision profiles constructed assuming 5% coefficient of variation in measuring the concentration of tracer bound tended to predict much greater imprecision than was actually observed (Figure 5.10).

The generated precision profiles tended to exaggerate the imprecision at the lowest standard dose (1.95 pmol/L). While the chosen coefficient of variation of 2.5% was lower than the error calculated for the experimental data the greater precision observed experimentally at the lowest standard dose was due to the greater relative displacement at this dose than was predicted by the one binding site model. Similarly the sensitivity calculated using the one binding site model overestimated the minimum concentration of hormone which could be measured (legend, Figure 5.8).

The optimum tracer concentration was assessed at the antiserum dilution $1/(6 \times 10^6)$ using the one binding site model. As expected the percent tracer bound decreased with increasing tracer concentration (Figure 5.7). The precision profile of the tracer self-displacement curve had special significance. The variation in the concentration of tracer bound in the absence of unlabelled hormone enables the calculation of the sensitivity of the dose response curve at that antiserum dilution if the slope of displacement is known (Figure 3.21). As it has been assumed that binding of labelled and unlabelled hormone are equivalent, the sensitivity of any particular tracer concentration can be determined. The calculation suggested that there was little change in sensitivity over the entire tracer concentration range studied (up to 14.0 pmol/L). It is of some interest that the highest tracer concentration studied was 2.4/K.

It was concluded from the experimental and theoretical studies that the sensitivity of the serum-free

T_3 assay was determined principally by the concentration of the high affinity binding sites, while the concentration of tracer was of lesser importance.

A comparison of the experimental data and displacement curves generated using the two binding site model is depicted in Figure 5.9. Surprisingly, this model did not agree as closely with the experimental data as did the one binding site model, except at very high concentrations of hormone.

An analysis of the percent occupancy of the two binding sites (Appendix, equation A16) showed that the high affinity binding site was 90% occupied at the highest concentration used in the fT_3 assay, namely 62.5 pmol/L (Figure 5.9b). In contrast, the lower affinity binding site was about 20% occupied and accounted for 30% of the ligand bound at this concentration (Figure 5.9b). It would appear that the one binding site model describes the displacement of tracer more accurately where the displacement curve is most sensitive and where binding to the high affinity sites is predominant. Only at high concentrations of ligand, where these sites are nearly saturated does the two binding site model fit the experimental data more accurately.

(ii) Serum-Free T_4 Assay - The displacement curve for the antiserum dilution $1/(2 \times 10^6)$ generated using the one binding site model agreed closely with that derived experimentally (Figure 5.14). Furthermore curves generated at the antiserum dilutions used in the experimental study

(Figure 5.13(b)) confirmed that little further sensitivity was gained by using dilutions greater than $1/(2 \times 10^6)$.

An analysis of the self-displacement by ^{125}I-T_4 at four antiserum dilutions confirmed the conclusion in the serum-free T_3 assay that the concentration of tracer was of secondary importance in optimising these assays for sensitivity (Figure 5.15b). Although sensitivity was dramatically improved by increasing the antiserum dilution from $1/(434 \times 10^3)$ to $1/(2 \times 10^6)$, further dilution did not appear to substantially increase the sensitivity.

An analysis of the coefficient of variation of the experimental measurement of the concentration of tracer bound suggested that the value of 2.5% was a minimum estimate for the purpose of generating precision profiles (Figure 5.16). Unlike the serum-free T_3 assays, the measurement tended to become more imprecise with increasing tracer displacement. Thus, while the generated precision profile at antiserum dilution $1/(2 \times 10^6)$ was similar to that derived experimentally, precision was overestimated at higher concentrations of unlabelled T_4.

Having optimized this antiserum, a later bleed of sheep #492 became available. Scatchard analysis revealed two binding species (Figure 5.12). Of particular interest was that one species had a similar K and q_o value to the earlier bleed, whereas the second species had a higher affinity of about half the concentration. This would suggest the appearance of a new high affinity binding species between the two bleeds. This antiserum was used for all the

assays reported in Section 5.4 and Chapter 6 at a dilution of $1/(2 \times 10^6)$ without further optimisation. The precision profile is presented in Figure 5.16.

(iii) Summary - The relationship between antiserum and tracer as described by the single binding site model agreed closely with the experimental data. For this approach to be useful, an accurate assessment of the assay errors must be made.

The major sources of error occur during the separation of free and antibody bound hormone, during pipetting, and to a lesser extent, during counting. While the contribution of these sources can be determined experimentally, the difficulty comes in predicting how the experimental error changes with variation in concentration of tracer bound. In this study, the assumption of constant coefficient of variation in the measurement of bound tracer concentration was justified in the serum-free T_3 assay. However, in the serum-free T_4 assay, the error in this measurement bore a complex relationship to the concentration of bound tracer.

The second criterion which must be satisfied if this approach is to be useful is that it reduce the amount of experimental work necessary to establish an assay. To provide the necessary data on affinity constants and binding site concentrations, a Scatchard analysis of the tracer displacement curve must be performed. The use of a Scatchard plot necessarily entails some initial experiments to find those combinations of tracer and antibody concentration which

give a satisfactory but not optimised displacement curve. This would usually be an antiserum dilution curve, and would include unlabelled hormone at a concentration in the physiological range to provide information on those antiserum dilutions showing best displacement.

The results presented here suggest that the single binding site model would conveniently predict those combinations of tracer and antibody concentration enabling assays of the required sensitivity and precision to be established. It is likely, however, that an experienced assayist, having provided a displacement curve for Scatchard analysis, could establish an assay of the required sensitivity with a minimum of experimental effort.

While the use of binding models did not provide combinations of tracer and antiserum markedly different to those used in the empirically established assays, it did give some insight into how both sensitivity and precision were related to tracer and antibody concentrations. Of particular interest was the demonstration that in the serum-free thyroid hormone assays at least, sensitivity was not critically related to tracer concentration. This may reflect the use of high specific activity tracers and Ekins and Newman (1968) have pointed out that there is a limit to the sensitivity which can be attained by increasing specific activity.

It seems likely that the use of the more sophisticated ligand binding models which incorporate the incubation time would considerably reduce the experimental work

required to optimise non-equilibrium assays.

SEPARATION OF FREE AND ANTIBODY BOUND HORMONE

It was decided to assess both the charcoal and double antibody separation methods. Charcoal separation was seen to be a relatively simple and cheap technique but with the disadvantage of large misclassification errors. It is particularly sensitive to the presence of iodide, leading to free misclassified as bound errors. The double antibody method is simple and, being specific for the bound fraction, is unaffected by changes in tracer with aging, the changes in tracer affecting only the first antibody reaction.

These studies were carried out with a sheep T_3 antiserum #395 which was later replaced by the higher affinity antiserum #478 (described in the assay optimisation studies). The T_4 antiserum used was the earlier bleed of #492, as used in the assay optimisation study.

1. Charcoal Separation

The adsorption of $^{125}I-T_4$ to varying concentrations of charcoal was assessed in the absence and presence of antibody, and the net binding to antibody at each charcoal concentration then calculated. Between 55 - 60% of the tracer was bound at very low concentrations of charcoal and increased to a maximum of 87% at very high charcoal concentration (Figure 4.17). The unbound fraction of tracer would most likely represent contaminating ^{125}I-iodide. Adsorption to charcoal in the presence of antibody increased slowly with increasing charcoal concentration. At concentrations greater than 1% charcoal, $^{125}I-T_4$ adsorption in the presence of

Figure 5.17 Charcoal separation of free and antibody bound tracer

(a) Optimisation of charcoal concentration

○——○ Adsorption of $^{125}I-T_4$ in the absence of antiserum

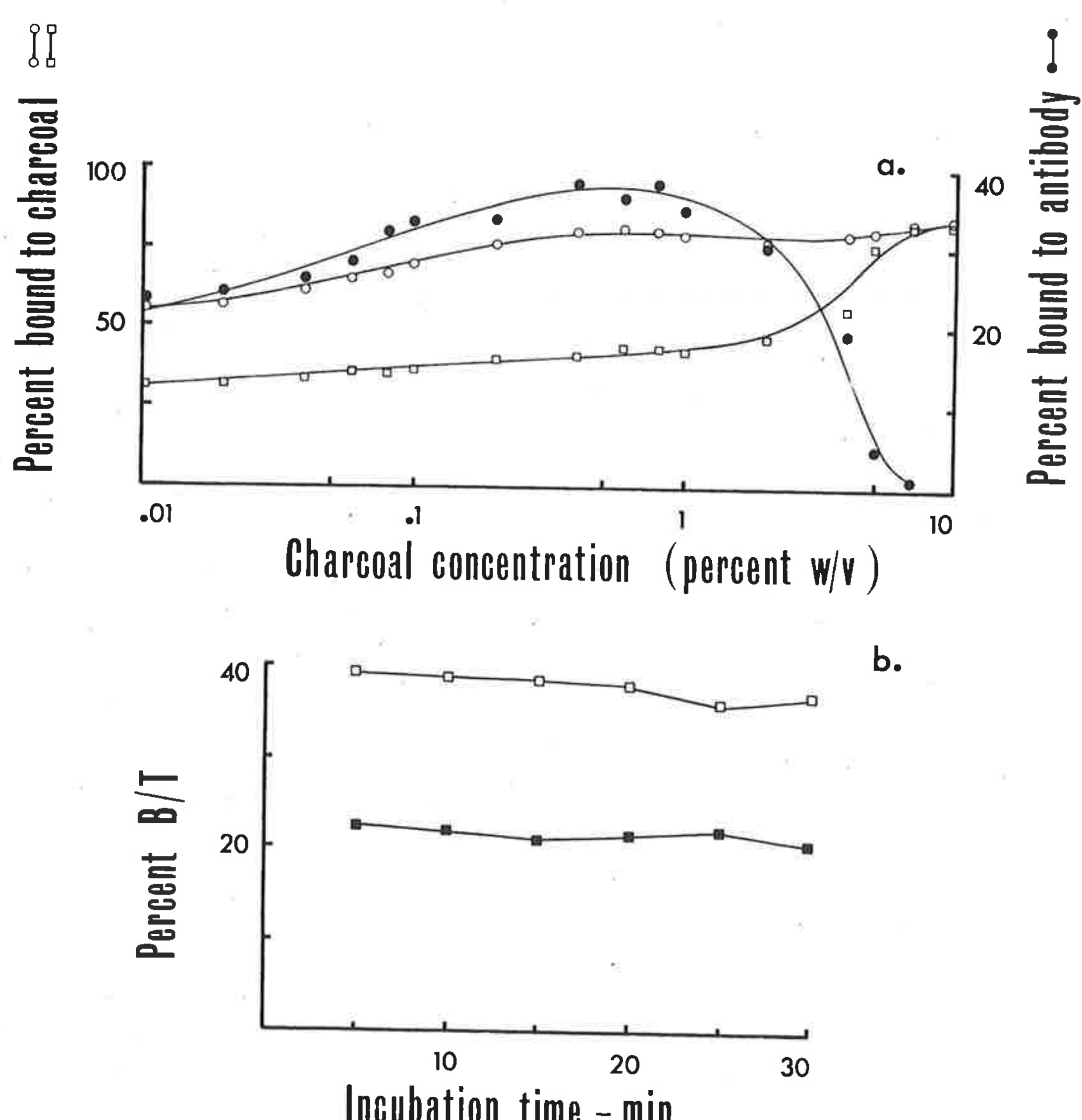
□——□ Adsorption of $^{125}I-T_4$ in the presence of antiserum

●——● Net binding of $^{125}I-T_4$ to antibody

(b) Effect of varying incubation time in the presence of charcoal

■——■ Adsorption of $^{125}I-T_4$ in the absence of antiserum

□——□ Net binding of $^{125}I-T_4$ to antibody.


Percent bound to charcoal
Percent bound to antibody
100
50
40
20
a.
.01
.1
1
10
Charcoal concentration (percent w/v)
b.
40
20
Percent B/T
10
20
30
Incubation time - min

antibody increased dramatically. This could reflect either binding of antibody to the charcoal or stripping of bound tracer from antibody by the charcoal. The presence of either 0.013% or 0.025% (w/v) dextran T-70 made no difference to the ^{125}I-T_4 adsorption characteristics. This would suggest that there is limited adsorption of the antibody to charcoal in the presence of 0.1% gelatine.

The net binding of tracer to antibody showed a broad optimum between 0.3% and 1.0% charcoal. A charcoal concentration of 0.42% was chosen, representing the addition of 250 µL of 2% charcoal to a 1200 µL assay volume.

The binding studies were done with 4.0 pmol/L of ^{125}I-T_4. To check that no displacement of charcoal adsorbed tracer occurred in the presence of standard, adsorption of ^{125}I-T_4 was measured in the presence of 62.5 pmol/L unlabelled T_4, with no antibody present. No displacement was apparent.

The adsorption of tracer to charcoal was extremely rapid being virtually complete in the time it took to add the charcoal, mix, place in centrifuge and begin centrifugation (Figure 5.17b). An incubation time of 5 min was chosen, giving sufficient time to handle a large number of tubes. Charcoal addition was synchronized by placing the charcoal suspension in caps and beginning the incubation by inverting the tubes.

The optimum separation conditions worked out for the fT_4 assay were adopted for the fT_3 assay without further changes.

2. Double Antibody Separation

The anti-gamma globulin and carrier serum concentrations were optimised by finding that combination which gave maximum Bo/T. That is, that combination giving rise to greatest precipitation of the anti-T_3 or anti-T_4 antiserum. A further consideration was the cost of precipitating antibody. Thus, the aim was to use the least amount of anti-gamma globulin whilst ensuring near maximum precipitation of bound tracer. Furthermore, optimum conditions were sought separately for the fT_3 and fT_4 assays, as the reaction between the precipitating antibody and either anti-T_3 or anti-T_4 could not be assumed to be similar.

As illustrated in Figure 5.18, increasing anti-gamma globulin concentration necessitated the use of increasing carrier serum (normal sheep serum) to obtain maximum separation. Optimum precipitating conditions for the fT_4 assay were considered to be 8 μL of donkey anti-sheep/goat gamma globulin (DASGG) and 0.25 μL of normal sheep serum (NSS) per tube. The fT_3 assay required 12 μL of DASGG and 1 μL of NSS per tube.

The precipitation reaction was found to be markedly affected by temperature (Figure 5.18). Maximum precipitation occurred at 4°C, but virtually none at 37°C. What is more, precipitation was complete within one hour and declined thereafter.

Figure 5.18 Optimisation of the precipitating reagent concentrations in the double antibody separation technique

(a) Serum-free T_3 assay - Selection of DASGG and sheep serum concentrations giving maximum precipitation of bound $^{125}I-T_3$.

T_3 antiserum # 395, 1/(2 x 10^6) dilution, was used.

The various volumes of DASGG (μL) per tube tested are indicated on the graphs.

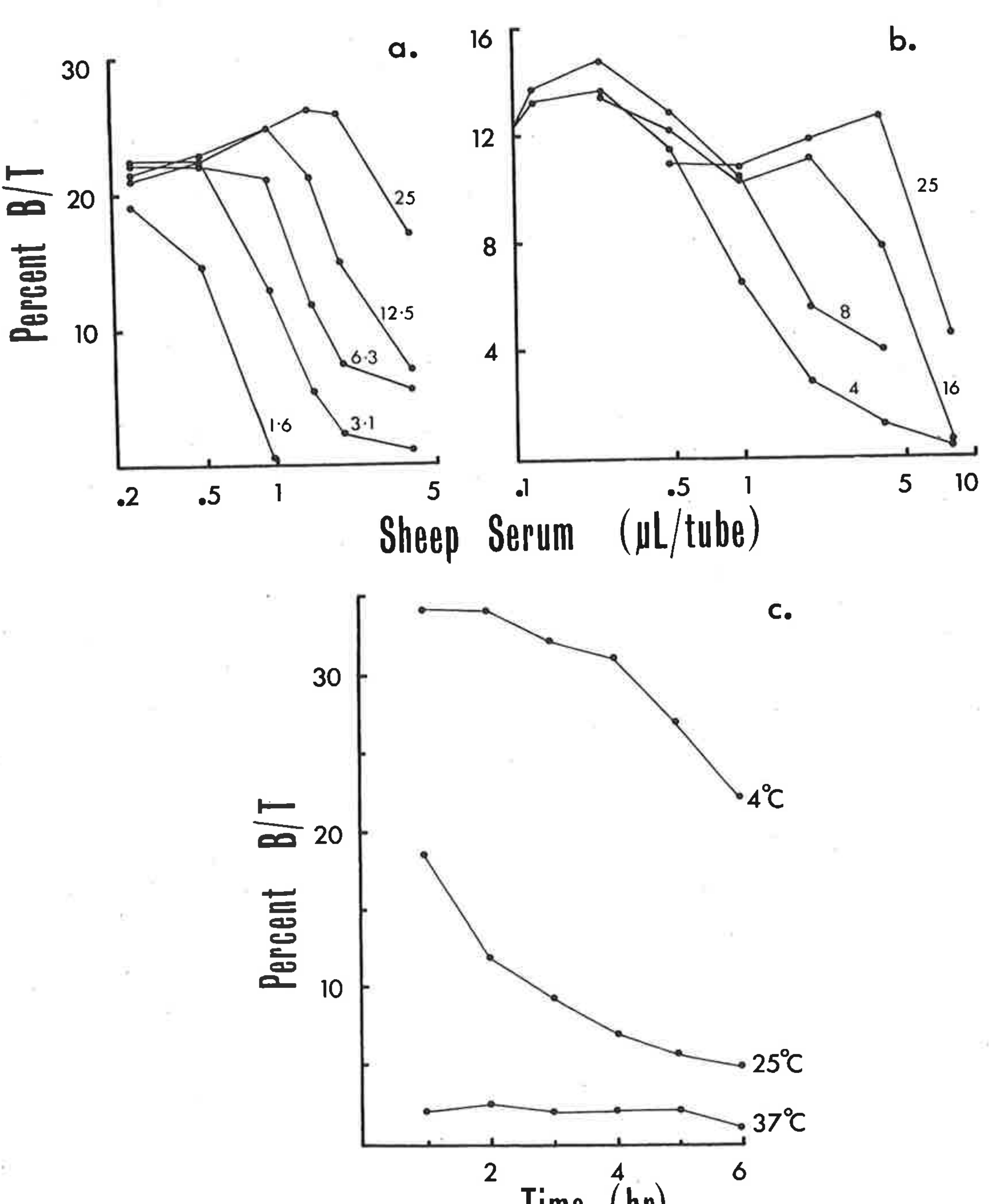
(b) Serum-free T_4 assay - selection of Donkey anti sheep gamma globulin (DASGG) and sheep serum concentrations giving maximum precipitation of bound $^{125}I-T_4$.

T_4 antiserum, bleed 1, 1/(2 x 10^6) dilution, was used.

The various volumes of DASGG (μL) per tube tested are indicated on the graph.

(c) Effect of temperature and incubation time on the precipitation of bound $^{125}I-T_4$.

T_4 antiserum # 492, bleed 1, 1/(2 x 10^6) dilution, DASGG 8 μL/tube, sheep serum 0.25 μL/tube.


a.
b.
c.
Percent B/T
30
20
10
16
12
8
4
25
12·5
6·3
3·1
1·6
25
16
8
4
.2
.5
1
5
.1
.5
1
5
10
Sheep Serum (µL/tube)
30
20
10
4°C
25°C
37°C
2
4
6
Time (hr)

3. Comparison of Separation Techniques

(i) Serum-Free T_3 Assay - A comparison of the precision profiles showed the charcoal method to be superior for the fT_3 assay (Figure 5.10). The precipitating technique had a $\Delta p/p$ of greater than 0.50 at the 2.0 pmol/L standard, suggesting the sensitivity to be higher than this standard. This was in agreement with the sensitivity as calculated from the Bo/T of 2.2 pmol/L. Although the profile of the charcoal method was in general better, precision at the 2.0, 3.9 and 7.8 pmol/L standards was still inadequate, $\Delta p/p$ being greater or equal to 0.20 (Figures 5.10(a) and 5.11). The sensitivity calculated from the Bo/T was 2.0 pmol/L, whilst the precision of the lowest standard was 2.0 $\pm$ 1.6 (2 S.D.) pmol/L.

As published estimates of mean fT_3 had been as low as 5.0 pmol/L (Table 5.6), the sensitivity of the fT_3 assay was considered only barely adequate. As was discussed in the section on optimising the fT_3 assay, a T_3 antiserum (#478) became available which had a higher affinity for T_3. The sensitivity of the assay was greatly improved by using this antiserum (Figure 5.10a). The Bo/T of 0.652 was considerably higher than the Bo/T of 0.261 using the sheep antiserum #395. Furthermore, the sensitivity of 1.4 pmol/L was well below the eventually determined normal range of 2.8 - 8.4 pmol/L.

(ii) Serum-Free T_4 Assay - There appeared to be little general difference between the two separation techniques for the fT_4 assay (Figure 5.16a). The sensitivities calculated

from the Bo/T precision were 1.3 and 0.2 pmol/L for the double antibody and charcoal assays respectively. The latter figure is extraordinarily low and, considering the similar precision of the two methods at the 7.8 pmol/L standard, is likely to be a gross underestimate.

A later bleed of sheep #492 was used for routine fT_4 measurement. The precision profile using this antiserum is only marginally better than the profiles determined in this study (Figure 5.16). This is despite an increase in Bo/T from 0.293 to 0.508. However, the sensitivity was well below the normal range (3.7 - 19.2 pmol/L) and a further standard was included, having a precision 3.9 ± 2.0 (2 S.D.) pmol/L. It would appear, though, that to reliably measure subnormal fT_4 concentrations, an antisera of even higher affinity is required.

DIALYSIS

The 0.01 M hepes, 0.11 M NaCl, 1.5 mM sodium azide, pH 7.4 buffer used by Ellis and Ekins (1975) was employed. Hepes has previously been shown to cause minimum disruption of T_4 binding to the thyronine binding proteins over the pH range 6.5 - 8.5 (Spaulding and Gregerman, 1972). Both phosphate and chloride at greater than physiological concentration cause increased percent free T_4. Other buffers have been successfully used including a modified Krebs-Ringer buffer by Weeke and Orskov (1975), which contained very low phosphate and physiological chloride concentration. No effect on T_3 binding to the thyroid hormone binding proteins could be demonstrated.

During the comparison of separation techniques it was noted that in several cells the dialysate gave B/T >> Bo/T in the charcoal assay and low binding in the double antibody assay (Figure 5.19a). This was thought to be most likely caused by leakage of protein through the membrane. Protein present in the dialysate will bind tracer and increase the apparent antibody bound tracer concentration in the charcoal assay. On the other hand, protein bound tracer in the double antibody method will not be precipitated and will tend to reduce the antibody bound fraction well below that expected.

The effect of adding serum directly to the serum free incubation tubes was investigated. The fT_4 assay was found to be exquisitely sensitive to the presence of serum in the dialysate (Figure 5.19b). The presence of 0.625 µL serum per incubation tube reduced the apparent B/T to near zero. The presence of 0.625 µL serum/mL dialysate is equivalent to 3.0 µL serum/4.8 mL dialysate buffer in the dialysis system. This is equivalent to a leakage of 1.5% (3 µL in 200 µL) of serum into the dialysate. Even 0.02 µL of serum/mL of dialysate was found to cause a significant reduction in measured B/T. Weeke and Orskov (1975) have previously reported that less than 0.007% of serum albumin leaked across the membrane during dialysis under normal conditions. That is, the membrane would be expected to prevent accumulation of protein in the dialysate at concentrations which would interfere in the assays.

It was concluded that protein leakage was most likely occurring because of strain put on the membrane when the top dialysand compartment was pushed into the lower dialysate

compartment. This problem was overcome by slightly reducing the outer diameter of the upper compartment with a resulting looser fit which did not excessively stretch the membrane.

The higher volume of serum required to significantly reduce B/T in the fT_3 assay (Figure 5.19) was thought to reflect the lower affinity of the serum binding proteins for T_3.

The problem of binding protein interference is also borne out in the double antibody method in which 0.25 μL of normal sheep serum is added to each tube. Over nine assays, the average B_o/T of the double antibody method was 0.246 as compared to 0.293 for the charcoal method. The B_o/T for the charcoal and double antibody fT_3 assays were not significantly different.

ASSAY IMPRECISION

Table 5.4 presents data on assay reproducibility. The coefficients of variation reflect the precision of both the dialysis step and the immunoassay step. Thus the variation is higher than would be expected from the precision profiles.

5.4 VALIDATION AND CLINICAL USE OF THE ESTABLISHED FREE THYROID HORMONE ASSAYS

This final section establishes the equilibrium dialysis-radioimmunoassay (E/D-RIA) method developed here as a valid procedure for assaying fT_4 and fT_3 concentration in serum.

Figure 5.19 The effect of serum in the serum-free T_3 and T_4 assays

(a) Binding data for dialysates simultaneously assayed in both the charcoal and double antibody serum-free T_4 assays.
Data from two different dialysis batches are shown (● and ○).

(b) Effect of serum in the serum-free T_4 assay
Double antibody assay

○——○ + antiserum # 492, bleed 1, $1/(2 \times 10^6)$ dilution

●——● - antiserum

Charcoal assay

□——□ + antiserum # 492, bleed 1, $1/(2 \times 10^6)$ dilution

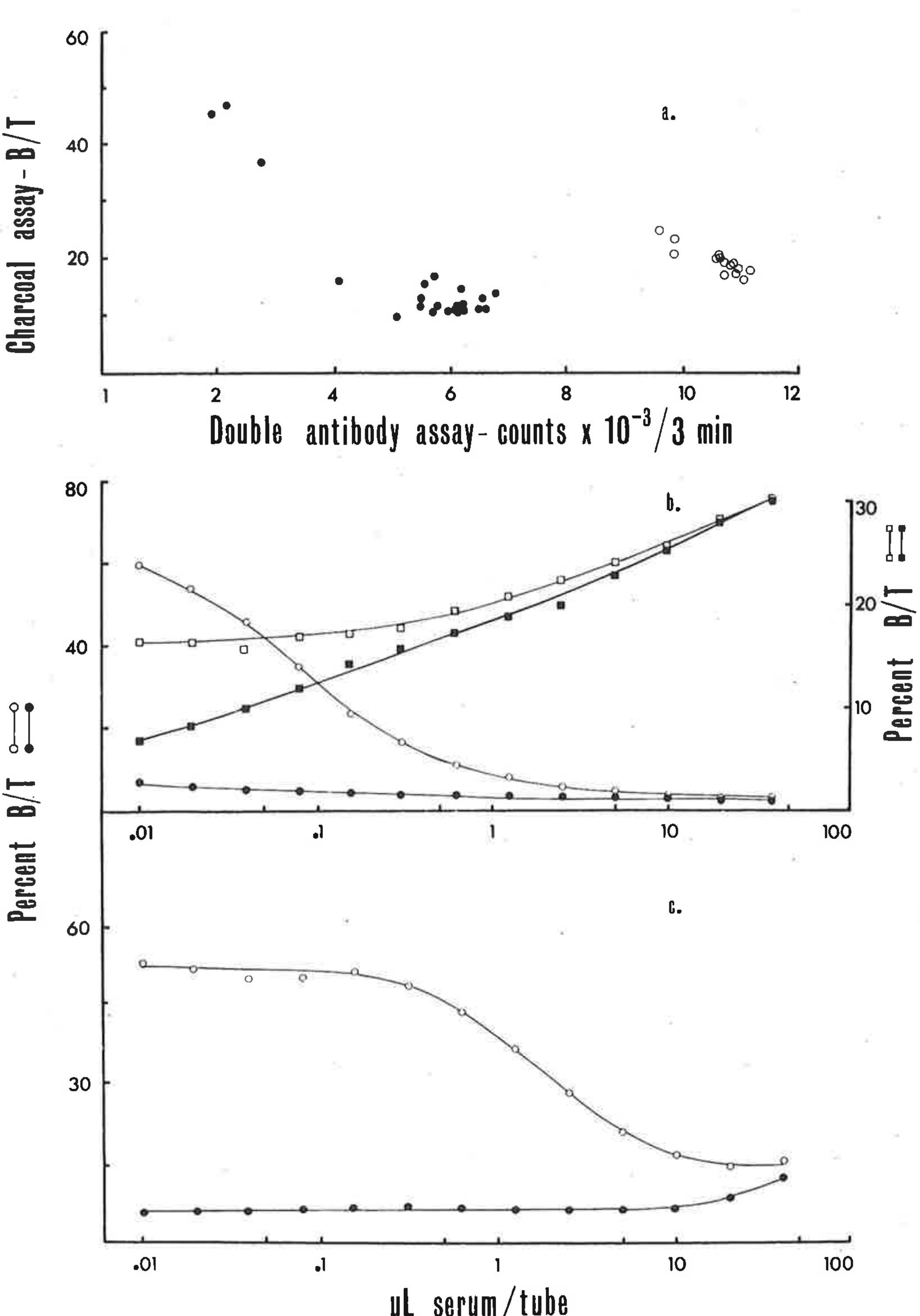
■——■ - antiserum

(c) Effect of serum in the serum-free T_3 assay

Double antibody assay

○——○ + antiserum # 478, $1/(6 \times 10^6)$ dilution

●——● - antiserum



a.
Charcoal assay - B/T
60
40
20
1
2
4
6
8
10
12
Double antibody assay - counts x 10^{-3}/3 min
b.
80
40
30
20
10
Percent B/T
.01
.1
1
10
100
c.
Percent B/T
60
30
.01
.1
1
10
100
µL serum/tube

TABLE 5.4 Assay Quality Control Data

	Intra-assay		Inter-assay	
Free T_4 Assay	a			
	15.2 ± 2.5	16.6	16.7 ± 2.4	14.3
			75.5 ± 8.6	11.4
Free T_3 Assay				
	5.2 ± 0.9	17.3	3.3 ± 0.5	14.8

[a]Expressed as $\bar{x}$ ± 1 S.D. % c.v.

