CLINICAL SIGNIFICANCE OF THYROXINE-BINDING GLOBULIN

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SYNOPSIS

The first chapter of this thesis comprises a review of thyroxine-binding proteins of man, with emphasis on TBG and, to a lesser extent, TBPA. The identification, isolation, physicochemical and physiological characteristics, and genetic variations of both proteins are included. The changes in protein concentration in disease are reviewed and the indications for the present study are presented.

Subsequent chapters describe the purification of TBPA and partial purification of TBG. The production of monospecific antisera to TBG and TBPA, and development of immunoelectrophoretic assays for both proteins are described.

TBG was measured in healthy persons, and the effects of age and sex on TBG were assessed, as was the effect of thyroid disease.

TBG, TBPA and thyroid hormones were measured in patients after surgery, myocardial infarction and starvation. The inter-relationships of protein and free hormone concentrations were explored.

Finally, nineteen families with inherited abnormalities of TBG concentration were studied. The typical case histories and biochemical findings of individuals with TBG abnormality were noted, especially when TBG abnormality coexisted with thyroid disease. Deficiencies of conventional thyroid function tests were found in TBG abnormality, and the use of T_4:TBG ratio as a means of assessing thyroid function was assessed retrospectively and prospectively.

I DEDICATE THIS THESIS TO

MY PARENTS, AND TO MY WIFE, JENNIFER

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COLLABORATIVE PROJECTS

The author wishes to make it clear that he was not responsible for all the practical work described in this thesis. All protein assays were performed by the author, who was also intimately involved in the development of these assays. The author was familiar with the techniques of radioimmunoassay and carried out such assays, but did not perform the hormone assays for this thesis.

In general, the work contributed by others is reflected in the authorship of the collaborative papers bound at the end of this thesis.

ABBREVIATIONS

ANS	8-anilino-1-naphthalene sulphonic acid
FT_4	free thyroxine
FT_3	free triiodothyronine
FTI	free thyroxine index
IEF	isoelectric focusing
EIP	immunoelectrophoresis
2DIEP	two-dimensional immunoelectrophoresis
K	metabolic clearance rate
Ka	association constant
PBI	protein-bound iodine
RIA	radioimmunoassay
T_4	thyroxine, 3, 5, 3', 5'-tetraiodothyronine
T_3	triiodothyronine, 3, 5, 3'-triiodothyronine
T_3U	T_3 uptake
rT_3	reverse-triiodothyronine, 3, 3', 5'-triiodothyronine
TBG	thyroxine-binding globulin
TBPA	thyroxine-binding prealbumin
TBP	thyroxine-binding protein
TRH	thyrotropin-releasing hormone
TSH	thyroid stimulating hormone, thyrotropin
TEMED	NNN'N'-Tetramethylethylenediamine
Tris	Tris(hydroxymethyl)methylamine

THE CLINICAL SIGNIFICANCE OF THYROXINE-BINDING GLOBULIN (TBG)

CHAPTER 1 INTRODUCTION

1) Identification of thyroxine-binding proteins

2) Isolation of TBP

3) Physicochemical characteristics of TBP

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4) Physiology of TBP

   a) TBP and thyroid hormones in plasma

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      ii) Measurement of TBP, their kinetics and distribution