Intra-assay variation was determined by assaying aliquots from 26 different dialysis cells in the same assay.

In establishing an assay procedure, the data derived by use of the method should comply with the following criteria. Firstly, the method must have a precision such that the derived data will be physiologically meaningful. This precision provides the basis for the interpretation of variations in concentration seen in different physiological conditions. Secondly, the assay must give 100% recovery within the limits of assay precision. If recovery is other than this ideal, the reasons should be investigated before considering the method to be of practical use. Thirdly, the data should be consistent with established procedures, preferably a reference method if one exists. Finally, and most importantly, the data must reflect the clinical state of the patient.

The criterion of adequate precision has been discussed in Section 5.3. The optimisation of the concentration of assay reagents was carried out with the express purpose of developing assays of adequate sensitivity and precision to be able to reliably measure physiological concentrations of fT_4 and fT_3. As there is no simple linear relationship between T_4 added to serum and the resulting fT_4 concentration, no adequate means of measuring recovery exists.

The final two criteria will be discussed here in some detail. Prior to the development of the E/D-RIA methodology, fT_4 and fT_3 were estimated by measuring the fraction free hormone and calculating the free hormone concentration using the total hormone concentration. The E/D-RIA is considered an improvement on this technique, for reasons

outlined in Chapter 4. Whilst a comparison can be made against these earlier techniques, the most important verification of the E/D-RIA method is the clinical assessment. In the absence of other suitable methods, this technique is then considered for use as a reference method.

A recently available kinetic measurement of fT_4 is assessed by both a clinical appraisal and comparison to the E/D-RIA data. Finally, a commonly used indirect estimation of fT_4 concentration, the FTI, is compared to both the E/D-RIA and kinetic procedures.

VALIDATION OF FREE THYROID HORMONE ANALYSIS BY EQUILIBRIUM DIALYSIS-RADIOIMMUNOASSAY

1. Free Thyroid Hormone Concentration in Healthy Euthyroid Subjects

Figure 5.20 and Table 5.5 present the fT_4 concentration data in euthyroid subjects with normal thyronine binding protein concentration. The mean concentration was 11.5 pmol/L, representing 0.011% of the mean T_4 concentration of 108 nmol/L.

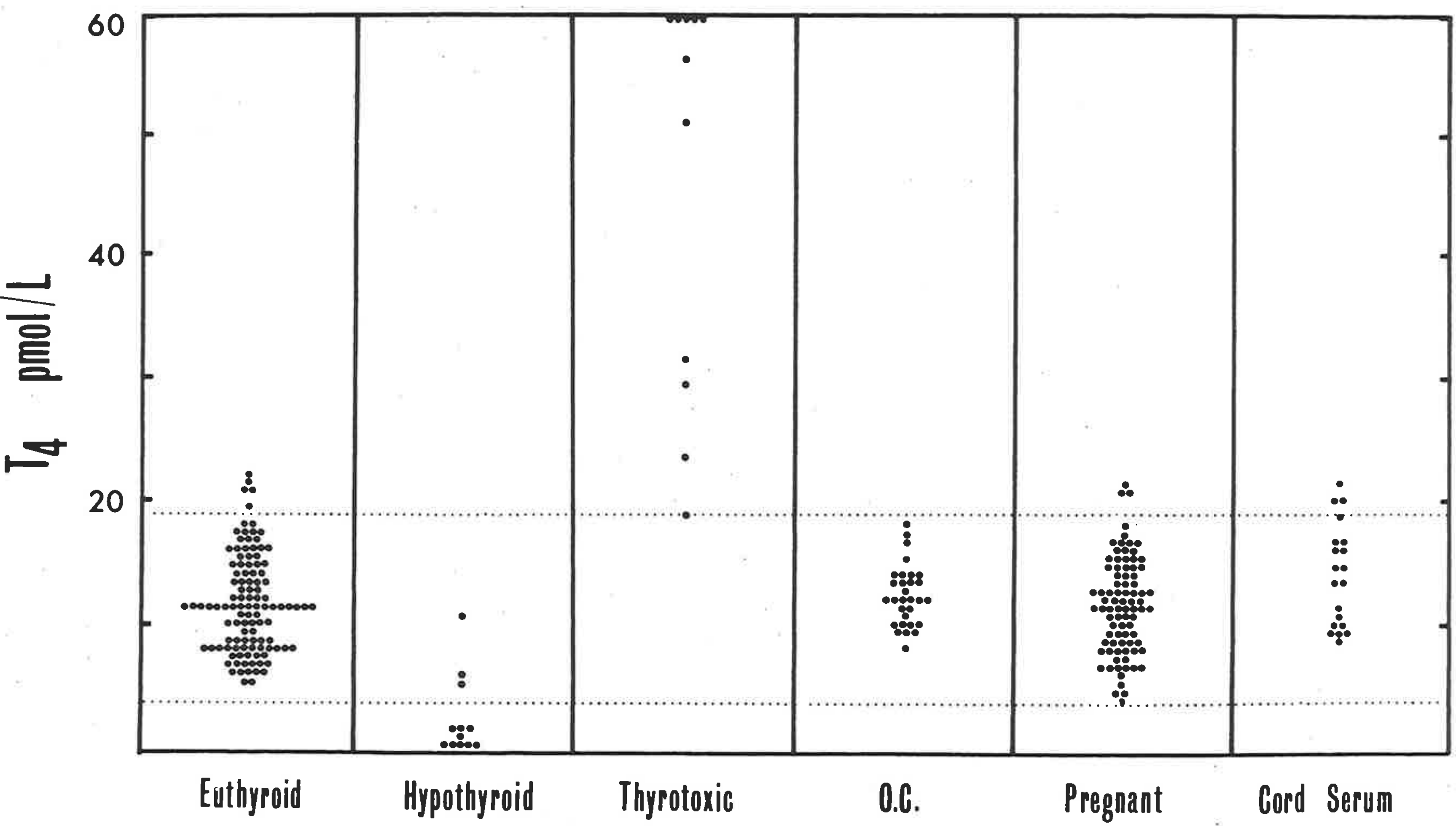
Mean euthyroid fT_3 concentration was 5.6 pmol/L. This was 0.34% of the mean T_3 of 1.9 nmol/L in the same group (Figure 5.20 and Table 5.5). The molar ratio of fT_4/fT_3 was 2.1 compared to the ratio of 57.4 for the total concentrations.

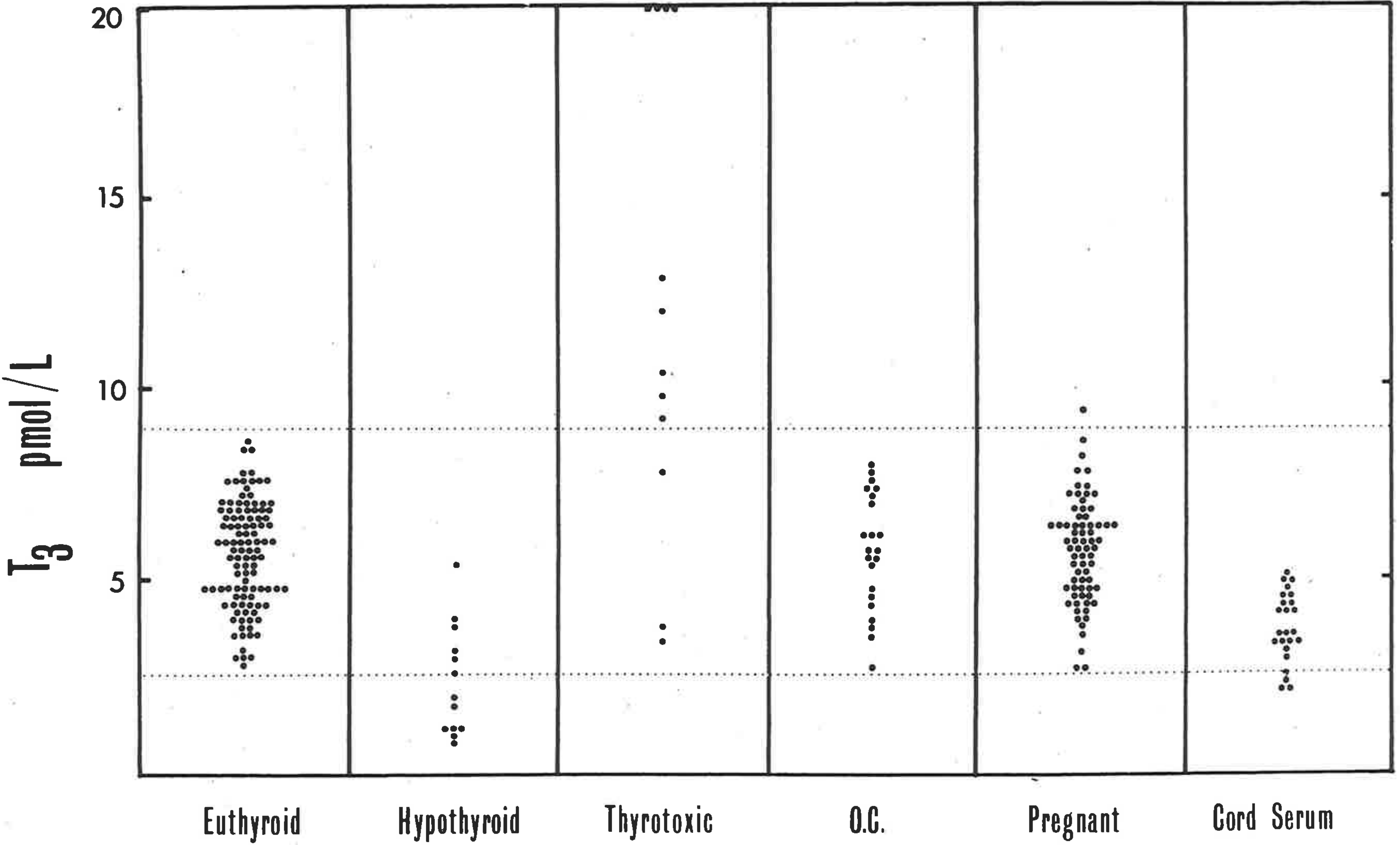
2. Free Thyroid Hormone Concentration in Euthyroid Subjects with Elevated Thyronine Binding Globulin Concentration

Both T_4 and T_3 rose to a maximum of 85% during

Figure 5.20 Free T_4 and free T_3 concentrations in euthyroid subjects and in subjects with thyroid disease

The euthryroid reference ranges are indicated on the diagrams.


60
40
20
T_4 pmol/L
Euthyroid
Hypothyroid
Thyrotoxic
O.C.
Pregnant
Cord Serum



T_3 pmol/L
20
15
10
5
Euthyroid
Hypothyroid
Thyrotoxic
O.C.
Pregnant
Cord Serum

TABLE 5.5 Total and free T_3 and T_4 concentrations and FTI in euthyroid subjects

Group	N	Total T_4 (nmol/L)	Free T_4 (pmol/L)	FTI	Total T_3 (nmol/L)	Free T_3 (pmol/L)	N
Normal euthyroid	108	108 ± 21	11.9 ± 3.9	106 ± 16	1.9 ± 0.5	5.6 ± 1.4	104
First trimester	29	156 ± 39	12.8 ± 4.3	128 ± 31	2.7 ± 0.7	5.5 ± 1.5	29
Second trimester	26	180 ± 32	11.0 ± 3.1	126 ± 28	3.5 ± 0.6	5.6 ± 1.2	26
Third trimester	20	183 ± 39	11.3 ± 2.7	120 ± 20	3.3 ± 0.6	6.1 ± 1.3	15
Total Pregnant	75	172 ± 38	11.8 ± 3.6	125 ± 27	3.0 ± 0.8	5.7 ± 1.4	70
Oral contraceptives	29	184 ± 36	12.2 ± 2.2	151 ± 21	2.9 ± 0.7	5.9 ± 1.6	23

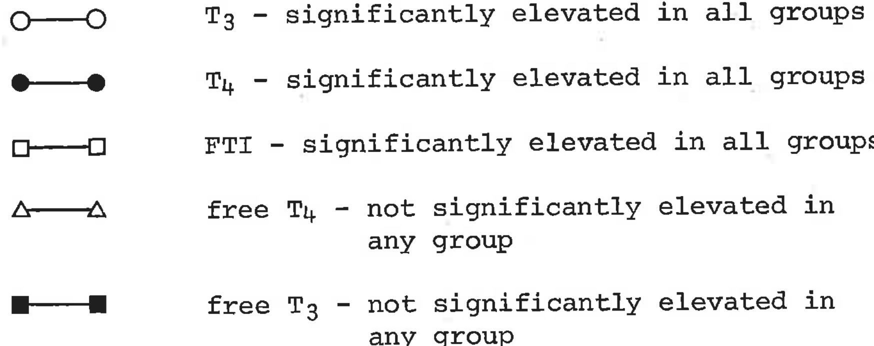
Mean total T_4, FTI and total T_3 were significantly elevated ($P < .001$) in all pregnant groups and the oral contraceptive group. Mean fT_4 and fT_3 were not significantly different to the normal euthyroid mean in any group.

pregnancy and by 41% and 53% respectively during regular ingestion of oral contraceptives (Figure 5.21). These increases reflect the elevated TBG in these states (Burr et al., 1977). The subjects in these groups showed no signs of thyroid dysfunction and were considered euthyroid. Neither mean fT_4 nor mean fT_3 concentration in any of these groups was significantly different to normal. However, in 2 first trimester subjects fT_4 concentration was above the euthyroid range (95% confidence limits) and one first trimester, one third trimester, and one oral contraceptive group subject had elevated fT_3 concentration (Figure 5.22).

Although the free hormone data can be considered to agree well with clinical euthyroidism in subjects with elevated TBG, there is evidence to suggest subtle changes in free hormone concentration during pregnancy. While early reports showed TSH to be elevated throughout pregnancy (Genazzani, Fioretti and Lemarchand-Beraud, 1971; Kannan, Sinha, Devi and Rastogi, 1973), Chan, Paraskevaides and Hale (1975) have recently shown TSH to be significantly elevated only in the first trimester.

Although Oppenheimer (1968) proposed that free thyroid hormone concentration was autoregulated during increased secretion of TBG, the combination of normal free hormone and elevated TSH concentration in the first trimester of pregnancy suggest pituitary involvement. It is significant that TSH is elevated during the period when TBG is most rapidly increasing, namely the first trimester. Alternatively, there may be an autonomous rise in TSH secretion

Figure 5.21 Relative changes in total and free T_3 and T_4 and in FTI in elevated TBG states.



There was no significant difference between the elevation in T_3 and T_4 in any group. The rise in T_3 and T_4 was significantly greater than FTI in all groups.

The groups are:

1 first trimester pregnancy

2 second trimester pregnancy

3 third trimester pregnancy

4 oral contraceptives

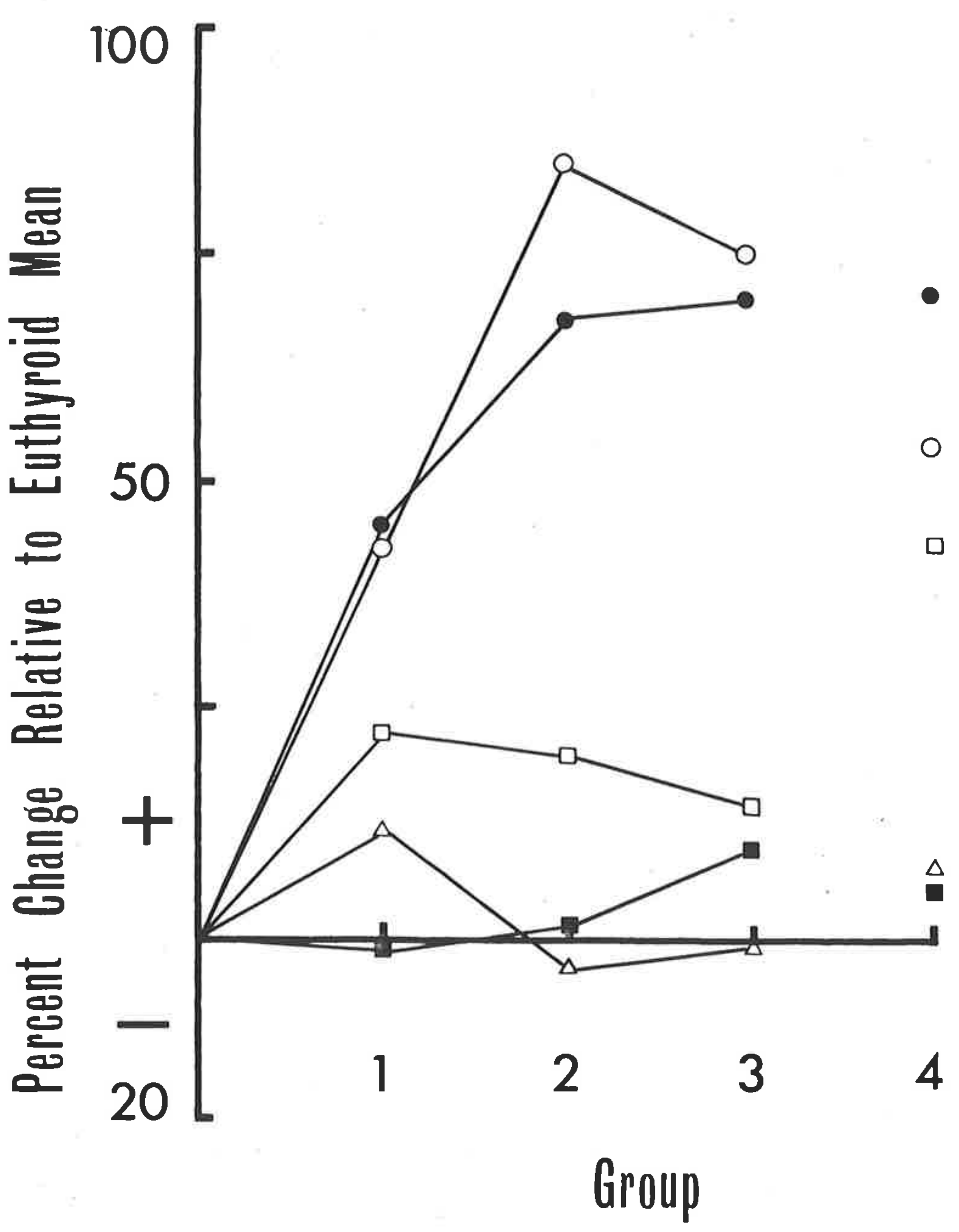

100
50
+
−
20
Percent Change Relative to Euthyroid Mean
1
2
3
4
Group

Figure 5.22 Percentage of subjects with elevated TBG with outlying fT_4 and fT_3 concentrations

The abcissa represents the percentage of subjects falling above or below the euthryoid reference range for that particular method.

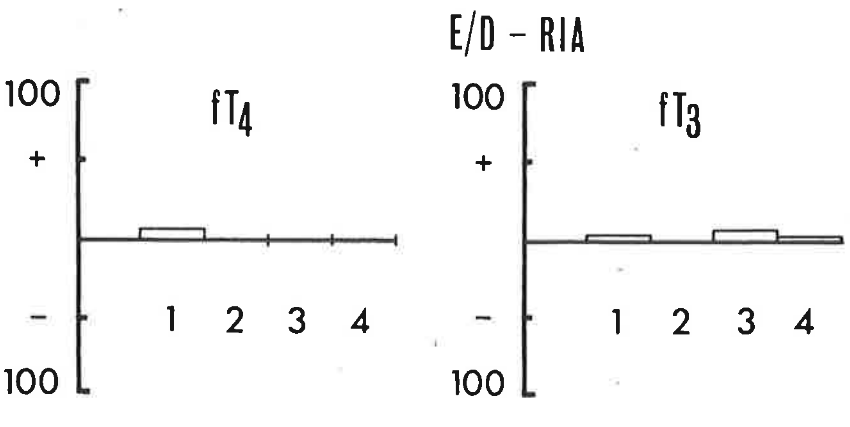
The groups are:

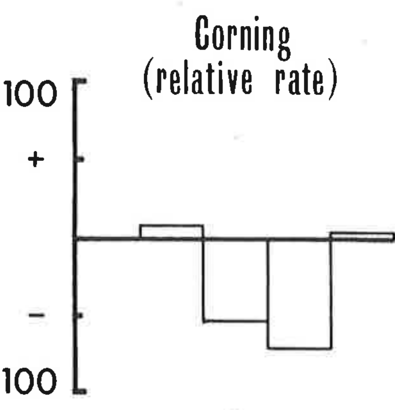
1 first trimester pregnancy

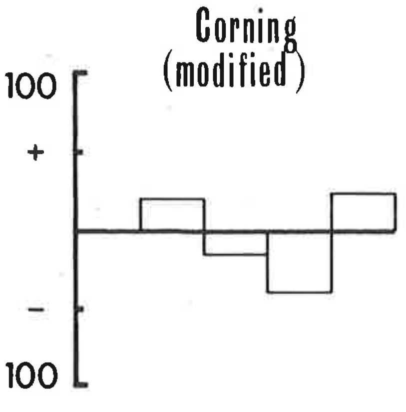
2 second trimester pregnancy

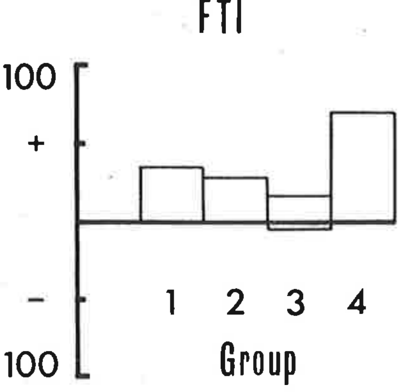
3 third trimester pregnancy

4 oral contraceptive group


E/D - RIA
100
+
−
100
fT_4
1 2 3 4
100
+
−
100
fT_3
1 2 3 4


Corning
(relative rate)
100
+
−
100


Corning
(modified)
100
+
−
100


FTI
100
+
−
100
1 2 3 4
Group

at this time in pregnancy which, together with an increased TBG concentration, does not result in elevated free hormone concentration.

In toto, the mean euthyroid fT_4 concentration was 11.7 $\pm$ 3.6 pmol/L and mean fT_3 concentration 5.7 $\pm$ 1.4 pmol/L.

3. Published Concentrations of Free Thyroid Hormone in Serum

In addition to being consistent with clinical euthyroid status, the data are in agreement with published results (Table 5.6). There is good general agreement on euthyroid fT_4 levels in the literature. The high mean reported by Petersen et al. (1977) may have been due to the use of 0.15 M phosphate buffer, pH 7.4 for dialysis. Spaulding and Gregerman (1972) found the percent fT_4 obtained by dialyzing against 0.15 M phosphate, pH 7.4 was double that when dialyzing against 0.01 M hepes at the same pH. Jiang and Tue (1977) used a 0.05 M phosphate buffer, pH 7.4, without apparent effect on fT_4, which was consistent with the observation of Spaulding and Gregerman (1972) that this concentration of phosphate had a lesser effect on protein binding of T_4.

It is of interest that 0.15 M phosphate, pH 7.4, had little effect on T_3 binding (Petersen et al., 1977). Due to the lower affinity of T_3 for the thyronine binding proteins, T_3 binding may be less sensitive to phosphate inhibition. Yeo et al. (1977) reported a higher level of fT_3 and concluded that fT_4 and fT_3 were present in equimolar

TABLE 5.6 Published data on free T_3 and free T_4 concentrations in euthyroid subjects

			fT_4 (pmol/L)	fT_3 (pmol/L)
1978	This study	E/D hepes/NaCl + RIA	11.7 3.4 - 20.0	5.6 2.7 - 8.4
1977	P.P.B. Yeo et al.	E/D hepes/NaCl + RIA	10.4 4.1 - 16.7	10.1 4.6 - 15.6
1977	B.A. Petersen et al.	E/D + Gas phosphate chrom.	24.4 13.2 - 35.6	6.5 4.7 - 8.3
1977	N-S Jiang et al.	E/D phosphate + RIA	9.8 6.3 - 17.0	No data
1976	R.D. Hesch et al.	E/D + RIA ?	No data	3.5 2 - 5
1975	S.M. Ellis and R.P. Ekins	E/D hepes/NaCl + RIA	11.7 7.4 - 16.0	5.0 3.3 - 6.7
1975	J. Weeke and H. Orskov	E/D + RIA modified Krebs-Ringer	--	8.2 5.2 - 11.2

concentrations. However, the consensus is that fT_4 is present at 2 - 4 times the concentration of fT_3.

In general, the concentrations of free thyroid hormones as determined by E/D-RIA or E/D-gas chromatography are lower than those determined by the earlier free thyroid hormone fraction methods (discussed in detail in Chapter 4 (4.2)).

4. Thyroid Disease

Despite the good correlation between the euthyroid status and free hormone concentration there were discrepancies with the clinically abnormal thyroid states (Table 5.7). This was particularly evident in the hypothyroid group in which 3 of 13 patients had normal fT_4 and 4 had normal fT_3 concentration. Yeo et al. (1977) have reported a similar problem. Of 21 hypothyroid subjects, they found 16 with normal fT_3 and 6 with normal fT_4 whereas all 21 thyrotoxic subjects had both fT_3 and fT_4 greater than normal. In this study, 2 of 14 fT_4 and 3 of 13 fT_3 values were normal in the thyrotoxic group.

This problem could reflect either the true physiological situation or technical limitations of the assays. Considering the precision profile of the fT_3 assay, a subnormal value of 2.2 pmol/L would have 95% confidence limits of 1.4 - 3.0 pmol/L and could be regarded as borderline normal (Figure 5.10). Similarly, the imprecision at low values of fT_4 could result in some subnormal fT_4 levels being measured as normal (Figure 5.16). Yeo et al. (1977) claim to have had less than 5% coefficient of variation at five points

TABLE 5.7 Thyroid disease

Patient	Total[b] T_4	Total[b] T_3	Free T_4[a] E/D-RIA	Free T_4[a] Corning	Free T_3[a] E/D-RIA	FTI	TSH[c]
			Hypothyroid				
396	26	1.0	< 1.0	< 6.5	3.9	< 10	-
400	18	0.6	< 1.0	< 6.5	3.0	< 10	-
402	27	1.1	< 1.0	< 6.5	5.3	16	-
500	< 10	0.2	< 1.0	-	0.9	< 10	32
501	14	1.0	< 1.0	-	1.0	12	46
502	< 10	0.8	< 1.0	-	1.8	< 10	75
Q2H	< 10	0.7	< 1.0	< 6.5	1.1	< 10	1.0
Q4H	< 10	0.5	< 1.0	< 6.5	0.7	< 10	> 40
Q5H	23	1.4	1.4	< 6.5	1.7	12	36
Q6H	27	0.4	1.2	< 6.5	1.1	18	> 40
Q8H	39	1.5	10.7	10.3	3.1	37	23
Q9H	42	0.9	5.0	-	1.5	43	6.5
Q10H	41	1.0	5.7	-	2.6	37	6.0
			Thyrotoxic				
395	200	5.0	59	66	34	301	UD[d]
399	248	7.0	96	74	49	428	UD
401	180	4.3	15	43	23	266	UD
403	218	-	94	68	-	413	UD
394	185	2.7	23	-	12	179	UD
503	> 400	11	175	-	59	> 600	UD
504	324	6.9	152	-	32	376	UD
Q1T	144	3.3	51	40	10	156	UD
Q3T	208	2.5	31	27	3.7	131	0.5
Q4T	242	6.0	56	41	13	196	UD
Q5T	155	3.7	29	-	9.2	195	1.0
Q6T	144	2.8	62	-	10	190	0.8
Q7T	164	3.0	18	-	3.4	182	UD
Q9T	169	4.0	66	-	7.7	236	UD

[a] pmol/L; [b] nmol/L; [c] mU/L

[d] < 0.4 mU/L

along the standard curve for both fT_3 and fT_4. Despite this very good precision, the spread of normal values is similar to that of data presented in this thesis. The ratio of the standard deviation to the mean was 0.26 and 0.36 for fT_3 and fT_4 respectively for the euthyroid data presented in this thesis compared with the ratios 0.28 and 0.31 for fT_3 and fT_4 respectively for data presented by Yeo et al. (1977). That is, the measurements of Yeo may have a higher imprecision than the intra-assay precision studies suggest, thus leaving open the question of technical limitations to the measurement of subnormal free hormone concentrations by E/D-RIA. Or, accepting the precision to be as good as reported, free hormone levels may in fact be normal in some hypothyroid individuals.

5. Equilibrium Dialysis-Radioimmunoassay as a Reference Method

Although the discrepancies between free hormone concentration as measured by E/D-RIA and clinically abnormal thyroid function remain to be clarified, the good correlation with the clinically euthyroid state in healthy subjects supports the use of this technique as a reference method.

The limitations to the use of the E/D-RIA method as a reference method for free hormone analysis should be recognised. The method reflects only the equilibrium conditions between the thyroid hormones and their binding proteins *in vitro*. Now that intracellular thyroid hormone receptors are well documented (Oppenheimer et al., 1972a), it is obvious that free hormone concen-

tration *in vivo* is determined by binding to both extracellular and intracellular binding proteins. T_3 has an affinity for the nuclear receptors of the same order of magnitude as for TBG (Schuster, Schwartz and Oppenheimer, 1979). When it is considered that the distribution space of T_3 is mainly intracellular, it becomes clear that nuclear binding of T_3 is an important determinant of fT_3 concentration. In contrast, affinity of the nuclear receptors for T_4 is an order of magnitude less than that for T_3. The distribution space of T_4 is confined principally to the circulation and the liver suggesting that fT_4 levels *in vivo* mainly reflect binding to the serum thyronine binding proteins.

Binding of the thyroid hormones *in vivo* need not necessarily be at equilibrium. It is possible that uptake of hormone is so rapid as to exceed the dissociation rate of thyroid hormone from the serum binding proteins. Serum free hormone concentration in these organs would be lower than that measured *in vitro*. If one considers whole body clearance of T_3, the clearance of free T_3 is about 1/6000 the dissociation rate of T_3 from the thyronine binding proteins (Appendix 2). That is, the system as a whole may be considered to be at equilibrium. However, the question still remains as to whether clearance of T_3 from the circulation in some organs exceeds the dissociation rate of T_3 from the serum binding proteins.

While these limitations are acknowledged, they are pertinent to all techniques measuring serum free thyroid hormone concentration. A comparison of the E/D-RIA method

with other procedures supports the conclusion that E/D-RIA most accurately reflects the concentration of the free thyroid hormones in a serum sample.

ANALYSIS OF FREE THYROID HORMONE CONCENTRATION BY A KINETIC METHOD

1. Free Thyroxine Concentration in Healthy Euthyroid Subjects

The principle of the kinetic measurement of free hormone concentration has been discussed in Section 4 (4.3). The Corning Free T_4-^{125}I Radioimmunoassay Test System, a commercially available kit using this principle, was evaluated for consistency with clinical status and compared to the E/D-RIA method (Table 5.8). Data using both the originally proposed relative rate method and the recently modified method (see Section 5.2 for a detailed description) are presented.

Using the relative rate method, the mean fT_4 concentration in both the first trimester and oral contraceptive groups were normal. However, during the second and third trimesters, fT_4 concentration was significantly diminished. A substantial number of subjects in these latter two groups had subnormal fT_4 (Figure 5.22). These results were clearly at variance with the euthyroid status of these individuals. Boss, Djahanbakhch and Kingstone (1978) and Hale (1979) have reported similar discrepancies and concluded that the elevated TBG in these euthyroid subjects was interfering in the Corning test system. These conclusions were verified by a theoretical analysis of the kinetic method

by Ekins (1979b). He suggested three different calculations to make the data more reliable, of which one was recommended by the manufacturer (Corning, 1979) and called here the modified method.

The precision profile of the Corning modified test system was particularly good at the normal and low fT_4 concentrations (Figure 5.24). The E/D-RIA method displayed higher imprecision in this range. While intraassay precision was good, inter-assay precision was no different to that of the E/D-RIA assays in this study (Table 5.9). In contrast to the relative rate method, the modified Corning test system gave data which was more consistent with the clinical status of individuals with elevated TBG (Table 5.8). When compared to the relative rate method data the modification appeared to have raised the mean of all groups relative to the healthy euthyroid mean. Thus the oral contraceptive and first trimester group means were now significantly greater than normal, while the second and third trimester means were still significantly lower than normal. Whereas the number of high fT_4 levels increased in the former two groups, the number of low fT_4 levels decreased in the latter two groups (Figure 5.22).

Free T_4 concentration in the serum of those patients on either salicylates or anticonvulsants was within the euthyroid range, as was the fT_4 concentration in serum derived from samples of cord blood (Table 5.8).

TABLE 5.8

Group	N	Free T_4			FTI	Total T_4	
		Corning		Equilibrium dialysis		Laboratory	Corning
		Relative rate	Modified				
Healthy euthyroid	60	17.9 ± 3.4	19.5 ± 2.1	10.2 ± 3.4	102 ± 19	103 ± 19	96 ± 16
First trimester	15	18.2 ± 4.0	21.1 ± 3.7[a]	12.4 ± 5.0[c]	124 ± 43[d]	137 ± 40[d]	139 ± 34[d]
Second trimester	14	11.1 ± 2.8[d]	17.3 ± 2.1[d]	9.7 ± 3.6	121 ± 38[b]	178 ± 40[d]	155 ± 28[d]
Third trimester	10	10.7 ± 3.2[d]	16.1 ± 2.1[d]	10.6 ± 2.6	116 ± 21	190 ± 35[d]	161 ± 16[d]
Oral contraceptives	25	17.4 ± 4.3	22.5 ± 2.7[d]	11.3 ± 2.2	150 ± 21[d]	169 ± 47[d]	141 ± 26[d]
Cord blood	9	17.1 ± 1.7	20.3 ± 1.7	15.0 ± 4.1[d]	-	-	117 ± 30[c]
Salicylate	4	17.7 ± 7.6	-	10.7 ± 4.7	74 ± 6	87 ± 7	92 ± 8
Anticonvulsants	7	13.6 ± 1.5[c]	-	8.2 ± 1.7	57 ± 7[d]	68 ± 10[d]	88 ± 10

All data expressed as mean ± 1 S.D. (pmol/L)

P values are given for the pregnant and oral contraceptive groups where the mean fT_4 concentration of these groups were significantly different to normal; [a] $P < .05$, [b] $P < .02$, [c] $P < .01$, [d] $P < .001$.

TABLE 5.9 Free T_4 quality control data[a]

Control	Corning test system		
	[b]Multiple Replicates	Pooled data	
	Intra-	Intra-	Inter-
Corning Low	19.1 ± 1.2 6.4	19.7 ± 0.3 1.3	19.7 ± 2.4 12.4
Corning High	35.9 ± 2.2 6.1	37.0 ± 2.5 6.6	37.0 ± 4.7 12.6
Euthyroid Serum pool		18.7 ± 1.2 6.4	18.7 ± 2.4 13.1
Thyrotoxic Control		73.4 ± 1.7 2.3	73.4 ± 10.5 14.3

[a] All data are expressed as mean (pmol/L) ± 1 S.D. with the % C.V. quoted below.

[b] Multiple replicate data were taken from an assay in which all controls were assayed as 20 replicates. Pooled data are the results of the one way analysis of variance of values pooled from 7 assays.

Figure 5.23 Comparison of fT_4 concentration assayed by the E/D-RIA and Corning (modified) techniques

● euthyroid

△ hypothyroid

□ thyrotoxic

○ fT_4 concentration obtained for the Corning standards measured by E/D-RIA plotted against the stated Corning fT_4 value for the particular standard.

There was no significant correlation between fT_4 concentrations measured by the two techniques in the healthy euthryroid and pregnant groups. There was a significant correlation between the two techniques in the oral contraceptive group ($P < 0.05$) and when all groups were considered (r 0.9196, $P < .001$).

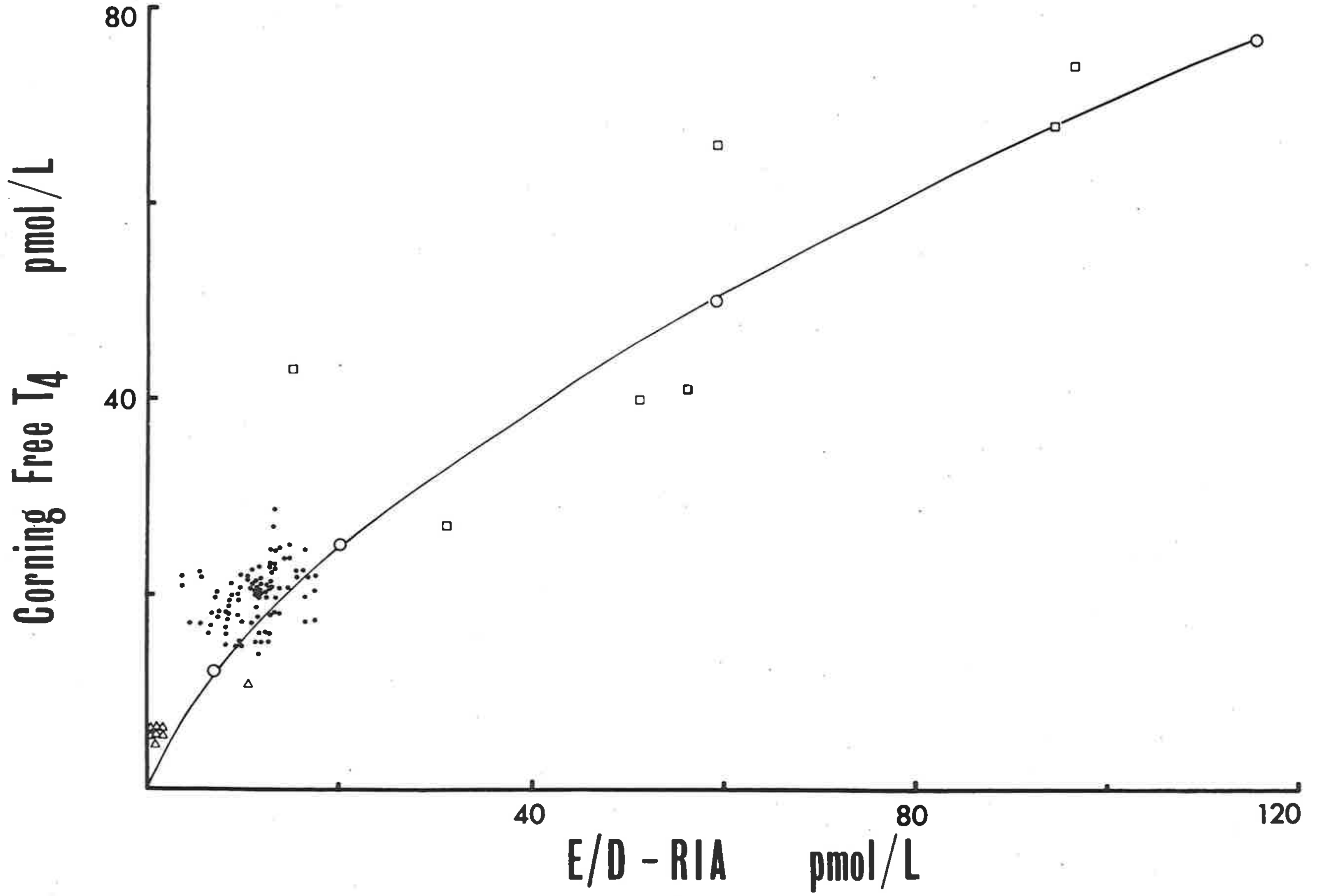
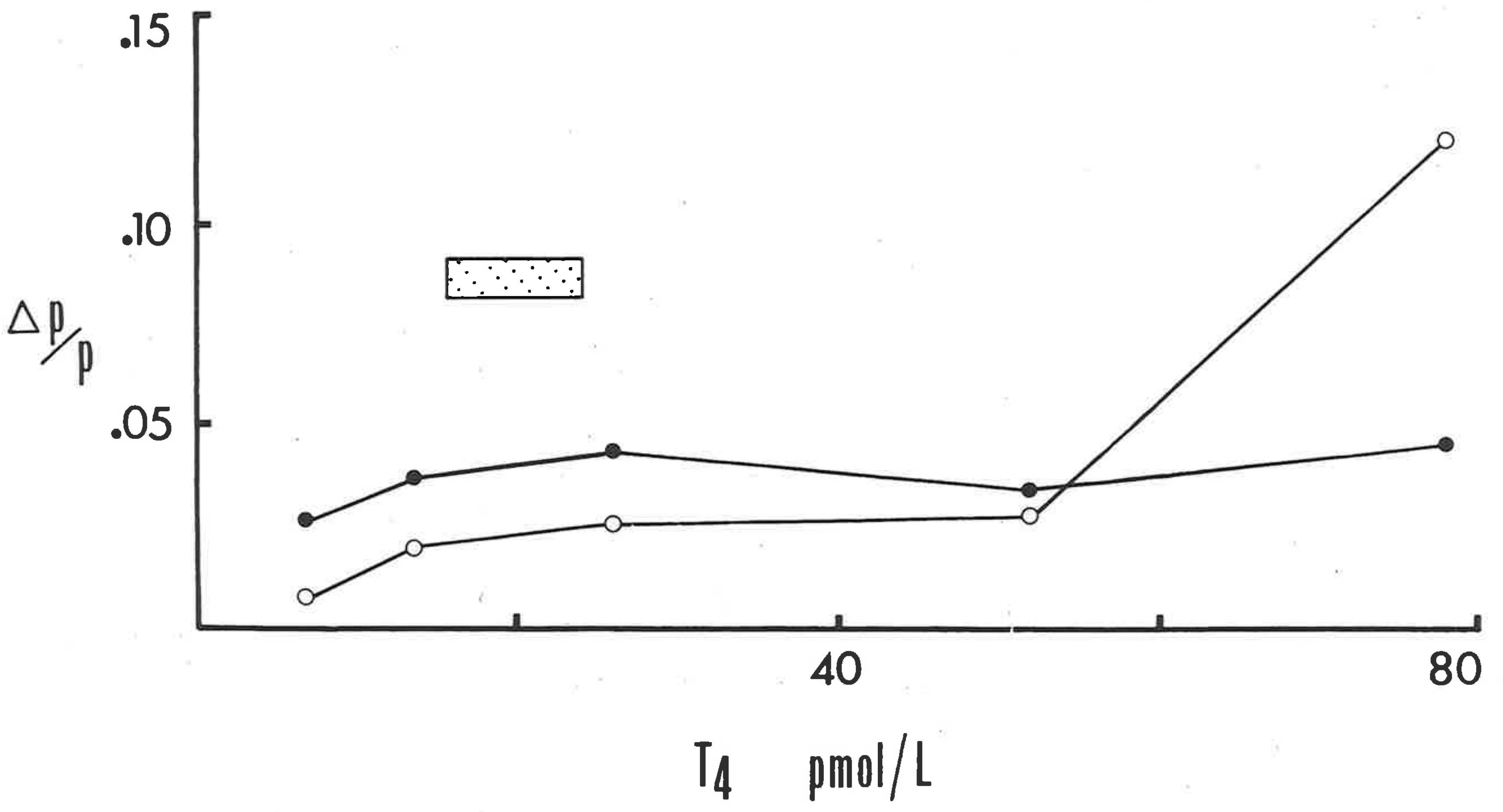

80
40
Corning Free T4 pmol/L
40
80
120
E/D - RIA pmol/L

Figure 2.24 Precision profile of the Corning (modified method) fT_4 assay

●——● Profile determined from data accumulated over seven assays

○——○ Profile determined by assaying each standard as ten replicates in the one assay.

[dotted box] Reference range



.15
.10
.05
Δp/p
40
80
T4 pmol/L

2. Free Thyroxine Concentration in Thyroid Disease

The Corning method differentiated all the abnormal thyroid function patients from normal (Table 5.7 and Figure 5.23). Of particular interest was one hypothyroid patient (Q8H) who had normal fT_4 by E/D-RIA. Although the Corning fT_4 value was considerably higher than other hypothyroid levels, it was below normal and agreed with the total T_4, FTI and clinical assessment. In one thyrotoxic patient (Q1T), total T_4 and FTI were borderline high, whereas fT_4 by both E/D-RIA and the Corning modified method was unequivocally elevated.

3. Investigation of the Discrepancy Between Clinical Euthyroid Status and Free Thyroxine Concentration

As was outlined in Chapter 4 (4.3), when anti-T_4 is added to serum, the rate of binding of T_4 to the antibody in the absence of binding inhibitors is determined by the fT_4 concentration. After a given incubation time the amount of T_4 bound to anti-T_4 is determined and related to a standard curve to determine the fT_4 concentration. In the Corning modified method, the amount of T_4 bound is determined by the use of ^{125}I-T_4. After a given time of incubation, the ^{125}I-T_4 bount to anti-T_4 is measured (A cpm). From the total ^{125}I-T_4 added to the tube (T cpm), the fraction of ^{125}I-T_4 bound to the antibody (A/T) is calculated. This fraction of ^{125}I-T_4 bound will be the same as the fraction of endogenous or unlabelled T_4 in the serum bound to the antibody. Thus, the absolute amount of serum T_4 bound to the antibody can be calculated knowing the serum total T_4 and the fraction of

$^{125}I\text{-}T_4$ bound and is expressed as $(A/T \times T_4)$nmol/L.

To further analyze the Corning data, the fraction $^{125}I\text{-}T_4$ bound to antibody (A/T) was plotted against T_4 for the standards (Figure 5.25a). For the purposes of the following discussion, it is assumed that TBG concentration is similar in all the standards. It is seen that as T_4 and fT_4 increase, A/T remains constant reflecting the constant fraction free T_4. In contrast, $(A/T \times T_4)$ increases, reflecting increasing fT_4. The constant A/T can be rationalized by referring to Table 4.5. With increasing fT_4, the rate of T_4 binding to anti-T_4 increases. However, $^{125}I\text{-}T_4$ binding does not increase because, with increasing endogenous T_4, the specific activity of the added $^{125}I\text{-}T_4$ diminishes.

The situation is different where fT_4 is constant and TBG variable as in the euthyroid subjects with elevated TBG. In these groups the constant fT_4 would be expected to be reflected by a constant $(A/T \times T_4)$. That is

$$A/T \times T_4 = \text{constant}$$

or

$$A/T = \text{constant} \times 1/T_4$$

That is, A/T varies inversely with T_4 in those subjects with variable TBG but normal fT_4. The mean $(A/T \times T_4)$ for the euthyroid subjects with normal binding protein concentration was 11.5 $\pm$ 1.3 nmol/L. That is, $(A/T \times T_4)$ values for these subjects had a 2 S.D. range of 8.9 - 14.1 nmol/L. Plotted in Figure 3.25 are the A/T vs T_4 curves representing $(A/T \times T_4)$ equal to 8.9, 11.5 and 14.1 nmol/L. Only 2 of the normal euthyroid subjects had $(A/T, T_4)$ outside these limits.

One would expect (A/T, T_4) for euthyroid subjects with variable TBG and normal fT_4 to fall within these limits also. Ideally, (A/T x T_4) would be expected to be the same as in the normal euthyroid group, that is, 11.5 ± 1.3 nmol/L. Thus, with increasing T_4 due to increasing TBG, A/T will decrease in proportion to the reciprocal of the T_4 concentration representing diminishing fraction free T_4. With reference to Table 4.5 this fall in A/T is seen to be due to diminished specific activity of the $^{125}I\text{-}T_4$ through dilution by increased endogenous T_4.

In this study, 18 of the 20 oral contraceptive subjects and 11 of the 15 first trimester subjects had A/T values higher than the expected mean A/T. On the other hand the A/T values of all the second and third trimester subjects were less than the expected mean A/T. That is, at any given T_4 concentration, the rate of binding of $^{125}I\text{-}T_4$ in the former two groups is higher than expected and in the latter groups, lower than expected.

One conclusion is that fT_4 is variable in these groups and that the better precision of the kinetic procedure allows these changes to be measured. This infers that these subtle changes in fT_4 are either not manifested clinically or that the clinical assessment is not sensitive enough to detect the clinical signs of slightly variable fT_4. An alternative conclusion is that fT_4 is in fact normal in these individuals and that the Corning method, despite the use of a modified method, is subject to interference by thyroid hormone binding proteins. Of interest here is that the pattern of changes in fT_4 as measured by the Corning method

Figure 5.25 Corning free T_4 assay: relationship between fraction $^{125}I-T_4$ bound to immobilized antibody and the serum T_4 concentration

●---● A/T vs T_4 for the healthy euthyroid group

A/T x T_4 = 11.5 (mean for the healthy euthyroid group)

●——● A/T x T_4 = 8.9 and 14.1 (the 2 S.D. limits for the healthy euthyroid group)

a.

○---○ A/T vs T_4 for the standards (with the 2 S.D. ranges marked in)

~~● healthy euthyroid~~

□ first trimester of pregnancy

■ oral contraceptives

▽——▽	dilution of serum 1	See Table 5.10
▼·····▽	calculated dilution curve for serum 1	
△——△	dilution of serum 2	See Table 5.10
▲·····△	calculated dilution curve for serum 2	

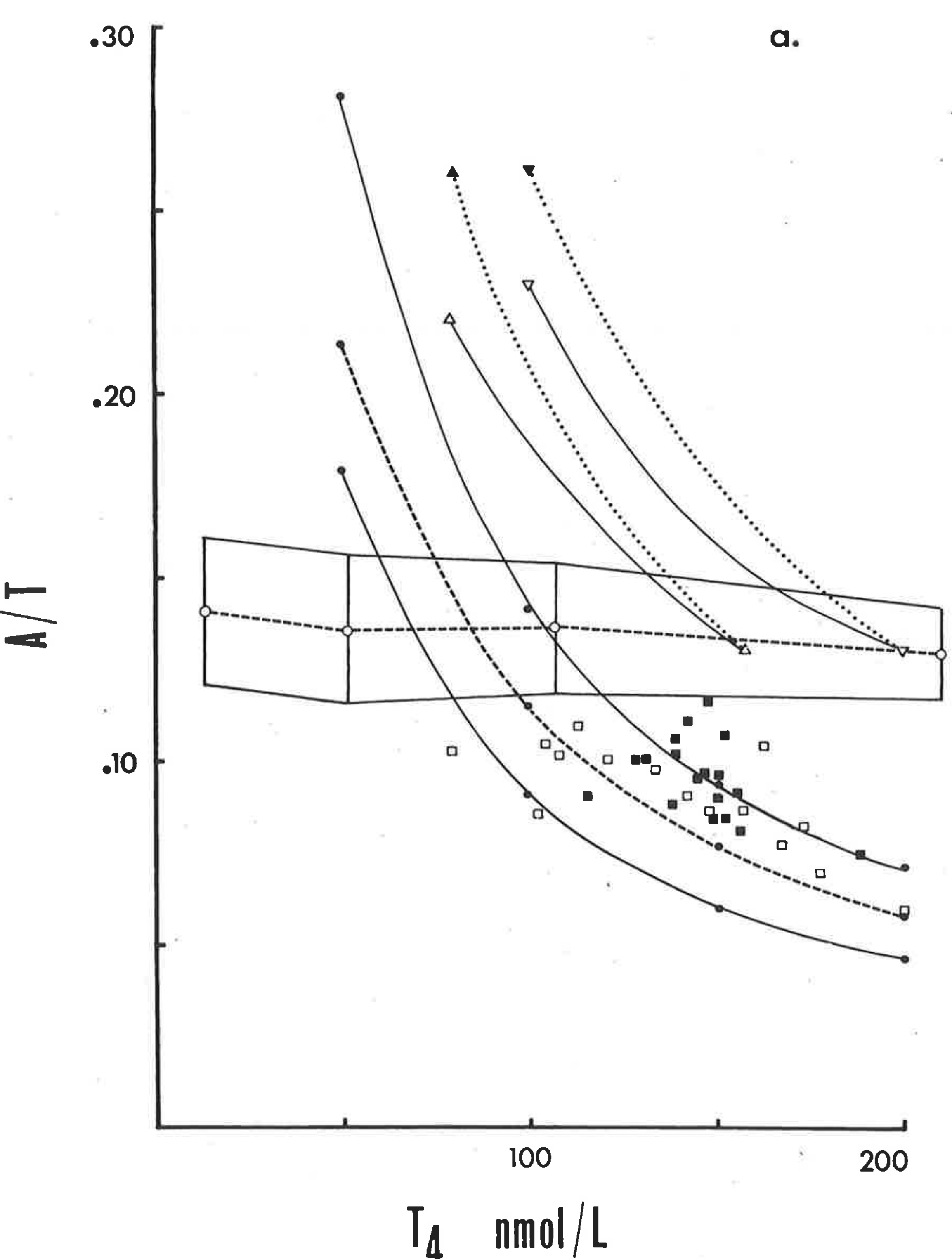
b.