      iii) Biosynthesis and metabolism

      iv) Significance

      v) Consequences of altered TBP concentration

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INTRODUCTION

1) IDENTIFICATION OF THYROXINE-BINDING PROTEINS

The strong association between thyroxine and serum components was apparent from work performed at the start of this century, which showed that serum iodine was not dialysable (Gley and Bourcet, 1900) and not ultrafiltrable (Trevorrow, 1939). Attempts to identify the molecules responsible by iodine analyses of plasma protein fractions were unsuccessful (Taurog and Chaikoff, 1948; Salter, 1949), and the existence of a specific thyroxine-binding protein was not appreciated until 1952, when Gordon and co-workers applied the technique of zone electrophoresis (Gordon et al, 1952). At about this time, several groups of workers (Gordon et al, 1952; Larson, Deiss and Albright, 1952; Robbins and Rall, 1952; Winzler and Notrica, 1952; Horst and Rösler, 1953) demonstrated that with small amounts of added thyroxine, most of the thyroxine in serum was bound to a protein which electrophoresed in barbital buffer, pH 8.6, to a position intermediate between the α-1 and α-2 globulins. This protein is now designated thyroxine-binding globulin (TBG). A smaller amount of bound T_4 was found in the albumin zone, but because the early workers were using barbital buffer, which inhibits T_4-binding to human prealbumin, the existence of thyroxine-binding prealbumin was not established until 1958 (Ingbar, 1958). Much subsequent work was performed using a reverse-flow paper electrophoretic system (Robbins, 1957) and glycine acetate buffer, pH 8.6. Using "tracer" amounts of T_4 ($\lesssim$ 40 nmol/l) added to normal human serum, approximately 60% of T_4 was bound to TBG, 30% to TBPA and 10% to albumin (Sterling and Tabachnik , 1961; Oppenheimer et al, 1963; Inada and Sterling, 1967b). The above conditions of buffer and pH can hardly be regarded as physiological, and experiments utilizing immunoprecepitation (Woeber and Ingbar, 1968) and electrophoresis at pH 7.4 (Lutz and Gregerman, 1969),

have since shown that the actual fraction of T_4 carried by TBPA is probably about 15%, with albumin carrying 10% and TBG 70 - 75%.

Binding in additional electrophoretic zones, demonstrated in a variety of buffer and electrophoresis systems in early studies, was variously attributed to artefact due to long-term freezing and storing (Blumberg and Robbins, 1960), to altered TBG mobility due to interaction with albumin (Refetoff, Robin and Fang, 1970), or to additional T_4-binding proteins (Tata, Widnell and Gratzer, 1961). It is now thought that the additional T_4-binding protein, with slower electrophoretic mobility than TBG, represents a partially desialylated TBG, or "slow" TBG (Premachandra, Perlstein and Blumenthal, 1970; Marshall, Pensky and Green, 1972).

Other T_4-binding proteins have been described in human serum, but elucidation of the nature and extent of these has required the advent of more sensitive techniques such as immunoelectrophoresis (Lightfoot and Christian, 1966; Freeman and Pearson, 1969), post electrophoresis-labelling (Freeman and Pearson, 1969; Hoch and Lewallen, 1974) and altered anodic mobility in T_4-containing gel (Hoch and Lewallen, 1974). These techniques have now clearly shown T_4-binding to α-1-lipoprotein and β-lipoprotein. Even though these proteins only account for 1% and 2% of total T_4 respectively (Miyai et al, 1968; Hoch and Lewallen, 1974), it is still possible that they may have an important rôle in thyroid physiology, although evidence for this is lacking.

T_4-binding by gamma globulins was first reported in a woman with thyroid carcinoma by Robbins and co-workers in 1956, (Robbins, Rall and Rawson, 1956) and seems to occur as part of a specific immune response. Gamma globulin binding of T_4, T_3, or both, has been noted in thyrotoxicosis (Staeheli, Valloton and Burger, 1975), Hashimoto's thyroiditis and primary

myxoedema (Premachandra and Blumenthal, 1967; Ochi et al, 1972; Staeheli et al, 1975; Herrmann et al, 1977), and nodular goitre (Wu and Green, 1976). Apart from gross derangement of thyroid hormone radioimmunoassays (Wu and Green, 1976; Jorgensen, Skovsted and Jensen, 1979), an important clinical effect of such antibodies may be the production of hypothyroidism (Karlsson, Wibell and Wide, 1977).

Other examples of anomalous T_4-binding proteins include diminished TBG-binding and increased albumin binding of T_4 in bisalbuminaemia (Barboree and Decker, 1971), and autosomal dominant inheritance of a protein with different T_4-binding and antigenic properties, and different electrophoretic mobility from TBG (Lee et al, 1979).

2) ISOLATION OF THYROXINE-BINDING PROTEINS

A number of problems were encountered during early attempts to purify TBG (see Robbins, 1976 for review). In part these were due to the low serum concentration of TBG, but other factors may have been:

a) The instability of T_4-TBG interaction at pH $\lesssim$ 5 (Marshall and Pensky, 1971) and the irreversible first-order structural transition which occurs at low pH (Gershengorn et al, 1977b).

b) The susceptibility of purified TBG to denaturation by mechanical agitation, and the loss of T_4-binding activity during a single freezing and thawing (Korcek and Tabachnik, 1974).

c) The desialylation of TBG preparations by neuraminidase-contamination of Sepharose columns (Marshall et al, 1972).

d) The variable T_4-contamination of TBG preparations (Gershengorn et al, 1977a).