△ second trimester pregnancy

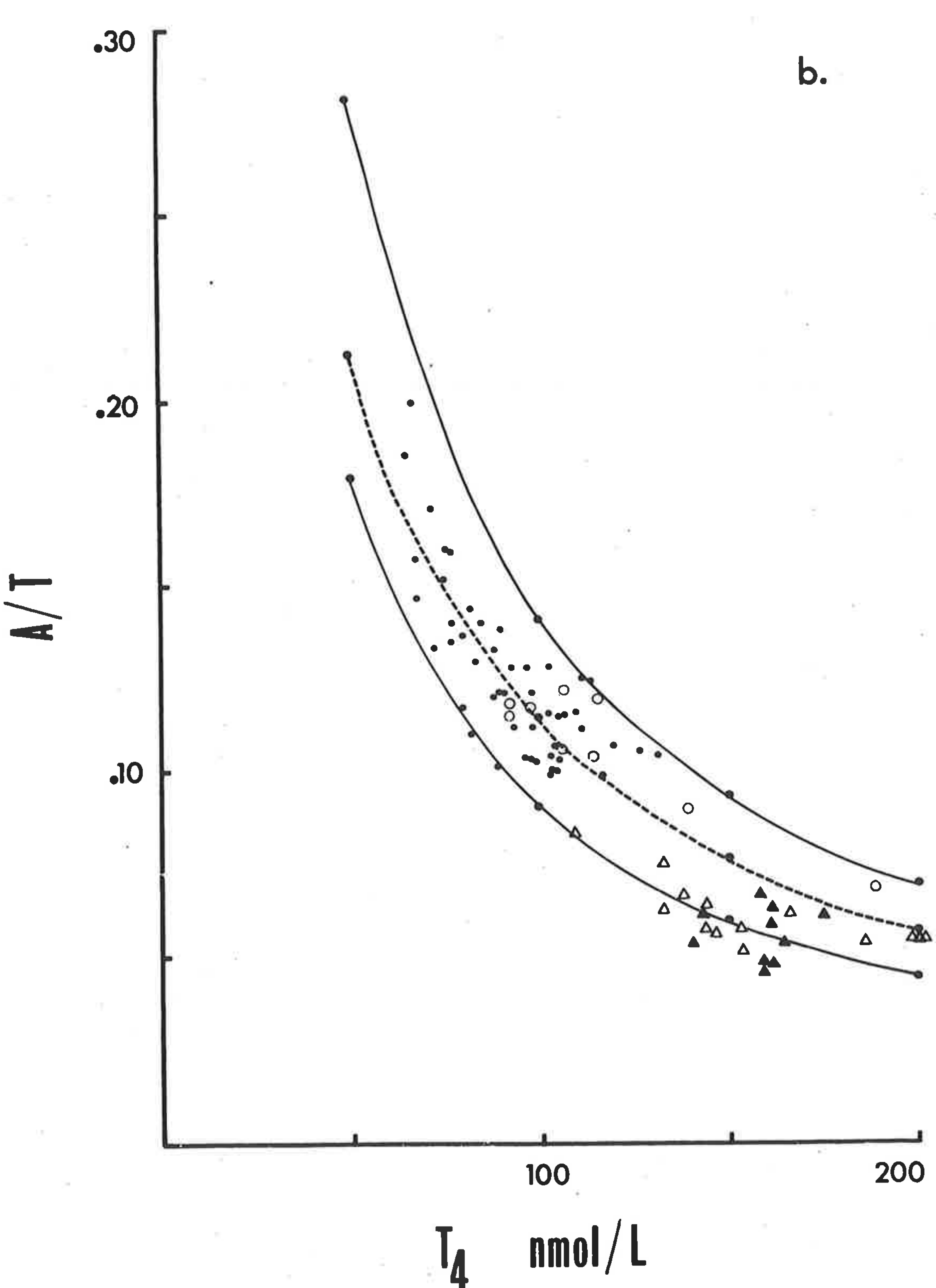
▲ third trimester pregnancy

○ cord sera

● healthy euthyroid



a.
.30
.20
.10
A/T
100
200
T_4 nmol/L



b.
.30
.20
.10
A/T
100
200
T_4 nmol/L

and the FTI are similar (Figure 5.22). As will be explained further on, the FTI is subject to binding protein interference. Both fT_4 (Corning) and FTI are raised in the first trimester and fall progressively in the second and third trimesters. What is more, both are higher in the oral contraceptive group than at any time during pregnancy.

4. Investigation of Thyronine Binding Protein Interference in Measuring Free Thyroxine Concentration

A disturbing feature of the Corning method was that up to 20% of the endogenous T_4 in serum may be stripped from the binding protein. This would make the method vulnerable to binding protein interference and it was recommended by Ekins (1979a) that not more than 5% of endogenous T_4 be stripped from the binding proteins if the kinetic procedure is to reliably measure fT_4 concentration.

On a theoretical study of binding of T_4 to TBG at various fT_4 and TBG concentrations, Roosdorp and Joustra (1979) found that at a given fT_4 concentration the ratio of T_4 to TBG decreased with increasing TBG. Burr et al. (1977) have shown TBG in the oral contraceptive group to be lower than in the pregnant groups. If one postulates that TBG in the first trimester is lower than that in the second and third trimesters (suggested by the total T_4 levels but as yet no direct comparative measurements of TBG have been reported) then the first trimester and oral contraceptive groups have similar and lower TBG concentrations than the late pregnancy groups. The data of Roosdorp and Joustra (1979) would infer the T_4/TBG ratio to be higher in the former two groups. It

is possible, because so much T_4 is stripped from the binding proteins during the incubation period in the Corning method that the T_4/TBG ratio becomes a determinant of binding of T_4 to the anti-T_4.

Thus the higher T_4/TBG ratio in the former two groups would tend to accelerate ^{125}I-T_4 binding to the anti-T_4, whereas the lower T_4/TBG ratio in mid to late pregnancy would tend to retard ^{125}I-T_4 binding to the anti-T_4. Little data is available on T_4/TBG in these groups. McDowell (1979) found T_4/TBG in pregnancy and during oral contraceptive use to be no different to normal. However, the kinetic fT_4 method may be more sensitive to changes in T_4/TBG.

The discrepancy between the estimates of fT_4 concentration in cord blood as measured by the kinetic and E/D-RIA methods can also be interpreted in this way. The E/D-RIA data suggest that fT_4 concentration in cord blood is elevated. In view of the high TBG concentration in cord blood as compared to the adult reference range (DeNayer, Malvaux, Van Den Schneck, Beckers and De Visscher, 1966), the fT_4 concentration may be underestimated by the Corning modified method in the same manner as the concentration is underestimated in the second and third trimester of pregnancy.

A further problem with the Corning method is the effect of serum dilution. The E/D-RIA method is insensitive to dilution of serum reflecting the expected constancy of fT_4 during serum dilution (see Chapter 4 (4.2)). Contrary to the manufacturer's claims, dilution caused a marked reduction in the measured fT_4 concentration (Figures 5.25a) and Table

5.10). In fact, for (A/T x T_4) to remain constant with dilution, the fraction of tracer bound to the anti-T_4 must exceed the amount added to the tube at serum dilutions of greater than 1/8.

5. Comparison of the Kinetic and Equilibrium Dialysis-Radioimmunoassay Data

Both the Corning methods gave fT_4 concentrations greater than the E/D-RIA procedure. Measurement of fT_4 in the Corning serum standards by E/D-RIA showed a curvilinear relationship between the two methods (Figure 5.26). The Corning standards are calibrated against an E/D-RIA method and the curvilinear relationship seen in Figure 5.26 may be due to differences between the manufacturer's E/D-RIA method and that used in this study. Furthermore, the buffer used in the Corning method is not stated and could have caused the higher levels of fT_4 obtained using this kit.

There was no significant correlation between the modified method and E/D-RIA data in any of the euthyroid groups except in the oral contraceptive group ($P < 0.05$; Figure 5.26). When the data in the abnormal thyroid function groups was included, there was significant correlation between the two methods (Figure 5.26).

6. Summary

While the kinetic method differentiated those subjects with thyroid disease, there were serious discrepancies between fT_4 concentration and clinical status in those euthyroid subjects with elevated TBG concentration.

TABLE 5.10 Effect of dilution on the estimate of fT_4 concentration by the Corning free T_4-^{125}I radioimmunoassay test system (modified method)

Dilution factor	Total T_4[b] (nmol/L)	Free T_4 (pmol/L)	Measured A/T[c]	A/T to give constant fT_4
		[a] Serum 1		
1	200	49	.13	-
2	100	35	.23	.26
4	50	24	.35	.52
8	25	15	.46	1.10
16	13	12	.56	2.10
		Serum 2		
1	158	33	.13	-
2	79	26	.22	.26
4	40	20	.33	.52
8	20	14	.44	1.00
16	10	9	.55	2.10

[a] The serum samples were diluted in 0.1% BSA, 0.01 M Hepes, 0.15 M NaCl, pH 7.4.

[b] Total T_4 (nmol/L) represents the idealized total T_4 after dilution and is equivalent to the (total T_4 concentration of the undiluted sample) ÷ (dilution factor).

[c] A/T is the fraction of counts bound to the immobilized antibody in the absence of thyroid hormone binding protein inhibitor.

Figure 5.26 Relationship between the T_4 concentration and the FTI

● normal euthyroid subjects

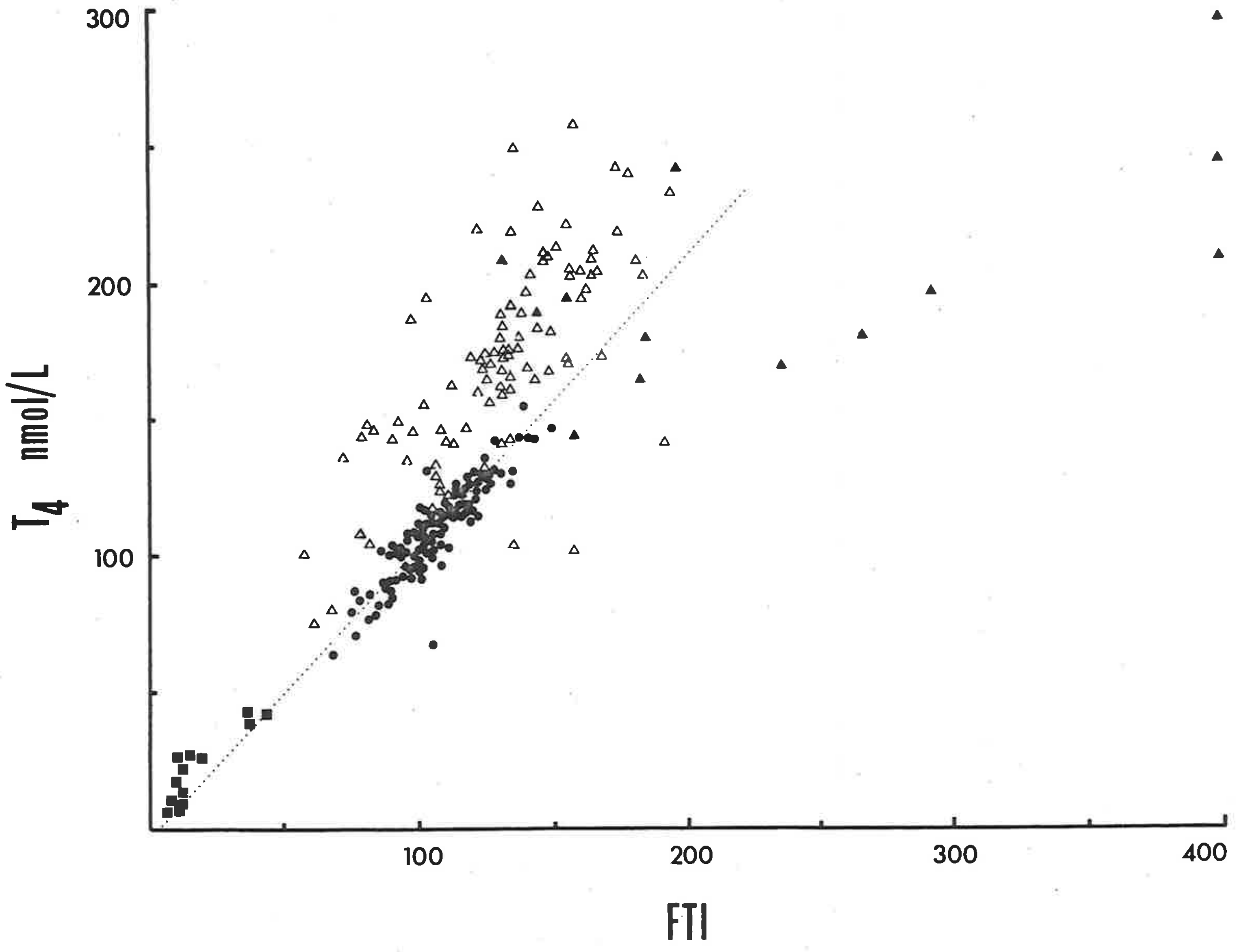
linear regression $r = 0.9115$, $P < 0.001$

△ euthyroid subjects with elevated TBG

linear regression $r = 0.7293$, $P < 0.001$

■ hypothyroid subjects

▲ thyrotoxic subjects


300
200
100
T_4 nmol/L
100
200
300
400
FTI

Although the principle of measurement of fT_4 concentration by this procedure is sound, the method used in the Corning test method is compromised by the excessive quantity of the endogenous T_4 which is taken up by the anit-body. While the assay may have been designed in this manner so as to ensure adequate precision, it would appear to have been done at the expense of rendering the assay susceptible to variations in TBG concentration.

AN INDIRECT MEASUREMENT OF FREE THYROXINE - THE FREE THYROXINE INDEX

1. Serum Free Thyroxine Index in Healthy Euthyroid Subjects

The FTI is a commonly employed calculation used to correct T_4 concentration for thyronine binding protein abnormalities (Chapter 4 (4.4)).

In this study the FTI was found to be significantly elevated in pregnancy and during oral contraceptive ingestion (Table 5.5). 40% of all euthyroid individuals with elevated TBG had abnormal FTI, all but one being higher than normal (Figure 5.22). The FTI was clearly quite inadequate in correcting for protein binding abnormalities in euthyroidism (Figure 5.22). There was a surprisingly high FTI in the oral contraceptive group, being significantly higher than the FTI at any time during pregnancy. Both Burr et al. (1977) and McDowell (1979) have shown TBG to be lower in oral contracep-tive users than in pregnant women. However, their data were not assessed for statistical significance. If these differ-ences are statistically significant then the finding in this study that T_4 in the oral contraceptive users is the same

as in the last two trimesters of pregnancy would suggest that T_4/TBG in the former group is higher than in mid to late pregnancy. The higher T_4/TBG ratio would lead to a greater T_3U and FTI. McDowell (1979) has shown a higher T_4/TBG ratio in the former group than in pregnancy, but there is no indication as to whether this was significant.

2. <u>Thyroid Disease</u>

The FTI discriminated the patients with abnormal thyroid function very well (Table 5.7). In only one patient, the thyrotoxic patient Q3T, was FTI normal whereas fT_4 by E/D-RIA and the Corning method was elevated.

3. <u>Correlation with Other Assays of Free Thyroxine Concentration</u>

There was a significant correlation between the FTI and the E/D-RIA data in the healthy euthyroid ($r = .3650$, $P < .001$, $n = 108$) and pregnancy groups ($r = .4534$, $P < .001$, $n = 74$), but not in the oral contraceptive group. Considering the data in both the euthyroid groups and in the thyroid disease, there was a significant correlation ($r = .8285$, $P < .001$, $n = 238$).

When compared with data from the modified kinetic method, the correlation was significant in all groups: healthy euthyroid ($r = .4663$, $P < .01$, $n = 34$), pregnancy ($r = .4467$, $P < .01$, $n = 33$), oral contraceptives ($r = .6229$, $P < .01$, $n = 19$) and total ($r = .9014$, $P < .001$, $n = 101$).

4. Thyronine Binding Globulin Interference

There is growing documentation that the FTI does not properly correct for binding protein variations. Despite claims that the FTI was a suitable estimate of fT_4 concentration (Anderson, 1968; Howorth and MacLagan, 1969; Stein and Price, 1972) more recent data dispute this conclusion. Both Souma, Niejadlik, Cottrell and Rankel (1973) and McDowell (1979) have reported significantly elevated FTI during pregnancy and Wellby, O'Halloran and Marshall (1974) found 13% of euthyroid subjects with elevated TBG to have abnormal FTI. There is also inadequate correction for low thyroid hormone binding protein concentrations. Wellby et al. (1974) found only 64% patients with hypoalbuminaemia of chronic illness or who were on methyl testosterome therapy had normal FTI. More recently McDowell (1979) has reported a significantly low mean FTI in a group of sick euthyroid patients with low mean TBG and Burr, Evans, Lee, Prince and Ramsden (1979) found only 4 of 12 patients with idiopathic low TBG had normal FTI.

The significant correlation between the FTI and T_4 in all groups studied ($P < 0.001$) supports the conclusion that FTI inadequately corrects for variations in thyronine protein concentration (Figure 5.26). In contrast, there was no correlation between the E/D-RIA fT_4 data and T_4 concentration (Figure 5.27) except in healthy euthyroid subjects without binding protein abnormalities ($r = 0.3396$, $P < 0.001$, $n = 107$). The Corning modified method data correlated significantly in both the healthy euthyroid subjects without binding protein abnormalities ($r = 0.3347$, $P < 0.05$, $n = 36$) and oral contraceptive group ($r = 0.4649$, $P < 0.05$, $n = 19$).

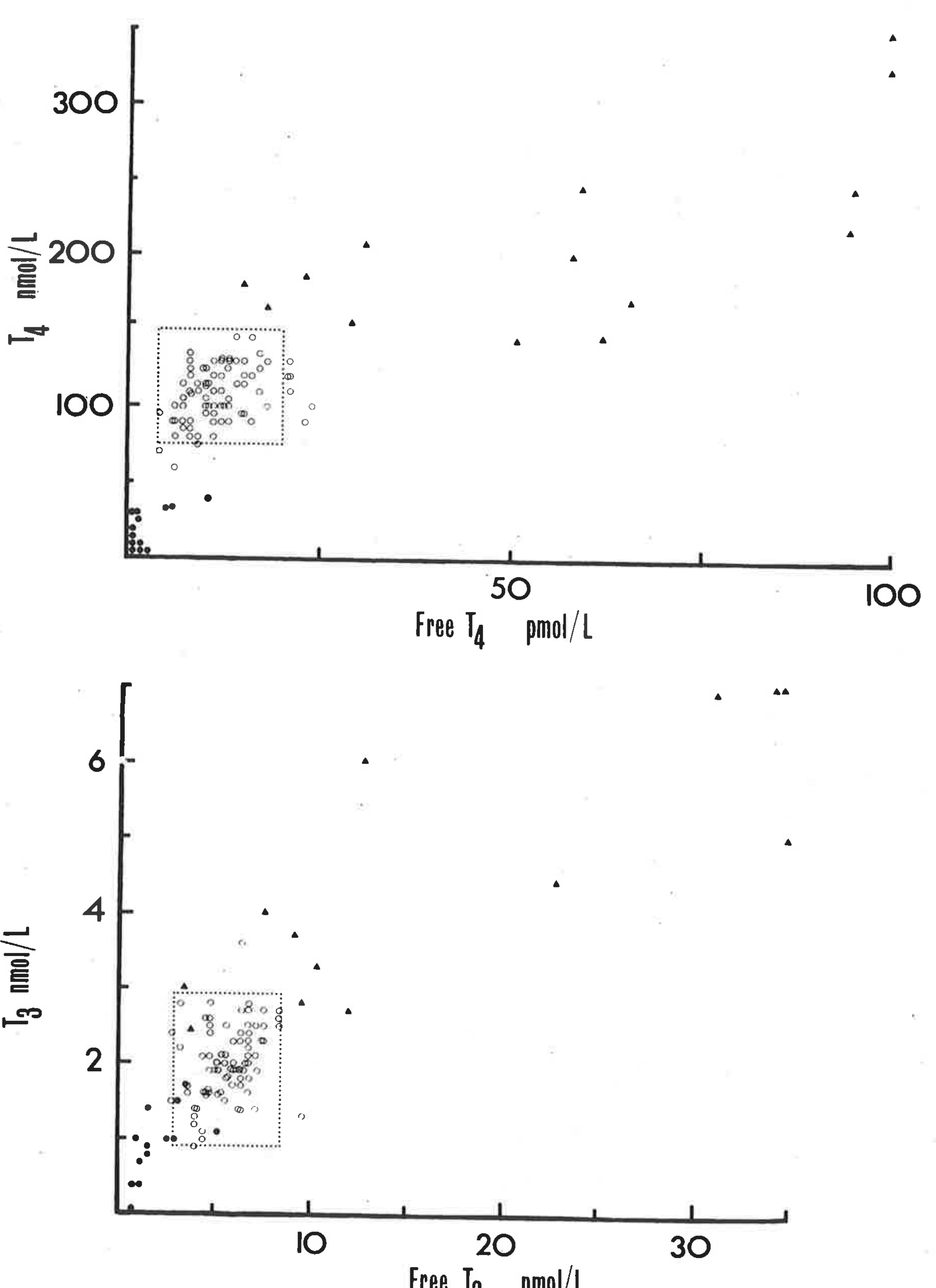
Figure 5.27 Relationship between total and free thyroid hormone concentrations

▲ thyrotoxic

○ euthyroid

● hypothyroid

The euthyroid reference ranges are marked.


300
200
100
T_4 nmol/L
50
100
Free T_4 pmol/L
6
4
2
T_3 nmol/L
10
20
30
Free T_3 pmol/L

Using the data of McDowell (1979) it is shown in Table 4.4(a) and Figure 4.1 that the concentration of unoccupied TBG binding sites [TBG] varies in concert with changes in [TBG_O] in the pregnant and oral contraceptive groups. The T_3U significantly underestimates the [TBG] in these groups, and so tends to be greater than would be expected if it accurately measured [TBG]. The result is an FTI value which undercorrects the elevated T_4. On the other hand, in the sick enthyroid group the T_3U does appear to reflect accurately the small drop in [TBG]. However, in this group the fall in [TT_4] is about double the fall in [TBG], most likely due to the fall in [TBPA] seen in chronically ill patients. One conclusion is that the problems with the T_3U are methodological. Thus, where changes in [TBG] are smaller, T_3U is accurate but where changes in [TBG] are larger (as in pregnant and oral contraceptive groups) T_3U is inaccurate. There is the additional problem that the T_3U does not reflect variations in unoccupied TBPA binding site concentrations. This is most likely due to the lower affinity of ^{125}I-T_3 for TBPA than TBG.

A comparison of T_3U and TBG data published by Burr et al. (1979) leads one to the same conclusion that the T_3U does not adequately reflect the concentration of unoccupied binding sites (Figure 4.1).

THE DIAGNOSTIC USE OF FREE THYROID HORMONE MEASUREMENT

In searching for a thyroid function test which accurately reflects the thyroid function of the patient one must keep in mind the spectrum of thyroid disease. It is

unlikely that any one test will ever give complete diagnostic accuracy. However, by pinpointing the chemical entities which determine thyroid function it should be possible to develop a minimum of tests which will together provide a satisfactory diagnosis in every patient.

At present, the free thyroid hormone concentration is considered to be the determinant of thyroid function. However, as has been discussed, this concept now appears too simplistic an interpretation of thyroid hormone action. Despite this, the analysis of free thyroid hormone appears at present to provide the most accurate initial assessment of thyroid function.

In summarising the results in this study, the E/D-RIA method, considered by many to be the reference method for free thyroid hormone analysis, showed poor diagnostic accuracy in abnormal thyroid function. There was considerable overlap of T_3, fT_3 and fT_3 values in the hypothyroid group with the respective reference ranges (Figure 5.20). In contrast, T_4 differentiated all hypothyroid subjects. Although the Corning method showed poorer correlation with the euthyroid state, every patient with abnormal thyroid function was discriminated. Similarly, the FTI differentiated all but one patient with abnormal thyroid function but had a very poor correlation with clinical euthyroid status in abnormal TBG states.

The poor diagnostic accuracy in subjects with primary thyroid disease, particularly hypothyroidism, does not appear to be entirely due to assay imprecision at low

free hormone concentrations. It is possible that clinical hypothyroidism might coexist with normal free hormone concentration, for example, in tissue resistance. While this problem requires further investigation, the method does exhibit very good correlation with the clinically euthyroid state in healthy subjects (the measurement of free thyroid concentration in moderate to severe non-thyroidal illness is discussed in Chapter 6 (6.3)).

The Corning modified method appears promising, particularly in measuring low fT_4 in hypothyroidism. It may be possible to modify the procedure so that fewer subjects with elevated TBG show abnormal fT_4 levels. This would entail reducing the amount of T_4 bound to the anti-T_4 during the incubation period although this may compromise the precision of the method. As it stands, the Corning method is rapid, simple and precise and superior to the FTI as a diagnostic test.

The FTI, however, is cheap and relatively simple but in view of its shortcomings, it is doubtful whether the advantages justify the use of the procedure when more satisfactory alternatives are available. Besides the Corning fT_4 method, the T_4/TBG ratio is becoming popular as an index of fT_4 concentration (Burr et al., 1977). In a diagnostic comparison of the FTI and the T_4/TBG ratio, Burr et al. (1979) found the T_4/TBG ratio to be marginally superior in that it gave less false negative and positive results. Although both tests were unreliable where TBG was low, the T_4/TBG ratio gave a much better indication of euthyroid status in subjects

with elevated TBG. Although hypothyroidism with low TBG would be a very rare event, diagnosis of hypothyroidism in the sick euthyroid groups (Chapter 6) requires a test which is reliable in the presence of low TBG.

Another popular indirect test, the effective thyroxine ratio or ETR, also appears to be unreliable in the hypothyroid range. Wellby, O'Halloran and Marshall (1973) found the mean ETR in the 21 hypothyroid patients was the same as the lower 95% limit of normal, whereas the thyrotoxic mean was considerably higher than the upper 95% limit of normal. Murray, Parkin and Gubanyi (1972) reported that 10% of both thyrotoxic and hypothyroid patients had normal ETR. Furthermore, 25% of the sick euthyroid patients studied had low ETR.

None of the tests reviewed is completely satisfactory in screening for thyroid disease. Diagnosis of hypothyroidism poses the greatest problem for the laboratory and at present it is still necessary to resort to measuring TSH in those patients with borderline low results. The kinetic method is promising and deserves further development and clinical assessment.

CHAPTER 6

PHYSIOLOGICAL STUDIES

6.1 INVESTIGATION OF THYROID PHYSIOLOGY DURING ACUTE STRENUOUS EXERCISE

INTRODUCTION

There are well documented changes in thyroid physiology in several stressful situations including severe non-thyroidal illness, starvation, and after surgery. The most characteristic alterations are the decline in serum T_3 and concomitant elevation of serum rT_3. The changes in T_3 and rT_3 concentration were ascribed to the diminished peripheral production of T_3 (Nomura et al., 1975; Lim et al., 1977) and diminished clearance of rT_3 (Chopra, 1976). Chopra et al. (1975a) suggested that these changes in the peripheral metabolism of T_4 may be an adaptive mechanism to enable the body to cope with the stress of illness and surgery, or reduced nutritional intake.