A major advance in the isolation of TBG was the application of affinity chromatography by Pensky and Marshall (1969), in which TBG in serum was

bound to covalently-linked T_4-agarose, and then eluted with 2mM KOH. Even after this procedure, multiple-step chromatographic or gel-filtration procedures were still necessary in order to obtain pure TBG (Marshall, Pensky and Williams, 1973; Korcek and Tabachnik, 1974; Gershengorn et al, 1977a). Early attempts to improve TBG yield by the use of carbon-chain spacers attached to agarose were not thought to be successful (Pages and Cahnman, 1972; Korcek and Tabachnik, 1974), although these have been utilised in recent studies (Horn et al, 1977; Kågedal and Källberg, 1977). Pure TBG preparations have recently been obtained in a 2-step procedure utilising affinity chromatography followed by ion-exchange chromatography with a buffer gradient (Cheng, Morrone and Robbins, 1979).

In contrast to the great difficulties encountered with TBG purification, TBPA has proved amenable to purification by conventional methods, and a number of workers have reported the successful isolation of TBPA (see Robbins, 1976 for review).

3) PHYSICOCHEMICAL PROPERTIES OF THYROXINE-BINDING PROTEINS

a) Molecular properties and structure

i) TBG

Some early studies on the molecular properties of TBG can no longer be regarded as reliable, for reasons mentioned above (e.g. Giorgio and Tabachnik, 1968; Sterling et al, 1971). Later studies utilising affinity chromatography are in general agreement that the molecular weight of TBG is between 53,000 and 64,000 Daltons (Marshall and Pensky, 1971; Korcek and Tabachnik, 1974; Nilsson and Peterson, 1975; Gershengorn et al, 1977a; Petek, 1979). Despite one claim that TBG is a tetramer composed of four identical subunits (Nilsson and Peterson, 1975), subsequent work by Gershengorn et al (1977a) has provided convincing evidence in support of

the claim that TBG consists of a single polypeptide chain (Marshall and Pensky, 1971; Korcek and Tabachnik, 1974).

TBG is a glycoprotein, and rather variable amino acid and carbohydrate contents have been estimated by different groups (Table 1.1). It is interesting to note however, that there is reasonably good agreement between the studies of Korcek and Tabachnik (1974), Gershengorn et al (1977a) and Zinn et al (1978a), who all used the affinity chromatographic separation procedure of Marshall et al (1973).

Gershengorn et al (1977b) suggested that the C-terminal sequence of TBG is (Ala-Ser)-Leu, while the N-terminal amino acid sequence is said to be Ala-Ser-Pro-Glu-Gly-Lys-Val-Thr-Ala-Asp-Ser-Ser-Ser-Glu-(Pro)-(Cheng, 1977).

The carbohydrate moiety of TBG is rich in sialic acid (Table 1.1), and this accounts for the low isoelectric point of pH 4.2 - 5.2 (Robbins, Petermann and Rall, 1955; Marshall et al, 1973). Varying degrees of desialylation account for the "slow" TBG found in obese patients (Premachandra et al, 1970) and in cirrhotics (Marshall et al, 1972 - and see Chapter 1 - 4.a.iv.). The glycopeptides and oligosaccharides of TBG have been subjected to further study by Zinn et al (1978a, b), who have proposed that TBG contains 4 oligosaccharide side chains. They have designated the chains Glycopeptide I, oligosaccharide A and oligosaccharide B . It is proposed that each TBG molecule contains one unit of glycopeptide 1, 2 units of oligosaccharide A and one unit of oligosaccharide B (Fig. 1.1).