A stressful stimulus invariably elicits the activation of the pituitary-adrenocortical axis with the release of corticotropin (ACTH) and consequent secretion of cortisol. It is not clear that these hormonal responses to stress mediate the changes in thyroid pathophysiology seen during severe illness, starvation or post surgery. While Chopra et al. (1975d) demonstrated increased serum rT_3 and reduced T_3 following ingestion of a large dose of dexamethasome, a similar response following acute physiological alterations in endogenous cortisol has not been reported.

The changes in thyroid pathophysiology characteristic of severe illness, starvation and after surgery would thus appear to be a consequence of other metabolic events common to these stimuli and which are not directly associated with stimulation of the pituitary-adrenocortical axis. While acute strenuous physical exercise does stimulate the pituitary-adrenocortical axis, it is also associated with a number of clearly defined metabolic changes which can be attributed to causes other than glucocorticoid secretion. The most dramatic metabolic event is the reversion to a catabolic state in which there is increased gluconeogenesis, accumulation of lactate, and accompanying metabolic acidosis (Bergstrom, Guarnieri and Hultman, 1971).

The purpose of this project was to investigate the changes in thyroid pathophysiology during acute strenuous exercise with the aim of more clearly defining those events during stress which lead to these changes.

METHODS

Fifteen healthy euthyroid males participated in the study. Ten of these subjects exercised on a bicycle ergometer for 15 minutes, during which time they maintained pulse rates greater than 150.

The remaining 5 subjects formed the control group. The control was designed to provide information not only on random changes in circulating iodothyronines during a period of time equivalent to the exercise period, but also to provide information on the effects of anticipation of strenuous exercises. These subjects had been told that they

would be exercising, but on mounting the ergometer they were informed that they were in the non-exercising control group. They dismounted after 15 minutes.

Blood sampling was via an indwelling catheter in the cubital vein. The catheter was inserted just prior to the first sample being taken at 5 min before commencement of exercise. 20 mL blood was taken 5 min prior to commencement of exercise or the control non-exercise period, at the commencement of the exercise or control period, and at 3, 6, 9, 12, 15, 20, 25, 30, 60, 90 and 120 min after the commencement of the exercise or control period.

The serum was separated and stored at -20°C until assayed for T_4, T_3, rT_3, T_3SU and TSH. The analytical procedures are outlined in Chapters 3 (3.8) and 5 (5.2). Total protein was assayed by refractometry. All samples from a particular subject were assayed in the one assay.

Changes in concentration of the assayed constituents during and after exercise were compared to the basal levels. Basal concentrations were obtained by pooling data from -5 min and 0 min. All comparisons were assessed for statistical significance using Students t test.

RESULTS

Total protein concentration rose rapidly after the onset of exercise and was significantly elevated above mean basal concentration (73 ± 3 g/L, mean ± 1 S.D.) by 6 min (Figure 6.1). A maximum concentration of 79 ± 5 g/L ($P < 0.01$) was attained at 9 min being 8% above the basal level. Total protein remained elevated for the duration of the

exercise period and returned to normal within 15 min of ceasing exercise. No significant changes were evident in the control group.

In view of these alterations in total protein, the changes in the circulating iodothyronines, T_3SU and TSH were assessed in two ways. Firstly, the temporal changes in concentrations were compared to the basal levels (Figure 6.1). Secondly, the percent changes at a given time were compared to the percent changes in total protein at that time (Figure 6.2). That is,

Change in concentration of compound X relative to change in total protein concentration at time t

$$= \left[\frac{\sum_{i=1}^{n} \left([t]/[basal] \right)_i^X \Big/ n}{\sum_{i=1}^{n} \left([t]/[basal] \right)_i^{TP} \Big/ n} \times 100 \right] - 100$$

where [t] is the concentration of X or total protein (TP) in the ith subject at time t, and [basal] is the basal concentration of X or total protein in the ith subject.

Serum T_4 rose significantly from a mean basal concentration of 112 ± 19 nmol/L to 119 ± 25 nmol/L after 6 min exercise ($P < 0.05$). Peak concentration (122 ± 23 nmol/L, $P < 0.01$) was reached at 15 min, the end of the exercise period. This represented an 8% rise above basal levels and was commensurate with the 8% rise in total protein concentration at that time.

Both T_3 and rT_3 rose rapidly during exercise. Serum

rT_3 (basal mean concentration 0.30 $\pm$ 0.10 nmol/L) was significantly elevated after 9 min exercise (0.37 $\pm$ 0.09 nmol/L, $P < 0.01$) and reached a peak concentration at 12 min (0.38 $\pm$ 0.13 nmol/L, $P < 0.05$). Although the percent rise in rT_3 was well in excess of the percent rise in total protein throughout exercise, only at 9 min was the percent rise in rT_3 significantly higher than the percent rise in total protein (relative change = 115%) (Figure 6.2). There was a more pronounced effect of exercise on serum T_3 levels. Serum T_3 was significantly elevated after 6 min of exercise (1.7 $\pm$ 0.3 nmol/L vs 1.4 $\pm$ 0.3 nmol/L, $P < 0.01$), reached a peak at 9 min (1.8 $\pm$ 0.4 nmol/L, $P < 0.01$) and was still elevated 5 min after ceasing exercise (1.6 $\pm$ 0.2 nmol/L, $P < 0.02$). The rise in T_3 was significantly higher than the rise in total protein at 6 and 9 min (relative change = 113% and 115% respectively). Both rT_3 and T_3 fell to a nadir after exercise and were later significantly elevated at 2 h after commencement of exercise.

The mean T_3SU did not change significantly during the test period (basal mean T_3SU was 95 $\pm$ 7). The FTI was significantly elevated only toward the end of exercise (113 $\pm$ 21 at 12 min vs 107 $\pm$ 16 basal, $P < 0.02$), and fell to a nadir significantly lower than the basal level at 45 min after completion of exercise (98 $\pm$ 18, $P < 0.05$).

Thyrotropin concentration showed a response to exercise, being significantly elevated from 9 min (1.4 $\pm$ 0.7 mU/L vs 1.1 $\pm$ 0.6 mU/L, $P < 0.01$) until 5 min after completion of exercise (1.4 $\pm$ 0.7 mU/L, $P < 0.01$). The rise

Figure 6.1 Iodothyronine, TSH, and total protein concentrations, T_3SU and FTI during and after acute strenuous exercise

.... non-exercised controls

——— exercised subjects (vertical bars represent 1 S.D.) strenuous exercise was maintained between 0 and 15 minutes.

Marked in each panel is a bar representing a 10% rise above the basal concentration.

Total protein and FTI were significantly different to the basal levels at the following times,

min	Total protein	FTI
+6	$P < .001$	
+9	$< .01$	
+12	$< .001$	$< .02$
+15	$< .001$	$< .05$
+20	$< .01$	
+25	$< .05$	
+30		
+60		$< .05$

Significant changes in rT_3, T_3 and TSH relative to total protein are shown in Figure 6.2.

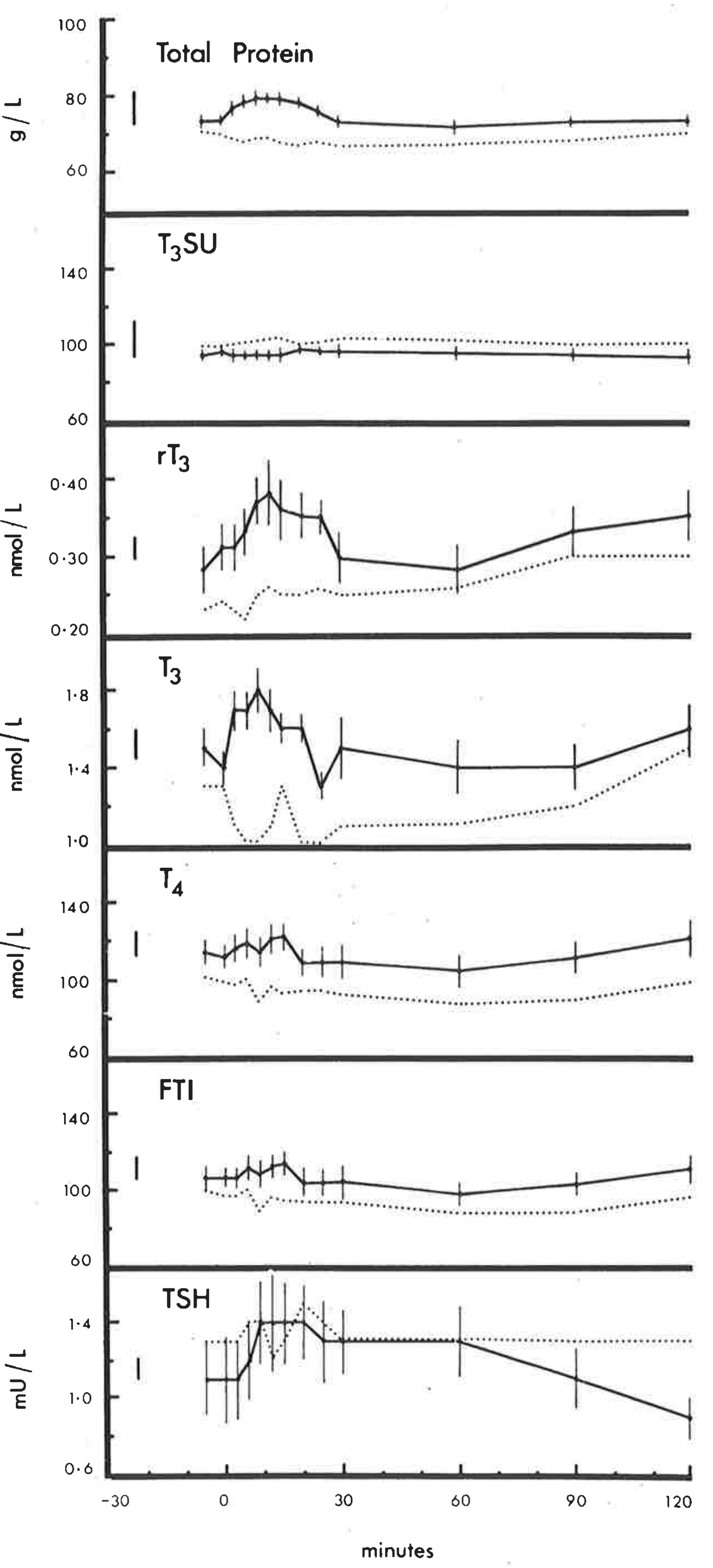

Total Protein
g/L
100
80
60
T3SU
140
100
60
rT3
nmol/L
0·40
0·30
0·20
T3
nmol/L
1·8
1·4
1·0
T4
nmol/L
140
100
60
FTI
140
100
60
TSH
mU/L
1·4
1·0
0·6
-30
0
30
60
90
120
minutes

Figure 6.2 Changes in rT_3, T_3 and TSH concentrations relative to alterations in total protein concentration during and after acute strenuous exercise

Percent relative change in serum concentration of X at time, t

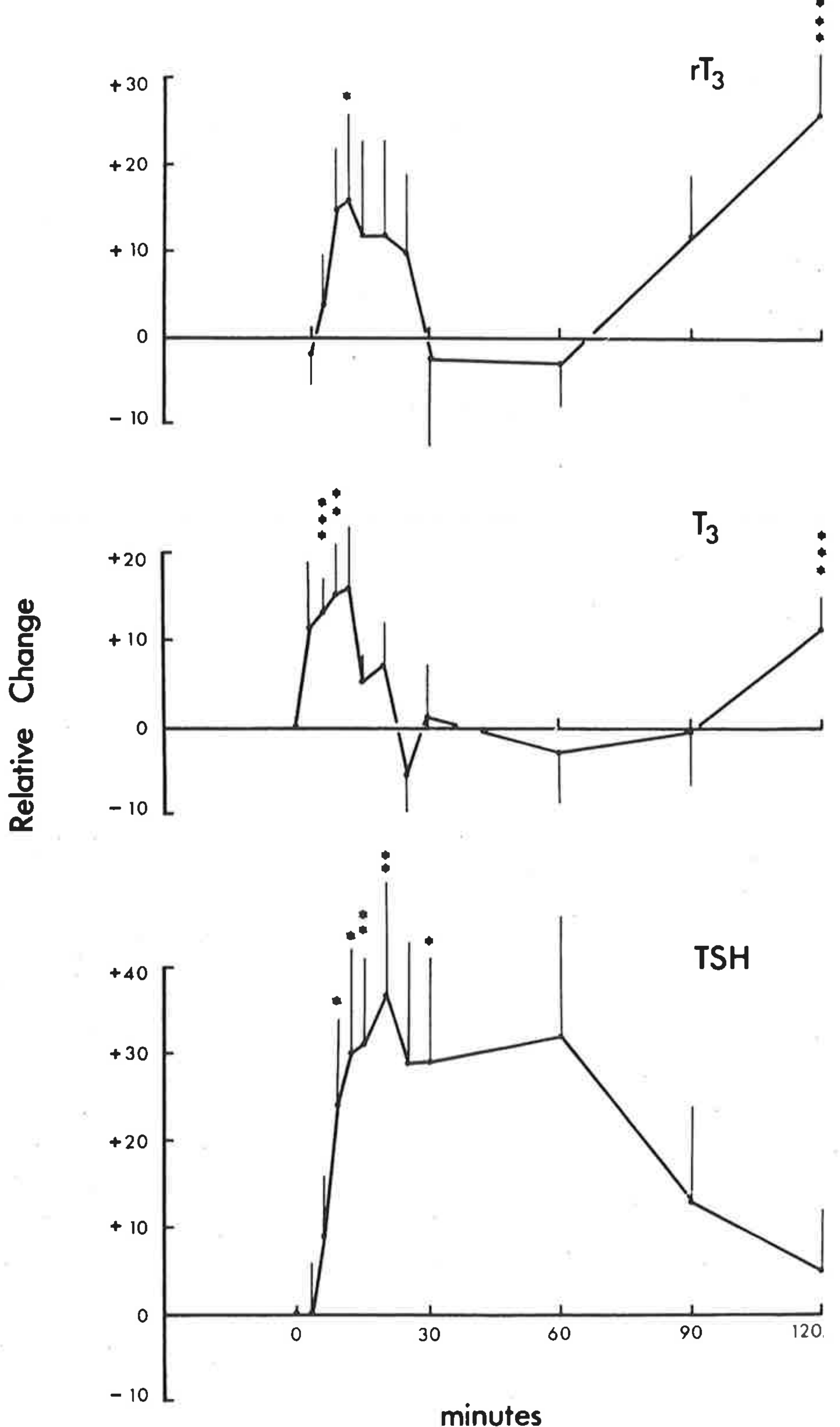
$$= \left[\frac{\sum_{i=1}^{n} \left([t]/[basal]\right)_i^{X} \Big/ n}{\sum_{i=1}^{n} \left([t]/[basal]\right)_i^{TP} \Big/ n} \times 100 \right] - 100$$

(see page 300)

Vertical bars represent 1 S.D.

Statistically significant relative changes are marked

*	$P < .05$
**	$P < .02$
***	$P < .01$


rT3
+30
+20
+10
0
-10
T3
+20
+10
0
-10
Relative Change
TSH
+40
+30
+20
+10
0
-10
0
30
60
90
120
minutes

in TSH was significantly greater than that of total protein between 9 and 30 min. The relative change in TSH reached 137% at 20 min (Figure 6.2).

No significant changes were noted in the measured constituents in the control group.

DISCUSSION

1. Effect of Exercise on Serum Protein

The 8% rise in total protein during the ergometric exercise reflects the well documented fluid shift from the circulation into the interstitial space during periods of acute strenuous exercise. This is due to both an increased systematic pressure and dilatation of the peripheral resistance vessels of the muscles (Smith, Guyton, Manning and White, 1976) and to the movement of water into the interstitium in response to the production and accumulation of lactate in the tissue (Bergstrom et al., 1971; Lundvall, Mellander, Westling and White, 1972).

Of particular importance was the question of how to assess the changes in plasma volume so as to interpret the changes in circulating hormones. Plasma volume changes are monitored by measuring haematocrit. In this study it was decided that the changes in total protein would best reflect changes in the thyronine binding proteins and the resulting alterations in circulating iodothyronine. The validity of this approach is borne out by the recent demonstration of a dissociation between the relative changes in plasma total protein and plasma volume during exercise

(Greenleaf, van Beaumont, Brock, Morse and Mangseth, 1979). The changes in total protein concentration were less than expected considering the changes in plasma volume and they concluded that protein was moving into the interstitium. In considering the thyronine binding proteins, it is possible that there may be a differential transfer of the binding proteins into the interstitial space. That is, it would have been preferable to measure the concentration of TBG, TBPA and albumin.

2. Serum Iodothyronine Concentration During Acute Strenuous Exercise

That the changes in T_4 concentration were similar in magnitude to those of total protein supported the view that the alterations in circulating iodothyronines due to fluid shifts could be evaluated by comparison with these total protein variations. Caralis, Edwards and Davis (1977) have observed concomitant changes in TBG binding capacity and albumin concentration during strenuous treadmill exercise. What is more, the elevation in circulating T_4 was similar to that of albumin and TBG binding capacity.

Both T_3 and rT_3 were present in concentrations greater than could be accounted for in terms of increased thyronine binding protein concentration during exercise. There was no significant difference between the relative increase of each suggesting that acute strenuous exercise does not influence peripheral T_4 metabolism.

There is little documentation of changes in serum

T_3 and rT_3 during and after exercise. Caralis et al. (1977) found no alteration in circulating T_3 following exercise. A similar power output during exercise in their study was inferred from the 11.4% rise in albumin, this rise being comparable to the 8% change in total protein seen in the study reported here. In keeping with the lack of change in T_3, Caralis et al. (1977) observed no alteration in fT_3. Unfortunately fT_3 was not measured in this study, as an elevated fT_3 would have helped to confirm the rise in T_3 above that due to fluid shifts.

3. Changes in Glucose Metabolism During Exercise and the Effect on Peripheral T_4 Metabolism

The 8% change in total protein observed during the period of acute strenuous exercise confirmed that changes in glucose metabolism were occurring. The production of lactate in the musculature during exercise is followed by a rising lactate concentration in the blood and falling blood pH (Nielsen, Christiansen, Hartling and Trup-Jensen, 1977). Although circulating lactate and pH were not measured, the rise in total protein was indicative of pronounced lactate production; that is, that the body was in a catabolic state. This conclusion is corroborated by the recently observed correlation between muscle intracellular water and intracellular lactate (Sahlin, Alvestrand, Brandt and Hultman, 1978), suggesting a direct relationship between the fluid shift into the intracellular compartment and the intracellular lactate accumulation.

The accumulation of lactate during short strenuous exercise is accompanied by a marked fall in intracellular pH from pH 7.00 to as low as pH 6.04 (Sahlin et al., 1978). They reported a rise of 14% in plasma protein concentration suggesting that the exercise in their study was more severe than reported here. The 8% rise in total protein would suggest a more moderate fall in intracellular pH.

Hoffken et al. (1977) have shown the conversion of T_4 to T_3 and rT_3 in rat liver microsomes to be pH dependent. rT_3 production was maximal at pH 9.5 and T_3 production at pH 6.0. They concluded that intracellular pH was an important regulating mechanism of T_4 metabolism. It would thus be expected that the fall in intracellular pH during acute exercise would induce a preferential synthesis of T_3 in the peripheral tissues. That this was not seen suggests that physiological changes in pH do not in fact regulate T_4 metabolism to a significant extent or that such changes have been masked by other metabolic and circulatory changes occurring during exercise.

That alterations in pH do not regulate T_4 metabolism *in vivo* is corroborated by the demonstration of the reduced peripheral production rate of T_3 in diabetic ketoacidosis (Suda, Pittman, Chambers and Ray, 1978b). Again, other metabolic changes in this pathophysiological state may be masking alterations in T_4 metabolism due to reduced intracellular pH. Visser (1980) has demonstrated that the pH optima of the 5'- and 5-deiodinases are not easily interpreted, and that the pH optima of 5'-deiodinase is substrate concen-

tration dependent. That is, some caution is required in inferring a regulatory role of pH on T_4 metabolism *in vivo*.

In addition to the pronounced accumulation of lactate during strenuous exercise, glucose metabolism via the hexose-monophosphate shunt is reduced (Montgomery, Dryer, Conway and Spector, 1974). Visser (1978) has recently pointed out the close relationship between the generation of NADPH by the hexose-monophosphate shunt and 5'-deiodinase activity in the liver. 5'-deiodinase activity is dependent on the presence of glutathione which in turn is maintained at adequate concentrations by a sufficient supply of NADPH (Chapter 1 (1.3)). With the shutdown of the hexose-monophosphate shunt during exercise, 5'-deiodinase activity would be expected to decrease, resulting in reduced peripheral production of T_3 and clearance of rT_3. This sequence of events is certainly observed during starvation in the rat (Balsam et al., 1979; Chopra, 1977). In contrast, 5-deiodination of T_4 to rT_3 is relatively insensitive to changes in reduced glutathione and NADPH concentration.

That these changes in T_4 metabolism do not occur during exercise may reflect the diminished perfusion of the liver and kidney and the markedly increased blood flow through the active skeletal muscle (Mitchell and Blomquist, 1971). The hexose-monophosphate shunt is of only minimal importance in muscle suggesting that 5'-deiodination of T_4 in the musculuture is not only lower in activity than that in the liver and kidney (Chopra, 1977) but is also qualitatively different to the activity in the liver and kidney.

That is, where muscle metabolism of T_4 might be expected to predominate quantitatively, as in exercise, the response to various pathophysiological states will be different to the response where liver metabolism predominates.

Furthermore, although the skeletal muscle has a much lower deiodinating activity than liver or kidney (Chopra, 1977), its bulk gives it a very large deiodinating capacity, especially where muscle perfusion is substantially and specifically increased. The increased T_3 and rT_3 may thus be a result of increased T_4 uptake by the muscle mass due to increased perfusion. If the raised FTI is an accurate reflection of changes in fT_4 concentration during exercise in this study, this would further increase the muscle uptake and consequent metabolism of T_4. Under these conditions, rT_3 and T_3 would be expected to increase in parallel. Jennings, Ferguson and Utiger (1979) have recently demonstrated that T_3 production may be increased by simply increasing organ perfusion, in this case perfusion of the liver. Unfortunately, no data was presented on the effect of increased perfusion on rT_3 production.

4. Response of the Hypothalamic-Pituitary-Thyroid Gland Axis to Exercise

There was a marked and prolonged stimulation of TSH secretion during and after exercise. The cause of increased TSH secretion is not known but may have been mediated by increased neural activity in the hypothalamus.

The rise in FTI would suggest that thyroid gland secretion of T_4 was increased. However, the FTI is not a

reliable guide to fT_4 concentration where thyronine binding protein concentration is elevated (Chapter 5 (5.4)) tending to overestimate fT_4 concentration. The lack of a similar rise in T_4 above that accounted for by increased total protein concentration would suggest that thyroid gland activity was not increased.

5. Conclusion

In conclusion, the changes in serum T_3 and rT_3 during acute strenuous exercise are very different to those seen during severe non-thyroidal illness, starvation and after surgery.

This would suggest that the metabolic responses to stressful stimuli of increased pituitary-adrenocortical activity and reversion to a gluconeogenic metabolic state, although being common to exercise, severe illness, starvation and the post-surgical period, do not elicit the thyroid pathophysiological changes seen in the latter three stress situations.

Alternatively, either or both these metabolic responses do effect the changes in thyroid pathophysiology seen in the latter three stress states, but are overridden by other metabolic events peculiar to exercise, for example, relative shutdown of kidney and liver perfusion.

Finally, there may be other unknown events which elicit the observed changes in thyroid pathophysiology during severe illness, starvation or after surgery, but which do not occur, or do not elicit these responses during strenuous exercise.

6.2 THE EFFECT OF THE RADIOPAQUE DYE, SODIUM IOPODATE, ON THYROID PHYSIOLOGY

INTRODUCTION

Burgi et al. (1976) first reported the reciprocal alterations in serum T_3 and rT_3 following oral cholecystography using sodium iopanoate. On the third day after ingestion of the radiocontrast agent, T_3 was significantly decreased and rT_3 significantly elevated in both euthyroid subjects and hypothyroid subjects on T_4 replacement. The demonstration of an effect in subjects with little functioning thyroid gland suggested that the agent acted by reducing peripheral tissue 5'-deiodination.

Two other iodine containing radiographic contrast agents used in urography and cholangiography had no effect on the concentrations of T_4, T_3 and rT_3. The conclusion was that iopanate acted by virtue of its structural similarity to the iodothyronines.

The study reported here was initiated in order to investigate the effects of another commonly used radiographic contrast agent used in cholecystography, namely, sodium iopodate (Biloptin). The study was designed to delineate more precisely the temporal sequence of events following ingestion of the compound and to examine the changes in free iodothyronine concentrations and the effects of these alterations.

METHODS AND MATERIALS

Eight healthy euthyroid adult male volunteers with an average age of 25 years were involved in the project. Blood samples were taken by venepuncture at 0900 on the days of collection. Samples were taken at 10 and 6 days prior to commencement of the study. On the day of commencement, a blood sample was taken at 0900 and three grams of sodium iopodate (Schering, Germany) was administered orally to six of the subjects as one gram doses between 0900 and 1200. An equivalent dose of potassium iodide was administered to the two control subjects. Samples were then collected at 1, 3, 4, 7, 14 and 21 days after the dose. The serum was separated and stored at -20°C until completion of the project. All samples from the same subject were assayed together.

The methods for analysis of T_4, T_3, rT_3, TSH, T_3U, fT_3 and fT_4 have been outlined. Total serum iodide was measured using the ceric ammonium sulphate catalytic reactin (Barker et al., 1951). Free rT_3 was assayed by equilibrium dialysis-radioimmunoassay. The procedure was similar to that described for fT_3 and fT_4 in Chapter 5. After overnight dialysis of serum against 0.01 M hepes, 0.11 M NaCl, 1.5 mM sodium azide, pH 7.4, 1 mL aliquots of dialysate were assayed for rT_3. Rabbit antiserum to rT_3-BSA conjugate was used at a final dilution of $1/(1 \times 10^6)$ and 2000 cpm ^{125}I-rT_3 (1.6 fmoles) was added to each tube. After 24 h incubation at 4°C, a final charcoal concentration of 0.34% (w/v) was used to separate antibody bound from free hormone.

There was no significant cross-reaction of sodium iopodate in the radioimmunoassay of the total and free iodothyronines at the concentrations of iopodate suggested present in the serum by the serum iodide concentrations.

The changes in the measured constituents following ingestion of iopodate were assessed by comparison to the mean basal concentrations. Mean basal concentration was obtained by pooling the data from days -10, -6 and 0 (Table 6.1). Student's t test was used to assess the statistical significance of the changes observed.

RESULTS

Serum total iodide concentration rose to 225 times basal level (Table 6.1) in the sodium iopodate treated subjects within 24 hours (Figure 6.3). The iodide concentration was still markedly and significantly elevated 21 days after ingestion of the radiocontrast agent. In the two control subjects, total serum iodide rose to 126 times basal concentration within 24 hours. The iodide was cleared very rapidly and was similar to the basal level on the fourth day.

Total T_3 concentration fell by 44% within 3 days of iopodate ingestion and reached a minimum concentration of 1.0 ± 0.4 nmol/L (mean $\pm$ 1 S.D.) on day 4. Serum T_3 was not significantly different to the mean basal level by the fourteenth day. While serum fT_3 concentration followed a similar pattern of changes, it was significantly depressed on only day 3 (2.5 ± 0.5 pmol/L).

Within 24 hours, both total and free rT_3 rose very rapidly to 3 and 2.3 times basal concentrations respectively. After attaining maximum concentrations on day 3 (rT_3 1.10 ± 0.21 nmol/L, frT_3 3.9 ± 0.9 pmol/L), rT_3 was rapidly cleared to basal level by day 7.

Although serum T_4, FTI and fT_4 rose to a maximum on day 4 (T_4 140 ± 26 nmol/L, FTI 135 ± 31, fT_4 12.7 ± 3.5 pmol/L), the elevations were not significant (Figure 6.4).

Considering only those samples with detectable TSH, mean TSH rose to a broad peak between days 3 and 7, the maximum value attained being 2.3 mU/L. This data was not tested for statistically significant changes due to the large number of samples with undetectable TSH concentrations.

No alterations in the serum total and free iodothyronines were evident in the two iodide treated control subjects. However, TSH did rise in a similar manner to that seen in the iopodate treated subjects.

DISCUSSION

The changes in thyroid hormone physiology following ingestion of sodium iopodate are similar to those reported by Burgi et al. (1976) after ingestion of sodium iopanoate (Telepaque). Wu et al. (1978) have also recently reported similar reciprocal alterations in T_3 and rT_3 in euthyroid, T_4 treated hypothyroid, and untreated thyrotoxic subjects after administration of sodium iopodate.

1. Clearance of Serum Total Iodide

The clearance of total iodide following sodium

TABLE 6.1 Basal Concentrations of the Total and Free Iodothyronines, TSH and Total Iodide

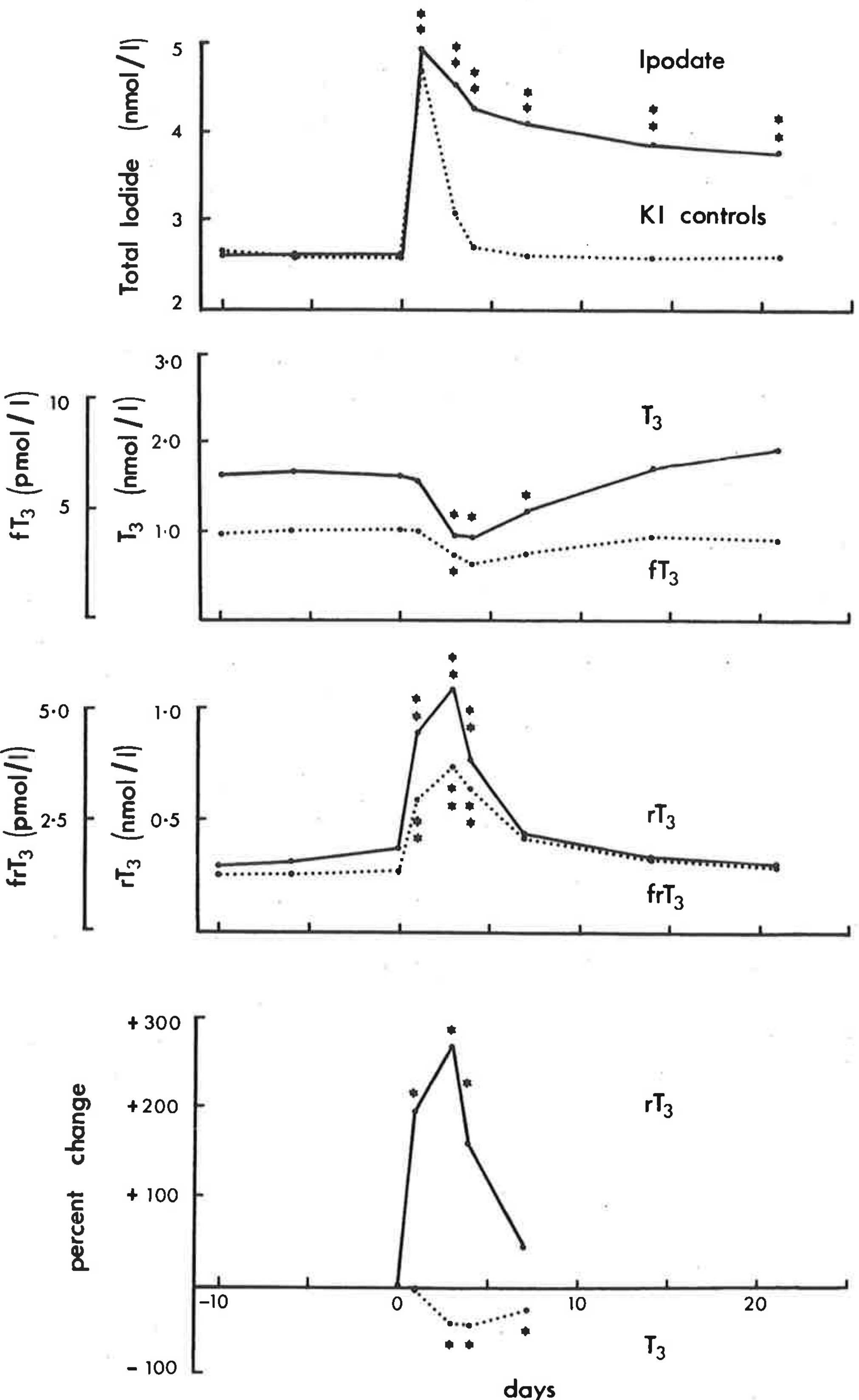
	Reference range	Pre-iopodate	
		Mean	S.D.
Total T_4	75 - 152 nmol/L	120	15
Free T_4	3.4 - 20.0 pmol/L	12.7	3.5
FTI	76 - 145	117	16
Total T_3	1.1 - 2.8 nmol/L	1.7	0.2
Free T_3	2.7 - 8.4 pmol/L	4.2	0.7
Total rT_3	0.12 - 0.38 nmol/L	0.32	0.11
Free rT_3	0.3 - 2.5 pmol/L	1.4	0.8
TSH	0.5 - 2.4 mU/L	Range	0.5-1.1
Total iodide		396	56

Figure 6.3 Total iodide, and total and free triiodothyronine concentrations after ingestion of sodium iopodate

The bottom panel represents percent changes in the triiodothyronine concentrations relative to their basal concentrations.

Those alterations which are significantly greater than basal concentration are marked,

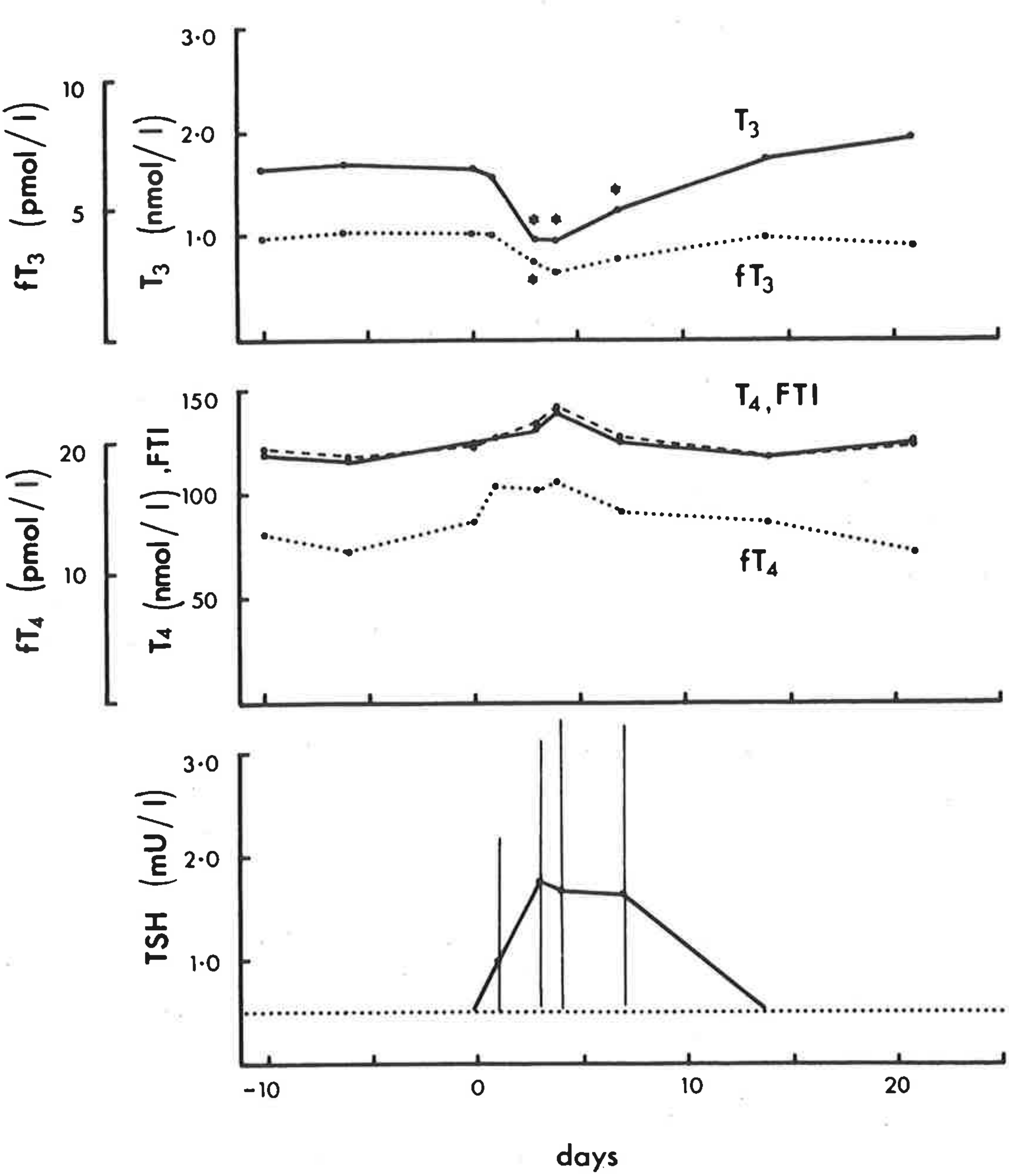
*	$P < 0.5$
**	$P < 0.1$



Total Iodide (nmol/l)
5
4
3
2
Ipodate
KI controls
fT3 (pmol/l)
10
5
T3 (nmol/l)
3·0
2·0
1·0
T3
fT3
frT3 (pmol/l)
5·0
2·5
rT3 (nmol/l)
1·0
0·5
rT3
frT3
percent change
+300
+200
+100
-100
rT3
-10
0
10
20
T3
days

Figure 6.4 Total T_4, free T_4, and TSH concentrations, and FTI after ingestion of sodium iopodate

Changes in total and free T_3 concentration are included for comparison.


3·0
10
2·0
5
1·0
T3
fT3
fT3 (pmol/l)
T3 (nmol/l)
150
T4, FTI
20
100
fT4
10
50
fT4 (pmol/l)
T4 (nmol/l), FTI
3·0
2·0
1·0
TSH (mU/l)
-10
0
10
20
days

iopodate administration was very slow. In contrast, iopanoate has a half life of 24 hours (Winthrop Laboratories Information Pamphlet on Telepaque, March 1976) and Burgi et al. (1976) noted that total iodide was down to 3.7% of peak levels within 7 days of iopanoate ingestion. There appeared to be two components to the clearance of sodium iopodate (Figure 6.3), with rapid disappearance of iodide between days 1 and 4, and slow clearance thereafter. This pattern could represent an initial rapid equilibrium of circulating iopodate with the tissues and subsequent slow release, or may reflect conversion to a more slowly cleared iodinated metabolite. Of particular interest is the observation that despite the prolonged clearance of iodide following iopodate ingestion, the temporal sequence of changes in circulating iodothyronines does not differ appreciably from those taking place after ingestion of the rapidly cleared iopanoate.

The absence of changes in rT_3 and T_3 in the iodide administered controls confirms the conclusions of Burgi et al. (1976) that the cholecystographic agents do not act through the liberation of iodide.

2. Mechanism of Action of Sodium Iopodate

Burgi et al. (1976) suggested a structural similarity between sodium iopanoate and the iodothyronines. Sodium iopodate is structurally related to iopanoate in being a triiodinated benzene derivative with amino- and propionate substituents.

Iopanoic acid

Sodium Iopodate

The demonstration of reciprocal alterations in T_3 and rT_3 levels in T_4 treated hypothyroid subjects suggests a peripheral action of these radiographic agents similar to that of an unrelated iodine containing drug, propylthiouracil (Westgren et al., 1977). They may therefore act by inhibiting peripheral 5'-deiodinative metabolism of T_4 to T_3 and clearance of rT_3. Chopra (1978b) has recently reported the effects of these radiocontrast agents on the 5'-deiodinative metabolism of $T_4 \rightarrow T_3$ and $rT_3 \rightarrow 3,3'\text{-}T_2$ in rat liver homogenates. Sodium iopodate and iopanoic acid inhibited

conversion of T_4 to T_3 *in vitro* with relative potencies of 2:1. Furthermore they were shown to be competitive inhibitors of both $T_4 \rightarrow T_3$ and $rT_3 \rightarrow 3,3'\text{-}T_2$ activities confirming their action through a structural resemblance to the iodothyronines.

Following ingestion of iopodate, there was a seeming temporal dissociation of the T_3 and rT_3 responses (Figure 6.3). Thus rT_3 rose very rapidly and was falling by at least day 3. On the other hand, T_3 reached a nadir between days 3 and 4 and slowly increased toward normal between days 4 and 7. That is, if the changes seen are due to inhibition of 5'-deiodinase, it would appear that $T_4 \rightarrow T_3$ and $rT_3 \rightarrow 3,3'\text{-}T_2$ are two separate activities. Wu et al. (1978) have shown a similar dissociation in T_4 treated hypothyroid and hyperthyroid subjects. Recent *in vitro* studies by Chopra (1978b) may clarify this. The K_i for $T_4 \rightarrow T_3$ and $rT_3 \rightarrow 3,3'\text{-}T_2$ were 0.04 μmol/L and 0.24 μmol/L sodium iopodate respectively. That is, the $T_4 \rightarrow T_3$ 5'-deiodinase activity is more sensitive to inhibition by iopodate and will remain inhibited until iopodate has fallen to appropriate levels.

It is of interest that on day 7, the concentration of iopodate in the circulation was far in excess of these estimations of K_i if one considers that all the circulating iodide, in excess of basal levels, is in this form. Although there may be discrepancies between the K_i values calculated in broken cell preparations and the concentration of iopodate required to inhibit 5'-deiodinase *in vivo*, and given that some caution is required in inferring intracellular concentrations from circulating concentrations, the rapid normal-

ization of rT_3 levels suggests that the concentrations of iopodate in the circulation are not as high as the serum total iodide concentrations would suggest. That is, the prolonged clearance of total iodide may represent the accumulation of slowly cleared iodinated metabolites of iopodate. Alternatively, most of the iopodate, assuming this to be the form present in the serum, may be protein bound with only a very low concentration of the free compound being available to the tissue.

3. Effect of Sodium Iopodate on Pituitary Function

The stimulation of TSH secretion may be secondary to the fall in fT_3 resulting from the lowered peripheral production of T_3. Alternatively, iopodate may have a direct effect on the pituitary secretion of TSH. Melmed, Nelson, Kaplowitz, Yamada and Hershman (1980) have recently shown that sodium iopodate inhibits $T_4 \rightarrow T_3$ conversion in the pituitary nonthyrotropic GH_3 tumor cell. In view of the demonstration by Silva et al. (1978) that one half of nuclear T_3 in the euthyroid rat pituitary derives from intrapituitary $T_4 \rightarrow T_3$ conversion, it is possible that sodium iopodate acts directly on the pituitary to reduce intrapituitary T_3. The diminished circulating fT_3 would further reduce the intra-pituitary T_3, resulting in TSH secretion.

Of particular interest in considering the intra-pituitary effects of iopodate is the question of rT_3 biological activity. Nicod et al. (1976a) have shown the pituitary response to thyrotropin releasing hormone (TRH) to be unaffected during the administration of rT_3 to

euthyroid subjects in doses sufficient to maintain mildly elevated serum concentrations. In contrast to the effect of exogenous rT_3 on the pituitary, the effect of iopodate on 5'-deiodinase in the pituitary provides a situation in which the effects of increased endogenous rT_3 may be studied. Although it has not been demonstrated directly, iopodate may lead to the intrapituitary accumulation of rT_3. The observation of TSH secretion under these conditions would argue against the intrinsic biological activity of rT_3 at physiological concentrations.

However, the effect of iodide in increasing TSH secretion in the control subjects makes it difficult to interpret the stimulation of TSH secretion following iopodate. Although massive doses can elicit increased secretion of TSH by blocking thyroid hormone secretion, no significant drop in T_3 or T_4 was observed in the two control subjects in the study. While there is no documented evidence of a direct effect of iodide on the pituitary gland, the changes observed may have been due to a direct effect of the massive dose of iodide ingested on thyrotrophe secretion or an effect at the hypothalamic level.

4. Interpretation of Thyroid Function Tests after Administration of Radiographic Agents

The results reported in this study do have clinical ramifications. Of particular interest is the acute effects of these cholecystographic agents in the patients after cholecystography. While the diminished total and free T_3 and the stimulated TSH secretion suggest a state of acute

hypothyroidism, it is very unlikely that this would be harmful to the patient, even in cases where the patient may be on T_4 replacement therapy.

The results do suggest that care must be taken in timing requests for thyroid function tests where the patient is also being assessed for thyroid disease. These would best be performed prior to cholecystography or at least 14 days after. However, in the event that the tests were performed shortly after cholecystography, the normal T_4 and FTI in association with low T_3 and elevated TSH should alert the clinician that profound hypothyroidism may not necessarily be present.

5. Conclusion

In conclusion, this study has shown sodium iopodate to cause marked changes in the pituitary-thyroid gland-target tissue axis. The results suggest that iopodate acts on the peripheral metabolism of T_4 by virtue of its structural resemblance to the iodothyronines.

Due to the marked decrease in serum T_3 concentration, thyroid function testing in patients undergoing cholecystography should be properly timed.

6.3 THYROID PATHOPHYSIOLOGY DURING SEVERE NON-THYROIDAL ILLNESS

INTRODUCTION

Moderate to severe non-thyroidal illness has a profound effect on thyroid pathophysiology. The most characteristic change taking place is a dramatic fall in

serum T_3, often to levels seen in hypothyroidism (Sullivan, Bollinger and Reichlin, 1973; Carter, Eastman, Corcoran and Lazarus, 1974). The primary effect of moderate to severe non-thyroidal illness appears to be at the level of peripheral T_4 metabolism. A reduction in the measured conversion of T_4 to T_3 has been reported in advanced alcoholic liver disease (Nomura et al, 1975). These reports also suggest that reduced peripheral T_3 production is a general systemic phenomenon and is not associated with any one particular diseased organ.

Often accompanying the fall in serum T_3 is a rise in rT_3, first described in general systemic illness by Chopra et al. (1975a). The peripheral origin of rT_3 (Chopra et al., 1975b) and demonstration of a reduced peripheral clearance rate of rT_3 in hepatic cirrhosis (Chopra, 1976) confirm the primary effect of severe non-thyroidal illness on peripheral T_4 metabolism.

While thyroid gland function and serum T_4 concentration are most often normal (Carter, Eastman, Corcoran and Lazarus, 1976), the reduced peripheral production of T_3 and associated reduction in serum T_3 levels would suggest that the tissue supply of the thyroid hormone with predominant biological activity is diminished. Despite the possibility of reduced tissue hormone availability, patients with moderate to severe non-thyroidal illness rarely show clinical signs of hypothyroidism.

The concentration of TSH is usually normal indicating a lack of response of the pituitary to the low

serum T_3 concentration. Although there is some evidence suggesting that pituitary function may be compromised during severe illness, there have been reports of normal pituitary function. Thus delayed and blunted TSH response to TRH has been reported in uremia (Gonzalez-Barcena, Kastin, Schalch, Torres-Zamora, Perez-Pasten, Kato and Schally, 1973; Lim et al., 1977), in hepatic cirrhosis (Chopra, Solomon, Chopra, Young and Chua-Teco, 1974; Green, Snitcher, Mowat, Ekins, Rees and Dawson, 1977), and in diabetic ketoacidosis (Naeije, Golstein, Clumeck, Meinhold, Wenzel and Vanhaelst, 1978). No change in TSH response to TRH was seen in infectious febrile illness (Wartofsky et al, 1977; Talwar et al., 1977) and an exaggerated response was reported in those patients with low FT_3I (Bermudez, Surks and Oppenheimer, 1975).

Despite the evidence suggesting that the pituitary may often be normally responsive to thyroid hormone levels during moderate to severe illness, this does not necessarily indicate that supply of hormone to the rest of the body is sufficient. Recent studies by Silva et al. (1978) suggest that the thyrotrophe may be responsive to both T_3 and T_4 concentrations, whereas other organs are mainly responsive to the T_3 concentration.

The tissue availability of thyroid hormone is most accurately reflected by the serum free hormone levels. While fT_4 concentration is usually normal or moderately elevated, reported fT_3 levels are either low normal or below the euthyroid reference range (Table 6.2). Although the earlier

reports of Carter et al. (1974) and Chopra et al. (1974) showed mean fT_3 concentration to be significantly below the euthyroid mean fT_3 concentration, recent reports have indicated the mean fT_3 concentration during illness is not significantly different to the mean euthyroid fT_3 level. This is despite the very low T_3 concentrations reported, with mean T_3 concentration often being below the euthyroid reference range.

To date all reported determinations have been made using the percent dialyzable ^{125}I-T_3 and total T_3 by radio-immunoassay to calculate the fT_3 concentration. This technique is susceptible to inaccuracies tending to over-estimate the concentration of free thyroid hormone (see Chapter 1 (1.2)). Although the quoted reference ranges (Table 6.2) are consistent with those reported using the E/D-RIA technique (Table 5.5) it was felt that the changes in fT_3 during severe illness should be validated with the E/D-RIA technique. A further aim was to also derive data from other thyroid function tests so as to assess their usefulness in diagnosing thyroid disease in moderate to severely ill patients.

SUBJECTS

The patients were selected from the requests coming into the routine thyroid laboratory. These patients had no clinical symptoms of thyroid disorder and thyroid function tests had been requested as part of a general biochemical assessment.

TABLE 6.2 Published Total and Free T_3 in Non-Thyroidal Illness

Results expressed as, mean $\pm$ 1 S.D.

95% range

The difference between the normal and ill groups were tested using Students t test, and are indicated where P values were quoted.

NTI .. .. non-thyroidal illness

UD .. .. undetectable

Each group except Green et al. (1977) assayed fT_3 by measuring the percent dialyzable ^{125}I-T_3 and calculating the free hormone concentration from the total T_3 concentration. Green et al. (1977) assayed fT_3 directly using E/D-RIA).

Reference	Illness	Free T_3 (pmol/L)		Total T_3 (nmol/L)	
		normal	NTI	normal	NTI
Carter et al. 1974	general severe illness	8.1±2.8 2.6-13.5	3.9±2.0 UD -7.8 $P<.001$	2.1±0.6 1.2-2.8	UD-2.3 .001
Chopra et al. 1974	hepatic cirrhosis	5.8±1.6 2.7-8.9	2.5±1.1 0.3-4.7 .001	2.0±0.5 0.8-3.3	0.5±0.2 0.1-0.9 .001
Nomura et al. 1975	hepatic cirrhosis	4.3±0.3 3.7-4.9	4.2±8.1 UD -20.1 N.S.	2.0±0.4 1.3-2.7	1.3±0.8 UD-2.8 .001
Burger et al. 1976	acute and subacute illness	3.1-12.4	6.2±1.1 4.0-8.4 N.S.	2.6±0.4 1.8-3.3	1.8±0.7 0.4-3.1
Talwar et al. 1977	acute infectious febrile illness	7.8±2.3 3.2-12.3	6.5±3.9 UD - 14 N.S.	2.2±0.4 1.3-3.0	1.0±0.4 0.1-1.8 .001
Green et al 1977	hepatic cirrhosis	7.5±1.5 4.5-10.5	7.0±1.8 3.4-10.6 N.S.	2.3±0.5 1.3-3.3	1.4±0.6 0.2-2.6 .005
Chopra et al. 1979	general illness	6.2±1.7 3.7-9.6	3.5±2.5 U.D -8.3	2.0±0.5 0.8-3.3	0.5±0.4 UD -1.3

The primary non-thyroidal illness groups and the number of subjects in each were cardiac disease 6, hepatic disease 5, renal disease 3, duodenal ulcer 1, malignancy not involving the above organs 2, dementia 1, and weight loss 2.

METHODS

All serum samples were stored at -20°C. The assays of rT_3, T_4, fT_4, T_3, fT_3, T_3SU, FTI and TSH have been described in Chapters 3 (3.8) and 5 (5.2). The normal euthyroid reference ranges are tabulated in Table 6.1. Comparison of data in the non-thyroidal illness group with the euthyroid reference data was made using Students t test.

RESULTS

In the group with non-thyroidal illness, serum T_3 ranged from undetectable levels to 1.0 nmol/L, all patients having T_3 concentration lower than the reference range (Figure 6.5).

Despite the low T_3 concentration, fT_3 was normal in 36% of the samples (Figure 6.5). The mean fT_3 concentration of 3.1 ± 1.6 pmol/L was significantly lower than the euthyroid mean of 5.6 ± 1.4 pmol/L ($P < 0.001$) but not significantly different to the hypothyroid mean of 2.2 ± 1.4 pmol/L.

To provide information on the variability of measurements at low fT_3 concentrations, a sample with low fT_3 was run with every assay. This consistently assayed at ≤ 1.3 pmol/L. Furthermore, a sample with normal fT_3 measured 3.8 ± 0.8 pmol/L. These results confirmed the

ability of the assay to discriminate between low and normal fT_3 concentration.

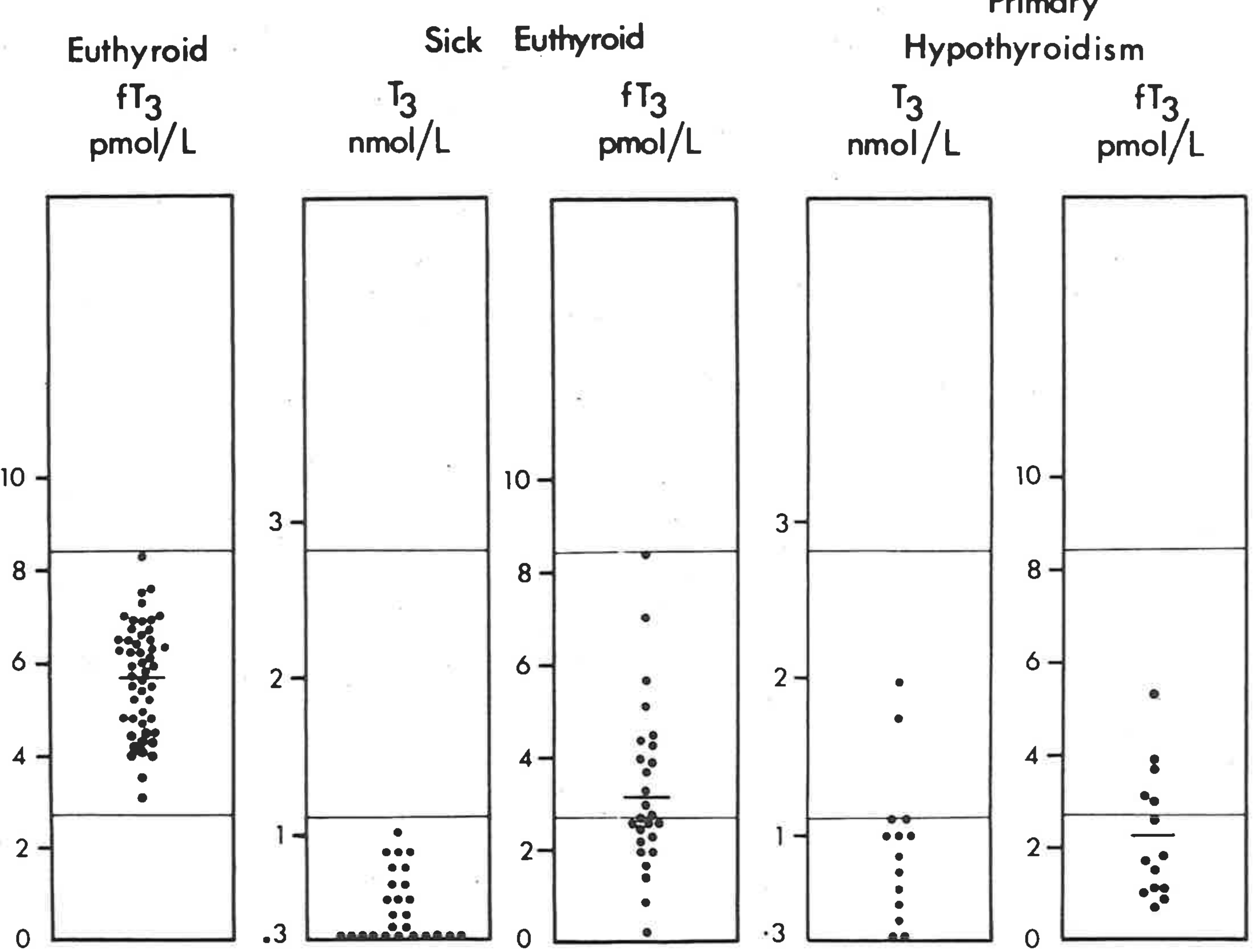
Serum rT_3 concentration was raised in 70% of the patients and reduced in 9%. Mean rT_3 (0.70 ± 0.54 nmol/L) was significantly raised above the euthyroid mean rT_3 ($P < 0.001$). The T_4/rT_3 ratio was normal (euthyroid reference range 219 - 714) in the 2 patients with low rT_3. Although the T_4 concentration was elevated in 4 patients with high rT_3, the low T_4/rT_3 suggested that rT_3 was higher than would be expected for the given T_4 concentration.

Serum T_4 concentration was variable with 17% above the euthyroid reference range and 42% below the range. The mean concentration (92 ± 44 nmol/L) was significantly higher than the euthyroid mean (114 ± 20 nmol/L, $P < 0.001$). All patients with low T_4 had elevated T_3SU, the mean T_3SU in this group being significantly higher than the T_3SU in those patients with normal or high T_4 concentration (135 ± 17 vs 108 ± 13, $P < 0.001$). Furthermore, T_4/T_3 in patients with low T_4 was high (euthyroid reference range 29 - 77) suggesting that T_3 was reduced below those levels which could be accounted for by reduced serum binding protein concentration.

Free T_4 concentration was either elevated (18%) or within the euthyroid reference range. The mean fT_4 (16 ± 6 pmol/L) was not significantly different to the euthyroid mean fT_4. The FTI was variable. Although the eight patients with low T_4 had normal fT_4, 5 had low FTI. Of the four patients with elevated T_4, two had elevated fT_4 and all had

Figure 6.5 Total and free T_3 concentrations in euthyroid, untreated hypothyroid subjects, and in patients with non-thyroidal illness

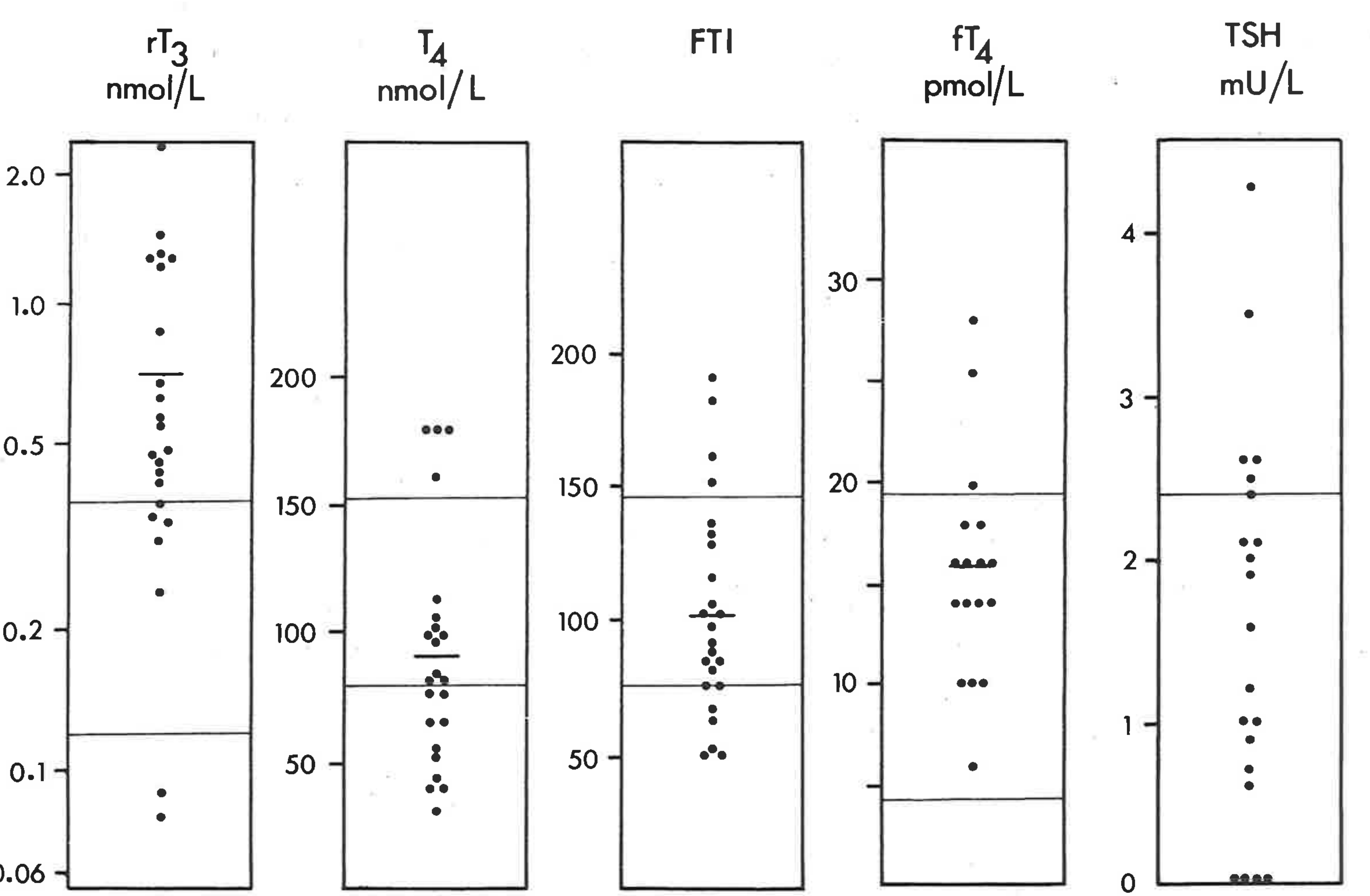
The healthy euthyroid reference ranges are marked.



Euthyroid
fT3
pmol/L
Sick Euthyroid
T3
nmol/L
fT3
pmol/L
Primary
Hypothyroidism
T3
nmol/L
fT3
pmol/L
10
8
6
4
2
0
3
2
1
.3

Figure 6.6 Concentrations of rT_3, T_4, fT_4 and TSH, and FTI in patients with non-thyroidal illness

The healthy euthyroid reference ranges are marked.



Sick Euthyroid
rT3 nmol/L
T4 nmol/L
FTI
fT4 pmol/L
TSH mU/L
2.0
1.0
0.5
0.2
0.1
0.06
200
150
100
50
200
150
100
50
30
20
10
4
3
2
1
0

elevated FTI.

Serum TSH concentration was slightly elevated in 24% of the patients, the highest level observed being 4.3 mU/L.

DISCUSSION

1. Total and Free T_3 Concentration in Moderate to Severe Non-Thyroidal Illness

Despite the reduction in serum T_3 concentration in non-thyroidal illness, fT_3 concentration was normal in 36% of the patients studied. This confirms previous reports in which the range of fT_3 values in non-thyroidal illness showed considerable overlap with normal values, despite the mean fT_3 being lower than the euthyroid reference range (Table 6.2).

That the T_3 concentration was below the sensitivity of the assay in 44% of the patients underscored the importance of substantiating the reports using the percent dialyzable T_3 technique. This technique requires a knowledge of the serum T_3 concentration to calculate the fT_3 and where T_3 concentration is undetectable, fT_3 can only be classified as undetectable. That is, there would be a tendency to underestimate the mean fT_3 concentration in the severe non-thyroidal illness group.

Other than this objection to the indirect dialysis technique, the euthyroid mean fT_3 concentrations reported (Table 6.2) are similar to that reported here (5.6 ± 1.4 pmol/L) confirming that the indirect dialysis technique

provides reliable data. This most likely reflects the commercial availability of relatively pure and high specific activity tracer which, in the past, has not been widely available and which has led to the development of assays with limited reliability.

A further limitation to the published data is the number of patients in these reports with normal T_3 levels. That is, there was the possibility that the patients were not severely ill or that, for some reason, the illness had not had a significant effect on peripheral T_4 metabolism. In the study reported here, serum T_3 concentration was below the reference range in all patients, confirming that the illness had influenced peripheral T_4 metabolism. Also characteristic of altered peripheral T_4 metabolism is the accumulation of rT_3. In this study 30% of the patients had normal or low rT_3. A similar dissociation has been osbserved following acute myocardial infarction where rT_3 was found to be normal despite low T_3 (Geisthovel, Perschke, Hehrmann, Kodding and Von zur Muhlen, 1976). Chopra, Solomon, Hepner and Morgenstein (1979) have also observed normal rT_3 in 7 of 19 patients with normal T_4 and low T_3.

2. <u>Availability of Thyroid Hormone to the Tissues in Moderate to Severe Non-Thyroidal Illness</u>

The low fT_3 concentration in 64% of the patients suggests that both peripheral intracellular production of T_3 and T_3 availability to the tissues is reduced. In view of the conclusions of Silva et al. (1978) that thyroid hormone action in the liver, kidney and heart muscle is dependent

more on the T_3 concentration rather than the T_4 concentration, it might be concluded that these patients are biochemically hypothyroid. There was no clinical evidence of hypothyroidism in the patients in this study, however.

The group of patients with low fT_3 in this study showed no differences in other thyroid function tests when compared to the group of patients with low T_3 but normal fT_3 concentration.

There are very few reports on basal metabolic rate during non-thyroidal illness. Spector et al. (1976) did find that although 54% of a group of patients with renal disease had low fT_3, only one had associated low basal metabolic rate. This observation confirms that thyroid hormone availability is adequate.

The hypothyroid group showed several important differences to the patients studied here. Although the range of fT_3 concentrations were similar (Figure 6.5), free and total T_4 were most often low in hypothyroidism, in the presence of normal thyronine binding protein concentration. What is more, there was a clear pituitary response and clinical symptoms were evident.

There are a number of reasons why hypothyroidism may not be apparent despite the low fT_3 concentration.

(a) Reduced thyroid hormone availability - there may be tissue hypothyroidism which is not manifest in clinical symptoms and reduced basal metabolic rate. This could be due to other metabolic stimuli specific to severe non-thyroidal illness maintaining euthyroid metabolic

rates and masking the clinical features of reduced tissue T_3 concentration. Alternatively there may be a greater sensitivity of the peripheral tissues to T_3. That is, adequate metabolic rate may be maintained at a lower tissue T_3 concentration.

(b) Adequate thyroid hormone availability - the conclusions of Silva et al. (1978) may be overinterpreted. That is, despite low fT_3, sufficient hormone may be available due to normal or elevated fT_4. Peripheral deiodination of T_4 may be sufficient to maintain the basal metabolic rate. This is consistent with the conclusions of Ingbar and Braverman (1975) that a number of clinical and physiological conditions conformed more closely with the balance between the concentration of T_4 and T_3, or even with the concentration of T_4 alone.

3. Inhibitors of Thyroid Hormone Binding to Thyronine Binding Proteins

Brown-Grant, Brennan and Yates (1970) and Lutz et al. (1972) raised the possibility of the appearance of inhibitors of thyroid hormone binding in severe illness and acute infectious illness. It was postulated that the inhibitors caused increased percent dialyzable T_4 where alterations in thyroid hormone binding proteins did not account for these changes. This postulate would account for the tendency to high fT_4 often seen in sick euthyroid subjects in whom there are normal binding protein and T_4 concentrations (Oppenheimer et al., 1963; Lutz et al., 1972), and normal fT_4 in patients in whom mean thyroid hormone binding

capacity is unaltered but the T_4 is significantly reduced (Carter et al., 1974; Chopra et al., 1979). The presence of a binding inhibitor would also explain the tendency to normal fT_3 concentration where T_3 is low.

In 7 of the 8 patients with low total T_4, fT_4 was normal but FTI low. Although T_3SU was elevated in these samples, this does not appear to correct adequately for the low T_4. If an inhibitor of T_4 binding were present, the T_3SU would be expected to increase and correct for the altered T_4 binding. Chopra et al. (1979) have reported a similar discrepancy between fT_4 and FTI. They found that TBG concentration tended to be lower in those subjects with low T_4 but that T_3RU, although elevated, did not adequately correct for the diminished T_4. They suggested the inhibitor was ineffective at the acid pH of the resin typically used in the T_3RU tests. In the study reported here, the T_3 uptake test utilizes Sephadex G-25 to bind protein unbound ^{125}I-T_3 at physiological pH. In this case inhibitor, if present, may compete with the ^{125}I-T_3 for binding to the Sephadex, or the inhibitor may partition itself between binding protein and Sephadex, making available a higher concentration of unoccupied protein binding sites for ^{125}I-T_3 binding. In those patients with low T_4, there is the possibility that both TBPA and TBG are lowered. The T_3SU test may be relatively insensitive to changes in TBPA and may thus underestimate the concentration of unoccupied protein binding sites.

Chopra et al. (1979) have reported the inhibitor to be non-dialyzable and heat labile. It was removed by

lowering the pH to 4.5 and separating the precipitated material. However, the heat and acid lability of TBG (Robbins, Cheng, Gerschengorn, Glinoer, Cahnmann and Edelnoch, 1978) casts some doubt on their conclusions as to the nature of the inhibitor.

4. Pituitary Function During Moderate to Severe Non-Thyroidal Illness

Silva et al. (1978) concluded that the thyrotrophe was responsive to both T_3 and T_4 in the circulation. T_4 was effective by way of intrapituitary deiodination to T_3 and binding of the T_3 so generated to the pituitary nuclear T_3 receptors.

In those patients with low fT_3, intrapituitary nuclear T_3 would be expected to be very low in view of both the diminished T_4 monodeiodination and the low circulating fT_3. There is some evidence suggesting a difference between the pituitary T_4-5'-deiodinase and the kidney and liver T_4-5'-deiodinases. Iopodate has been shown to inhibit the activity of both pituitary and liver 5'-deiodinase *in vitro* (Melmed et al., 1980). In the same study, PTU inhibited liver 5'-deiodinase activity but not that of the pituitary. Acute iopodate administration stimulates TSH secretion (Figure 4.4) whereas TSH response to acute PTU administration is equivocal, despite the fall in both total and free T_3 (Siersbaek-Nielsen et al., 1978). The possibility exists then, that pituitary 5'-deiodinase activity may be sufficient to ensure adequate intrapituitary T_3 from circulating T_4.

In this study there were no patients with both low

fT_3 and low fT_4. Chopra et al. (1979) reported 3 of 47 patients who had both low fT_3 and fT_4 but normal TSH. Kolendorf, Moller and Rogowski (1978) reported a considerable number of patients with chronic renal failure who had both low FT_4I and FT_3I but normal TSH. In view of the discrepancy between fT_4 and FT_4I in the presence of reduced binding protein concentration, caution is needed in interpreting the results of this latter group. The lack of pituitary response to diminution of both fT_3 and fT_4 strongly suggests an alteration in the hypothalamic-pituitary axis. That pituitary function may be compromised during non-thyroidal illness has been shown by TRH stimulation tests (see introduction to this section) and has been unequivocally borne out in a patient with untreated primary hypothyroidism with ketoacidosis (Hooper, 1976). In this case TSH became elevated only after the diabetes had been brought under control.

5. The Investigation of Thyroid Disease in the Presence of Moderate to Severe Non-Thyroidal Illness

The investigation of thyroid disease in the presence of severe non-thyroidal illness presents a challenging diagnostic problem. A number of patients with non-thyroidal illness exhibit some biochemical features of hypothyroidism, and hypothyroidism per se may be masked by accompanying severe illness. Chopra et al. (1979) have suggested the use of rT_3 estimations where fT_4 is low in order to rule out the possibility of secondary hypothyroidism. They found elevated rT_3 in 3 patients with low total and free T_4, and concluded that the increased rT_3 suggested

sufficient supply of T_4 to the tissue. They suggested that this would not be expected to be the case in secondary hypothyroidism. However, in the study presented here, rT_3 was low in 2 patients with low T_4 and normal fT_4. One could only come to the conclusion in these patients that secondary hypothyroidism was not present only if one had assayed not only rT_3 but also fT_4. Furthermore, it is possible, in view of reported low fT_4 in non-thyroidal illness, that one may see the combination of low T_4, low rT_3 and low fT_4. A diagnosis of secondary hypothyroidism would not necessarily be correct in these cases.

Engler, Donaldson, Stockigt and Taft (1978) have reported two severely ill patients with coexisting hyperthyroidism. Although T_3 was low during the period of illness, both patients exhibited features of toxicity. In the study presented here, several patients had elevated free and total T_4 and low T_3, but no clinical evidence of thyrotoxicity. One would have to be cautious in diagnosing hyperthyroidism in these patients on the biochemical parameters alone.

The difficulties in diagnosing thyroid disease in severe non-thyroidal illness are evident in the interpretation of Recant and Riggs' (1952) observations on thyroid pathophysiology during renal disease. The development of assays to measure serum fT_4 concentration has provided substantial experimental support for their hypothesis that T_4 delivery to the tissues can be adequate despite low serum T_4.

However, their observation of reduced basal

metabolic rate is difficult to interpret. Assuming T_4 supply to be adequate and considering the lack of clinical symptoms of hypothyroidism, they postulated that basal metabolic rate was suppressed by other unknown mechanisms unrelated to hypothyroidism. This would suggest that the clinical manifestations of hypothyroidism are due not so much to a low basal metabolic rate, but primarily to a lack of thyroid hormone.

The reduction in peripheral T_3 production observed in severe non-thyroidal illness provides a mechanism by which basal metabolic rate may be reduced in the presence of sufficient T_4 supply to the tissues. In the study reported in this thesis it was concluded from the lack of pituitary stimulation and clinical signs of hypothyroidism that thyroid hormone supply to the tissues was adequate despite the reduction of peripheral T_3 production to levels causing low serum fT_3 concentration. Although no measurement of basal metabolic rate was made in this study, Spector et al. (1976) made similar biochemical observations to those reported here and found no reduction in basal metabolic rate. In view of these results it might be concluded that the group of patients studied by Recant and Riggs (1952) differ in the severity of non-thyroidal illness. Thus peripheral T_3 production may have been reduced to such an extent that reduced basal metabolic rate ensued but clinical symptoms were masked by other facets of the illness. Alternatively, thyroid hormone concentration in the tissues may have been adequate, with basal metabolic rate reduced by other unknown mechanisms.

6. Conclusions

The investigation of thyroid function during non-thyroidal illness presents an interesting physiological problem. Severe illness appears to interact with the complete hypothalamic-pituitary-thyroid gland-target cell axis. Well characterized changes take place in the target cell or peripheral T_4 metabolism and more subtle and uncharacterized changes appear to occur in the hypothalamic-pituitary axis. It was concluded from this study that:

(a) Thyroid hormone availability to the tissues in moderate to severe non-thyroidal illness, as determined by measuring serum free hormone concentration, was not as low as was suggested by the very low serum T_3 concentration.

(b) Even where fT_3 concentration was low in moderate to severe non-thyroidal illness, tissue supply of hormone was maintained by way of normal fT_4 levels.

There was no evidence to suggest that any one particular test, or combination of tests might be of value in detecting thyroid disease during severe illness. It would appear that an unequivocal demonstration of thyroid disease can only be made after the non-thyroidal disease has subsided.

6.4 DISCUSSION

Although the mechanism of peripheral T_4 deiodination is now well characterized, less is known of its regulation and how this affects peripheral tissue thyroid hormone supply and metabolic action, and pituitary secretion

of TSH. This is highlighted by the apparent discrepancy between the last two studies reported in this chapter. Although fT_3 was low and fT_4 normal after iopodate ingestion and during non-thyroidal illness, increased TSH secretion was evident only in the former study.

The work of Silva and Larsen (1978) suggests that the thyrotrophe plays a unique role in monitoring and regulating both thyroid gland secretion of T_4 and T_3 production in the peripheral tissues. They have postulated that the peripheral tissues are responsive mainly to circulating T_3 while the pituitary is sensitive to both circulating T_4 and T_3 levels. Thus, although T_3 concentration may often be normal in the initial stages of thyroid gland failure and iodine deficiency, the diminished T_4 concentration signals thyroid failure. The pituitary detects the reduced T_4 by way of reduced intrapituitary T_4 deiodination to T_3 and responds by increasing TSH secretion. After the ingestion of iopodate, thyroid gland secretion is normal but peripheral T_3 production reduced, leading to diminished levels of the biologically active hormone in the tissues. The pituitary responds to both the reduced circulating fT_3 and intrapituitary T_4 deiodination by increasing the secretion of TSH in order to boost supply of the precursor T_4 to the tissues.

Although these phenomena are conveniently explained by the hypothesis of Silva and Larsen, the thyroid pathophysiology of non-thyroidal illness is not so easily reconciled. While thyroid gland function is normal and peripheral T_3 production reduced, as after iopodate ingestion,

there is no pituitary response. It has been suggested that there is either tertiary hypothyroidism or that the intrapituitary deiodination of T_4 is unaltered during severe non-thyroidal illness, in contrast to the reduced T_4 deiodination in the peripheral organs. The latter suggestion infers that adequate intrapituitary T_3 concentration is maintained by intrapituitary T_4 deiodination despite low circulating fT_3, whereas during the early stages of thyroid failure, normal circulating fT_3 does not maintain adequate intrapituitary T_3 concentration where intrapituitary deiodination of T_4 is reduced due to low circulating fT_4.

Chopra et al. (1975a) and Vagenakis et al. (1975) speculated that reduced peripheral conversion of T_4 to T_3 may represent an adaptation to non-thyroidal illness and starvation. The insensitivity of the pituitary to low T_3 and conditions reducing peripheral T_3 production may be similarly interpreted. It may be speculated that reduced T_3 supply to the peripheral tissues may be maintained by a mechanism which ensures that the pituitary does not respond by increasing secretion of the precursor T_4.

The lack of data on thyroid pathophysiology during non-thyroidal illness, starvation and surgery highlights the need for an adequate experimental model of the effects of stress on thyroid hormone metabolism and its regulation. Unfortunately, the exercise study showed the stress of acute strenuous activity not to be a satisfactory model of these conditions.

APPENDIX 1

A.1 EFFECT OF SERUM DILUTION ON FREE HORMONE CONCENTRATION DURING EQUILIBRIUM DIALYSIS

SINGLE BINDING PROTEIN

Consider the binding of hormone to a hormone binding protein P under equilibrium conditions

$$F + P \rightleftharpoons B$$

where [F] is the concentration of free hormone F, [P] the concentration of unoccupied sites on the binding protein P, and [B] is the concentration of bound hormone or unoccupied protein binding sites. Then

total concentration of binding protein $[P_o] = [B] + [P]$

moles of hormone in serum volume $V_s = [B].V_s + [F].V_s$

At equilibrium

$$K = \frac{[B]}{[F][P]}$$

where K is the association constant.

When a volume of serum V_s is dialysed against dialysis buffer of volume V_D, a new equilibrium is attained.

serum (V_s)		dialysate (V_D)
F'	$\leftrightarrow$	F'
P'		
B'		

This describes the general situation in which the concentration of free and bound hormone may be changed by the

conditions of dialysis to [F'] and [B'] respectively. The concentration of free hormone will be the same in dialysand and dialysate. In this system,

$$F' + P' \longrightarrow B'$$

moles of hormone in the dialysis system $= [B'] . V_S + [F'] . V_S + [F'] .V_D$ (A2)

At equilibirum,

$$K = \frac{[B']}{[F'] [P']}$$

Equating equations A1 and A2,

$$[B] V_S + [F] V_S = [B'] V_S + [F'] V_S + [F'] V_D$$

Rearranging

$$V_S \left[[B] + [F] \right] = V_S \left[[B'] + [F'] \right] + [F'] .V_D$$

$$[B] + [F] = [B'] + [F'] + [F'] .V_D/V_S$$

that is,

$$[B'] + [F'] \left[1 + V_D/V_S \right] - \left[[B] + [F] \right] = 0 \qquad \text{(A3)}$$

Wosilait and Nagy (1976) have provided a general solution for the concentration of ligand bound to multiple binding sites on different proteins in plasma. For a single binding site on a single plasma binding protein,

$$\frac{K[F][P_o]}{1 + K[F]} = [B]$$

Substituting for [B'] in equation A3,

$$\frac{K [F'] [P_o]}{1 + K [F']} + F' \left[1 + \frac{V_D}{V_S} \right] - \left[[B] + [F] \right] = 0$$

Solving for [F']

$$K[F'][P_o] + [F']\left[1 + K[F']\right]\left[1 + \frac{V_D}{V_S}\right] - \left[1 + K[F']\right]\left[[B]+[F]\right] = 0$$

$$K[F'][P_o] + [F']\left[1 + \frac{V_D}{V_S}\right] + [F']^2\left[K(1 + \frac{V_D}{V_S})\right] - \left[[B] + [F]\right] + K[F']\left[[B] + [F]\right] = 0$$

$$[F']^2\left[K(1 + \frac{V_D}{V_S})\right] + [F']\left[(1 + \frac{V_D}{V_S}) + K[P_o] - K[B] - K[F]\right] - [T] = 0$$

where the total concentration of hormone [T] = [B] + [F]
and [B] = [T] - [F]

$$[F']^2\left[K(1 + \frac{V_D}{V_S})\right] + [F']\left[(1 + \frac{V_D}{V_S}) + K[P_o] - K[T]\right] - [T] = 0 \quad \text{(A4)}$$

Using equation A4, solutions are provided in table 4.2 for free T_4, free T_3 and free cortisol concentrations at various V_D/V_S. Data used to solve the equation are tabulated in table 4.1. Also tabulated is [F'] relative to [F]. [F] is the concentration of free hormone prior to dialysis.

The fraction free hormone at any V_D/V_S was calculated from the [F'] at that V_D/V_S.

Thus,

$$\text{fraction free hormone} = \frac{\text{mol/unit volume dialysate}}{\text{mol/unit volume dialysand}}$$

Now,

mol/unit volume dialysate $= [F']$

and mol/unit dialysand $= \dfrac{[T]V_S - [F']V_D}{V_S}$

that is, the total concentration of hormone remaining in the dialysand after removal of $[F']V_D$ moles to the dialysate compartment.

Thus,

$$\text{fraction free hormone} = \frac{[F']V_S}{[T]V_S - [F']V_D}$$

$$= \frac{[F']}{[T] - [F']V_D/V_S}$$

and percent free hormone $[D']$ $= \dfrac{[F'].100}{[T] - [F']V_D/V_S}$

The percent free hormone in undiluted serum before dialysis ($V_D/V_S = 0$) is designated D. Table 4.2 tabulates D and D' at various V_D/V_S for free T_4, free T_3 and free cortisol. Also tabulated is D' relative to D (D'/D).

The fraction free hormone can be determined experimentally using radiolabelled hormone in which case,

$$\text{fraction free hormone} = \frac{\text{cpm/unit colume dialysate}}{\text{cpm unit volume dialysand}}$$

THREE BINDING PROTEINS

Considering the binding of T_3 or T_4 to the thyroid hormone binding proteins, TBG, TBPA and albumin,

$$F + P + Q + R \rightleftharpoons B_1 + B_2 + B_3$$

Where P represents TBG, Q represents TBPA and R represents albumin. In a derivation analogous to that for equation A3, describing the total concentration of hormone in the dialysis system,

$$[T'] = [B'] + [F'] \left[1 + \frac{V_D}{V_S} \right] \qquad (A5)$$

where $[B'] = [B_1'] + [B_2'] + [B_3']$

Using the solution of Wosilait and Nagy (1976) for the concentration of hormone bound,

$$[B'] = \frac{K_1 [F'][P_o]}{1 + K_1[F']} + \frac{K_2[F'][Q_o]}{1 + K_2[F']} + \frac{K_3 [F'][R_o]}{1 + K_3 [F']}$$

where $K_1 = \dfrac{[B'_1]}{[F'][P_o]}$ or $\dfrac{[T.TBG']}{[F'][TBG_o]}$

$K_2 = \dfrac{[B'_2]}{[F'][Q_o]}$ or $\dfrac{[T.TBPA']}{[F'][TBPA_o]}$

and $K_3 = \dfrac{[B'_3]}{[F'][R_o]}$ or $\dfrac{[T.albumin']}{[F'][albumin_o]}$

Substituting for [B'] in equation A5,

$$\frac{K[F'][P_o]}{1 + K_1[F']} + \frac{K[F'][Q_o]}{1 + K_2[F']} + \frac{K[F'][R_o]}{1 + K_3[F']}$$

$$+ [F']\left[1 + \frac{V_D}{V_S}\right] - [T] = 0$$

Solving for [F'],

$$K_1\Big[[F'][P_o]\Big]\Big[1 + K_2[F']\Big]\Big[1 + K_3[F']\Big] +$$

$$K_2\Big[[F'][Q_o]\Big]\Big[1 + K_1[F']\Big]\Big[1 + K_3[F']\Big] +$$

$$K_3\Big[[F'][R_o]\Big]\Big[1 + K_1[F']\Big]\Big[1 + K_2[F']\Big] +$$

$$\left[[F'](1 + \frac{V_D}{V_S}) - [T]\right]\Big[1 + K_1[F']\Big].$$

$$\Big[1 + K_2[F']\Big]\Big[1 + K_3[F']\Big] = 0$$

expanding,

$$K_1[F'][P_o] + K_1K_3[P_o][F']^2 + K_1K_2[P_o][F']^2$$

$$+ K_1K_2K_3[F']^3[P_o] +$$

$$K_2[F'][Q_o] + K_2K_3[Q_o][F']^2 + K_2K_1[Q_o][F']^2$$

$$+ K_1K_2K_3[F']^3[Q_o] +$$

$$K_3[F'][R_o] + K_1K_2[R_o][F']^2 + K_3K_2[R_o][F']^2$$

$$+ K_1K_2K_3[F']^3[R_o] +$$

$$[F']\left[1 + \frac{V_D}{V_S}\right] + K_2[F']^2\left[1 + \frac{V_D}{V_S}\right] + K_1[F']^2\left[1 + \frac{V_D}{V_S}\right] +$$

$$K_1K_2[F']^3\left[1 + \frac{V_D}{V_S}\right] + K_3[F']^2\left[1 + \frac{V_D}{V_S}\right] + K_2K_3[F']^3\left[1 + \frac{V_D}{V_S}\right] +$$

$$K_1K_2[F']^3\left[1 + \frac{V_D}{V_S}\right] + K_1K_2K_3[F']^4\left[1 + \frac{V_D}{V_S}\right] -$$

$$[T] - K_2[F'][T] - K_1[F'][T] - K_1K_2[F']^2[T] - K_3[F'][T] -$$

$$K_2K_3[F']^2[T] - K_1K_3[F']^2[T] - K_1K_2K_3[F']^3[T] = 0$$

collecting the coefficients,

$$K_1K_2K_3\left[1 + \frac{V_D}{V_S}\right] \qquad [F']^4$$

$$+ \quad K_1K_2K_3\left[[P_o] + [Q_o] + [R_o] - [T]\right] + \left[1 + \frac{V_D}{V_S}\right]\left[K_1K_2 + K_2K_3 + K_1K_3\right] \qquad [F']^3$$

$$+ \left[K_1K_3[P_o] + K_1K_2[P_o] + K_2K_3[Q_o] + K_2K_1[Q_o] + K_3K_1[R_o] + K_3K_2[R_o] - [T](K_1K_2 + K_2K_3 + K_1K_3)\right] + \left[1 + \frac{V_D}{V_S}\right]\left[K_1 + K_2 + K_3\right][F']^2$$

$$+ \left[K_1[P_o] + K_2[Q_o] + K_3[R_o] - [T](K_1 + K_2 + K_3)\right] + \left[1 + \frac{V_D}{V_S}\right][F']$$

$$- [T] = 0. \qquad \text{(A6)}$$

Equation A6 was solved for [F'] by Newtons method for solving f(x) = 0 using an HP-97 calculator (Hewlett Packard, U.S.A.). Solutions for free T_4 and free T_3 concentrations at various V_D/V_S are tabulated in table 4.2. Percent free hormone at various V_D/V_S is also tabulated.

A2 CALCULATION OF THE UNOCCUPIED TBG BINDING SITE CONCENTRATION, [TBG].

Considering only the binding of T_4 to TBG, then at equilibrium,

$$T_4 + TBG \rightleftharpoons T_4.TBG$$

and

$$K = \frac{[T_4.TBG]}{[F][TBG]} \qquad (A7)$$

where [F] represents the fT_4 concentration, $[T_4.TBG]$ the concentration of occupied binding sites and [TBG] the concentration of unoccupied binding sites. The total circulating concentration of TBG binding sites,

$$[TBG_o] = [TBG] + [T_4.TBG]$$

rearranging equation A7,

$$K[F][TBG] = [T_4.TBG]$$

$$= [TBG_o] - [TBG] \qquad (A8)$$

now

$$[F] = [TT_4] - [T_4.TBG]$$

$$= [TT_4] - [TBG_o] + [TBG]$$

where $[TT_4]$ represents the total circulating T_4 concentration. Substituting for [F] in equation A8.

$$K[TT_4][TBG] - K[TBG_o][TBG] + K[TBG]^2 = [TBG_o] - [TBG]$$

that is,

$$[TBG]^2 K + [TBG]\Big[K[TT_4] - K[TBG_o] + 1\Big] - [TBG_o] = 0 \qquad (A9)$$

TBG was calculated using the data for $[TBG_o]$ and $[TT_4]$ published by McDowell (1979) (Table 4.4(a) and Figure 4.1).

A3 $[TT_4]/[TBG_o]$ AS AN INDIRECT ESTIMATE OF fT_4 CONCENTRATION.

Consider only the binding of T_4 to TBG, at equilibrium,

$$K = \frac{[T_4.TBG]}{[F][TBG]} \qquad (A7)$$

then $$[F]K = \frac{[T_4.TBG]}{[TBG]}$$

$$= \frac{[T_4.TBG]}{[TBG_o] - [T_4.TBG]}$$

and $$\frac{1}{K[F]} = \frac{[TBG_o]}{[T_4.TBG]} - 1$$

$$\frac{1}{K[F]} + 1 = \frac{[TBG_o]}{[T_4.TBG]}$$

$$\frac{1 + K[F]}{K[F]} = \frac{[TBG_o]}{[T_4.TBG]}$$

now $$[T_4.TBG] = [TT_4] - [F]$$

that is $$\frac{K[F]}{1 + K[F]} = \frac{[TT_4] - [F]}{[TBG_o]}$$

$$= \frac{[TT_4]}{[TBG_o]} - \frac{[F]}{[TBG_o]}$$

solving for $[TT_4]/[TBG_o]$,

$$\frac{[TT_4]}{[TBG_o]} = \frac{K[F]}{1 + K[F]} + \frac{[F]}{[TBG_o]} \qquad (A10)$$

Solutions for $[TT_4]/[TBG_o]$ using data for $[TBG_o]$ published by McDowell (1979) are tabulated in Table 4.4(b).

A4 CALCULATION OF TRACER DISPLACEMENT BY UNLABELLED HORMONE.

ONE BINDING SITE MODEL

1. Calculation of Relative Displacement of Labelled Hormone

Consider the binding of ligand, P to antibody, Q, at equilibrium.

$$p + q \longleftrightarrow pq$$

Using the solution of Wosilait and Nagy (1976) for the concentration of bound ligand, pq,

$$\frac{K.p.q_o}{1+K.p} = pq$$

where p is the concentration of unbound ligand, p_o the total concentration of ligand ($p_o = p + pq$), and q_o is the total concentration of the antibody ($q_o = q + pq$).

now, $p = p_o - pq$

then, solving for pq,

$$\frac{K.q_o(p_o - pq)}{1 + K(p_o - pq)} - pq = 0$$

$$\frac{K.p_o.q_o - Kq_o.pq}{1 + K.p_o - K.pq} - pq = 0$$

$$K.p_o.q_o - K.q_o.pq - pq(1 + K.p_o - K.pq) = 0$$

$$K.p_o.q_o - K.q_o.pq - pq - K.p_o.pq + K.pq^2 = 0$$

$$pq^2(K) - pq(Kq_o + Kp_o + 1) + K.p_o.q_o = 0$$

$$pq^2 - pq(q_o + p_o + 1/K) + p_oq_o = 0 \qquad \text{(A11)}$$

This equation was used to construct theoretical displacement curves for the serum-free T_3 and T_4 assays (figures 5.8 and 5.14 respectively).

To construct the displacement curves it was assumed that the labelled and unlabelled hormone bound to the antibody with identical affinity. The concentration of labelled hormone was represented as p^*. Thus the total concentration of hormone in any particular tube, $p_o = p^* + p$, where p is the concentration of added standard. pq could then be calculated for each p.

Knowing pq,

$$\text{fraction of hormone bound to antibody} = pq/p_o = p^*q/p^*$$

where p^*q is the concentration of labelled hormone bound to the antibody.

$$\text{The relative displacement of labelled hormone} = \frac{(p^*q/p^* \text{ at a given } p\,)}{p^*q/p^* \text{ when } p = 0)} \times 100$$

(plotted in figures 5.8 and 5.14).

The self-displacement of labelled hormone can be determined by setting $p = 0$ and defining $p_o = p^*$. The fraction of hormone bound to the antibody (p^*q/p^*) is calculated as above.

2. Calculation of Percent Occupancy of Antibody Binding Sites by Hormone

percent occupancy at a given p $= pq/q_o \times 100$ (A12)

3. Calculation of the Precision Profile.

Knowing the standard deviation of the measurement of the fraction labelled hormone bound in the presence of a given concentration of hormone, p_o, and the slope of the displacement curve at that fraction of labelled hormone bound, the precision of the estimate of hormone concentration giving rise to that displacement can be calculated.

The fraction of labelled hormone bound $= p^*q/p^* = pq/p_o = B$

Multiply each term in equation A11 by $1/(p_o)^2$,

$$\left[\frac{pq}{p_o}\right]^2 - \left[\frac{pq}{p_o}\right]\left[\frac{1}{p_o}\right]\left[q_o + \frac{1}{K}\right] - \left[\frac{pq}{p_o}\right] + \left[\frac{1}{p_o}\right] q_o = 0$$

that is,

$$B^2 - B(p_o^{-1})\left[q_o + \frac{1}{K}\right] - B + (p_o^{-1})q_o = 0$$

differentiating with respect to p_o,

$$2B.\frac{dB}{dp_o} - (p_o^{-1})\left[q_o + \frac{1}{K}\right]\frac{dB}{dp_o} + B(p_o^{-2})\left[q_o + \frac{1}{K}\right] - \frac{dB}{dp_o} - (p_o^{-2})q_o = 0$$

Rearranging,

$$\frac{dB}{dp_o} = \frac{(p_o^{-2})\left[q_o - B\,(q_o + 1/K)\right]}{\left[2B - (p_o^{-1})\,(q_o + 1/K) - 1\right]} \qquad \text{(A13)}$$

where dB/dp_o is the slope of the displacement curve B vs p_o.

Let ΔB the standard deviation of the mean measured B at any given p_o. Then the precision of the estimate of p_o associated with any B can be calculated and is defined as

$$\Delta p_o = \frac{\Delta B}{\text{slope}}$$

The precision is usually expressed relative to the concentration of hormone, that is $\Delta p_o/p_o$. A precision profile, $\Delta p_o/p_o$ vs p_o can then be constructed (see Figures 5.10 and 5.16).

4. Calculating of Sensitivity

The sensitivity was calculated from the standard deviation of the mean measured B at $p_o = p^*$, that is where no unlabelled hormone is present ($p = o$). Knowing the slope at this point, the sensitivity is defined as

$$\Delta p_o = \frac{\Delta B_o}{\text{slope}} \qquad \text{(A14)}$$

where ΔB_o is the ΔB at $p_o = p^*$.

Equation A14 enables the calculation of the sensitivity at any concentration of labelled hormone at a given concentration of antibody binding sites, q (see Figures 5.7 and 5.15).

TWO BINDING SITE MODEL

1. Calculation of the Relative Displacement of Labelled Hormone.

Consider the binding of ligand, P to antibody species Q and R at equilibrium,

$$p + q + r \rightleftharpoons pq + pr$$

Then, according to Wosilait and Nagy (1976),

$$\frac{K_1 . p . q_o}{1 + K_1 . p} + \frac{K_2 . p . r_o}{1 + K_2 . p} = pq + pr$$

now,

$$p = p_o - (pq + pr)$$

Substituting for p in equation A12 and solving for (pq + pr),

$$K_1 q_o\left[p_o - (pq + pr)\right]\left[1 + K_2\left\langle p_o - (pq + pr)\right\rangle\right]$$

$$+ K_2 r_o\left[p_o - (pq + pr)\right]\left[1 + K_1\left\langle p_o - (pq + pr)\right\rangle\right]$$

$$- (pq + pr)\left[1 + K_2\left\langle p_o - (pq + pr)\right\rangle\right]\left[1 + K_1\left\langle p_o - (pq + pr)\right\rangle\right] = 0$$

$$K_1 q_o p_o - K_1 q_o(pq + pr)$$

$$+ K_1K_2q_o\left[p_o^2 - 2p_o (pq + pr) + (pq + pr)^2\right]$$

$$+ K_2r_op_o - K_2r_o(pq + pr) + K_1K_2r_o\left[p_o^2 - 2p_o (pq + pr) + (pq + pr)^2\right]$$

$$- (pq + pr)$$

$$- K_1q_o(pq + pr) + K_1(pq + pr)^2 - K_2p_o(pq + pr) + K_2(pq + pr)^2$$

$$- K_1K_2(pq + pr)\left[p_o^2 - 2p_o(pq + pr) + (pq + pr)^2\right] = 0$$

that is,

$$K_1p_oq_o - K_1q_o(pq + pr) + K_1K_2q_op_o^2$$

$$- 2K_1K_2p_oq_o(pq + pr) + K_1K_2q_o(pq + pr)^2$$

$$+ K_2r_oP_o - K_2r_o (pq + pr) + K_1K_2r_op_o^2$$

$$- 2K_1K_2p_or_o (pq + pr) + K_1K_2r_o (pq + pr)^2$$

$$- (pq + pr)$$

$$- K_1p_o(pq + pr) + K_1(pq + pr)^2 - K_2p_o (pq + pr) + K_2(pq + pr)^2$$

$$- K_1K_2p_o^2(pq + pr) + 2K_1K_2p_o (pq + pr)^2 - K_1K_2 (pq + pr)^3 = 0$$

isolating the coefficients,

$$- K_1K_2 \qquad (pr + pr)^3$$

$$K_1K_2(p_o + r_o + 2p_o) + (K_1 + K_2) \qquad (pq + pr)^2$$

$$- (K_1q_o + 2K_1K_2p_oq_o + K_2r_o + 2K_1K_2p_or_o$$

$$+ K_1p_o + K_2p_o + K_1K_2p_o^2 + 1) \qquad (pq + pr)$$

$$+ K_1p_oq_o + K_1K_2q_op_o^2 + K_2r_op_o + K_1K_2r_op_o^2 = 0 \qquad (A15)$$

Using the same technique as employed in the one binding site model, the concentration of hormone bound (pq+pr) can be calculated at any p_o ($p_o = p^* + p$)

Knowing (pq + pr)

$$\text{fraction of hormone bound} = \frac{(pr + pr)}{p_o} = \frac{(p^*q + p^*r)}{p^*}$$

Thus,

$$\text{the relative displacement of labelled hormone} = \frac{\left[\dfrac{p^*q + p^*r}{p^*} \text{ at a given } p\right]}{\left[\dfrac{p^*q + p^*r}{p^*} \text{ when } p = 0\right]} \times 100$$

(plotted in Figure 5.9).

2. Calculation of Percent Occupancy of Antibody Binding Sites by Hormone

At equilibrium,

$$p + q + r \longleftrightarrow pq + pr$$

Then,

$$K_1 = \frac{pq}{p.q}$$

and,

$$K_2 = \frac{pr}{p.r}$$

Rearranging

$$p = \frac{pq}{K_1 .q}$$

$$= \frac{pq}{K_1 (q_o - pq)}$$

$$= \frac{pq}{K_1 q_o - K_1 .pq}$$

$$K_1 .q_o .p - K_1 .\ pq.p = pq$$

$$K_1 .p_o .p = pq(1 + K_1 .p)$$

Now,

$$p = p_o - (pq + pr)$$

$$K_1 q_o \left[p_o - (pq + pr) \right] = pq \left[1 + K_1 \left\langle p_o - (pq + pr) \right\rangle \right]$$

$$pq = \frac{K_1 .q_o \left[p_o - (pq + pr) \right]}{1 + K_1 \left[p_o - (pq + pr) \right]}$$

thus,

Percent occupancy of Q antibody binding sites

$$= \frac{pq}{q_o} \times 100$$

$$= \frac{K_1\left[p_o - (pq + pr)\right]}{1 + K_1\left[p_o - (pq + pr)\right]} \times 100 \quad (A16)$$

Similarly,

Percent occupancy of
R antibody binding sites

$$= \frac{pr}{r_o} \quad \times \ 100$$

$$= \frac{K_2\left[p_o - (pq + pr)\right]}{1 + K_2\left[p_o - (pq + pr)\right]} \times 100 \quad (A17)$$

(see Figure 5.9).

APPENDIX 2

CLEARANCE OF T_3

Gavin, Castle, McMahon, Martin, Hammond and Cavalieri (1977) calculated the metabolic clearance rate of T_3 (MCR T_3) to be 24.0 = 5.9 L/d. Considering the fraction fT_3 to be 0.3%, then this clearance rate is equivalent to 8000 L/d of fT_3 or 45.6 nmoL/d (at a fT_3 concentration of 5.7 pmol/L).

If 180L/d of plasma are cleared through the glomeruli (Ganong, 1973), then 5.7 pmol/L x 180 L/d or 1.03 nmoles fT_3 are cleared/day. That is, (45.6 - 1.03) = 44.6 nmoles of T_3 is stripped from the circulating binding proteins per day.

Hillier (1975) has calculated that every 3 sec, an amount of T_3 equivalent to the entire circulating T_3 dissociates into solution. Assuming a circulating volume of 5.5L the dissociation rate is,

$$1.9 \text{ nmol/L} \times 5.5 \text{ L/3 sec}$$
$$= 273.6 \text{ umoL/d.}$$

This is 6140 x the quantity of T_3 stripped from the circulating proteins per day. That is, the dissociation rate of T_3 in the circulating is far in excess of the whole body clearance of T_3.

APPENDIX 3

ABBREVIATIONS

A	$^{125}I-T_4$ (cpm) bound to immcbilized antibody (Corning fT_4 assay)
ACTH	corticotrophin
[albumin]	concentration of unoccupied albumin binding sites
ANS	8-anilino-l-naphthalene sulphonic acid, sodium salt
A/T	fraction of $^{125}I-T_4$ bound to immobilized anti-T_4 antibody (Corning fT_4 assay)
B	counts, fraction or percent of tracer bound to antibody
B, [B]	concentration of hormone bound to serum thyronine binding proteins
B', B_{ab}	percent tracer bound to antibody
B'	concentration of hormone bound to serum thyronine binding proteins
ΔB	standard deviation of the measured counts, fraction or percent tracer bound to antibody
B_{ic}	intracellular iodothyronine binding sites
BMISF	bound tracer misclassified as free
B_o	counts, fraction or percent tracer bound to antibody in the absence of space unlabelled hormone
B_p,B'_p	percent of tracer bound to serum and buffer protein

BSA	bovine serum albumin
B_o/T	fraction or percent tracer bound to antibody in the absence of unlabelled hormone
CPBA	competitive protein binding assay
cpm	counts per minute
%CV	percent coefficient of variation
DAB	double antibody separation of free and antibody bound tracer
DASGG	donkey anti-sheep goat gamma globulin
DTT	dithiothreitol
E/D-RIA	equilibrium dialysis-radioimmunoassay
E-SH	active reduced iodothyronine deiodinase
E-SI	iodothyronine deiodinase enzyme-sulphenyl iodide intermediate complex
ETR	effective thyroxine ratio
F	counts, fraction of percent of tracer not bound to antibody (free)
F, [F]	concentration of hormone in serum not bound to serum thyronine binding proteins (free)
F'	percent of tracer not bound to antibody
F', [F']	concentration of hormone in serum not bound to serum thyronine binding proteins (free)

F/B	ratio of free tracer and antibody bound tracer
frT_3	rT_3 in serum which is unbound to thyronine binding proteins (free rT_3)
fT_3	T_3 in serum which is unbound to thyronine binding proteins (free T_3)
fT_4	T_4 in serum which is unbound to thyronine binding proteins (free T_4)
FTI	free thyroxine index
GARGG	goat anti-rabbit gamma globulin
GSH	glutathione
HSA	human serum albumin
k	reaction rate constant
K	binding affinity constant (association)
K_m	Michaelis constant
K_i	Michaelis inhibition constant
[L]	ligand
L	concentration of free ligand
NADPH	reduced nicotinamide adenine dinucleotide phosphate
p	unlabelled hormone concentration
Δp	standard deviation of the measured unlabelled hormone concentration
p*	labelled hormone concentration

P'	assay sensitivity
p_o	total hormone concentration (p_o=p+p*)
$\Delta p/p$	precision or coefficient of variation of p
pq	concentration of occupied binding sites on antibody Q
pr	concentration of occupied binding sites on antibody R
P,P'	iodothyronine binding proteins in serum
P	probability
[P]	concentration of unoccupied iodothyronine binding protein binding sites
$[P_o]$	total concentration of iodothyronine binding protein binding sites
PBI	protein bound iodine
[PBI]	serum concentration of protein bound iodine
PR	daily production rate
PTFE	polytetrafluoroethylene
PTU	propylthiouracil
q	concentration of unoccupied binding sites on antibody Q
q_o	total concentration of binding sites on antibody Q(q_o=q+pq)
Q	antibody species Q

r	concentration of unoccupied binding sites on antibody R
r	correlation coefficient
r_o	total concentration of binding sites on antibody R (r+pr)
R	antibody species R
R	binding reagent
[R]	concentration of unoccupied binding reagent binding sites
R_f	used in this text to refer to the distance of migration of a given compound during thin layer chromotography
$[R_o]$	total concentration of binding reagent binding sites $[R_o]=[R]+[RL]$
RL	ligand/binding reagent complex
[RL]	concentration of occupied binding reagent binding sites
rT_3	3,3',5'-triiodothyronine
SD	standard deviation
T	tracer (cpm) added to incubation tube
T_n	iodothyronine with n iodine atoms; used in the text to denote the total serum T_n
$[T_n]$	concentration of T_n in serum which is unbound to thyronine binding proteins (free)

$*T_n$	labelled iodothyronine
$3\text{-}T_1$	3-monoiodothyronine
$3'\text{-}T_1$	3'-monoiodothyronine
$3,3'\text{-}T_2$	3,3'-diiodothyronine
$3,5\text{-}T_2$	3,5-diiodothyronine
$3',5'\text{-}T_2$	3',5'-diiodothyronine
T_3	3,5,3'-triiodothyronine
T_4	3,5,3',5'-tetraiodothyronine
TBG	thyroxine binding globulin (when referring to earlier papers in which this term was used); thyronine binding globulin (comtemporary terminology)
$[TBG_o]$	total concentration of thyronine binding globulin
[TBP]	concentration of unoccupied thyronine binding protein binding sites
TBP	thyronine binding proteins
TBPA	thyronine binding prealbumin
$[T_n.\text{albumin}]$	concentration of albumin binding sites occupied by iodothyronine, T_n
$[T_n.\text{TBG}]$	concentration of thyronine binding globulin binding sites occupied by iodothyronine, T_n

$[T_n.TBP]$	concentration of thyronine binding protein binding sites occupied by iodothyronine, T_n
$[T_n.TBPA]$	concentration of thyronine binding prealbumin binding sites occupied by iodothyronine, T_n
TRH	thyrotropin releasing hormone
TSH	thyrotropin
$[TT_4]$	concentration of serum total T_4
$[TT_4]/[TBG_o]$	ratio of serum total T_4 and thyronine binding globulin concentration (referred to as T_4/TBG in the text)
T_3RU	T_3 resin uptake
T_3SU	T_3 sephadex uptake
T_3U	T_3 uptake (generic term for the various T_3 uptake methods)
$T_4.B_{ic}$	thyroxine/intracellular binding protein complex
$T_4.R$	thyroxine/binding reagent complex complex
$*T_4.R$	^{125}I-thyroxine/binding reagent
$*T_4.TBP$	^{125}I-thyroxine/thyronine binding protein complex
V_e	elution volume of a particular compound during gel filtration chromatography
V_D	dialysate volume
V_S	volume of serum being dialysed

V_t total volume of gel in gel filtration chromatography column (measured as V_e of ^{125}I-iodide)

V_e/V_t ratio of elution volume and total gel volume

V_{max} maximum enxyme reaction velocity

APPENDIX 4

Oral Communications

Symons, R.G., Wellby, M.L. and J.McEntee (1977), Assay of 3,3',5'-Triiodothyronine (Reverse T_3) and its Potential Clinical Application, Proceedings of the 16th Annual Conference of the Australian Association of Clinical Biochemists, Abstract 7.

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Manuscript in Preparation

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