The secondary and tertiary structure of TBG was investigated by Gershengorn et al (1977a), using circular dichroism in the near ultraviolet, and polarization of dansyl-TBG fluorescence. They found that approximately

Table 1.1

AMINO ACID	Moles per mole Reference* (see foot of Table) 1	2	3	4	5	6	7
Lysine	12	18	25	22	29	28	26
Histidine	11	7	10	14	12	11	11
Arginine	22	4	16	16	17	6	7
Aspartic acid	25	26	38	42	45	36	40
Threonine	18	20	25	26	28	25	28
Serine	19	24	34	32	49	29	35
Glutamic acid	34	30	52	63	61	42	46
Proline	27	16	30	29	20	15	13
Glycine	25	23	25	32	29	19	28
Alanine	27	32	31	35	30	28	ND
½-Cystine	5	3	8	11	8	5	8
Valine	12	21	23	27	25	27	26
Methionine	3	6	5	11	8	12	16
Isolencine	8	12	9	19	17	18	22
Lencine	43	28	44	47	37	38	44
Tyrosine	7	4	10	12	15	9	8
Phenylalanine	14	12	18	22	17	22	22
Tryptophan	4	3	4	ND	4	4	ND
Number of residues	316	289	407	464	440	380	387
Molecular weight	59,000	36,000	58,000		53,200	54,000	

AMINO ACID AND CARBOHYDRATE COMPOSITION OF THYROXINE-BINDING GLOBULIN PREPARATIONS FROM DIFFERENT LABORATORIES

Table 1.1 (cont.)

CARBOHYDRATE	Reference 1	2	3	4	5	6	7
Glucose		2		2	0	6	0
Galactose		6		6	7	13	9
Mannose		5		12	7	6	11
Fucose	19	0	0.2	0	1	0	0
Xylose		1		0	0	0	0
Glucosamine		11	12	12	7	22	16
Galactosamine		0	3	4?	0	0	0
Sialic acid	9	4	5	6	0	10	10
Total % Carbohydrate	32	15		13	7.5	23	15

* 1 = Seal and Doe, 1964

2 = Sterling et al, 1971

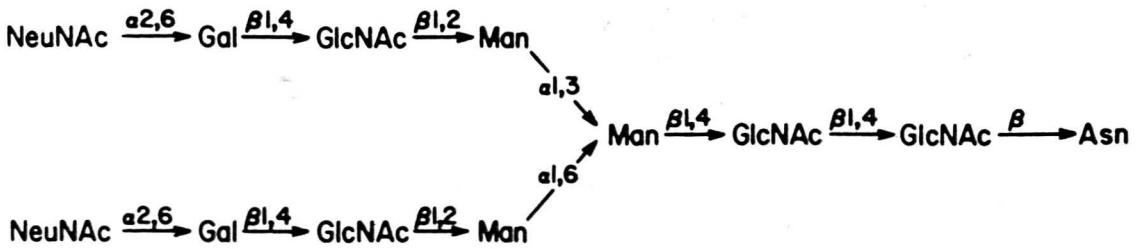
3 = Giorgio and Tabachnik, 1968

4 = Korcek and Tabachnik, 1974

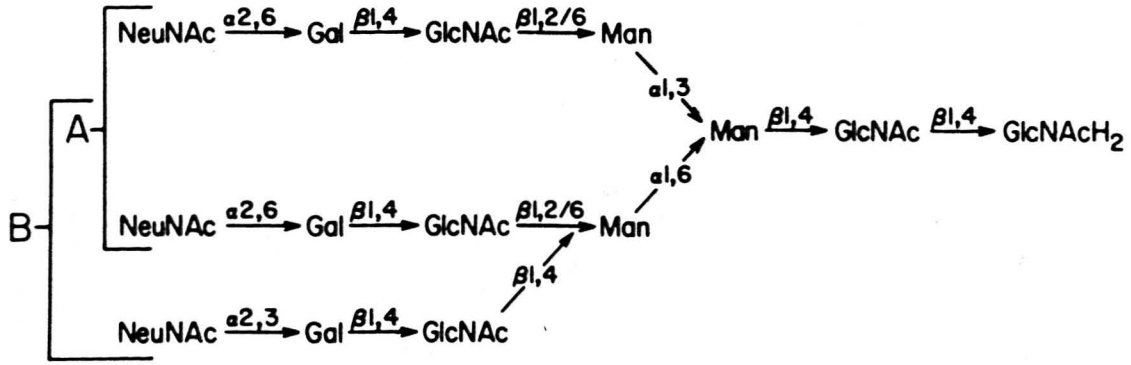
5 = Nilsson and Peterson, 1975

6 = Gershengorn et al, 1977a

7 = Zinn et al, 1978a



Proposed structure for glycopeptide I.



Proposed structures for oligosaccharides A and B.

Figure 1.1

(From Zinn et al, 1978a)

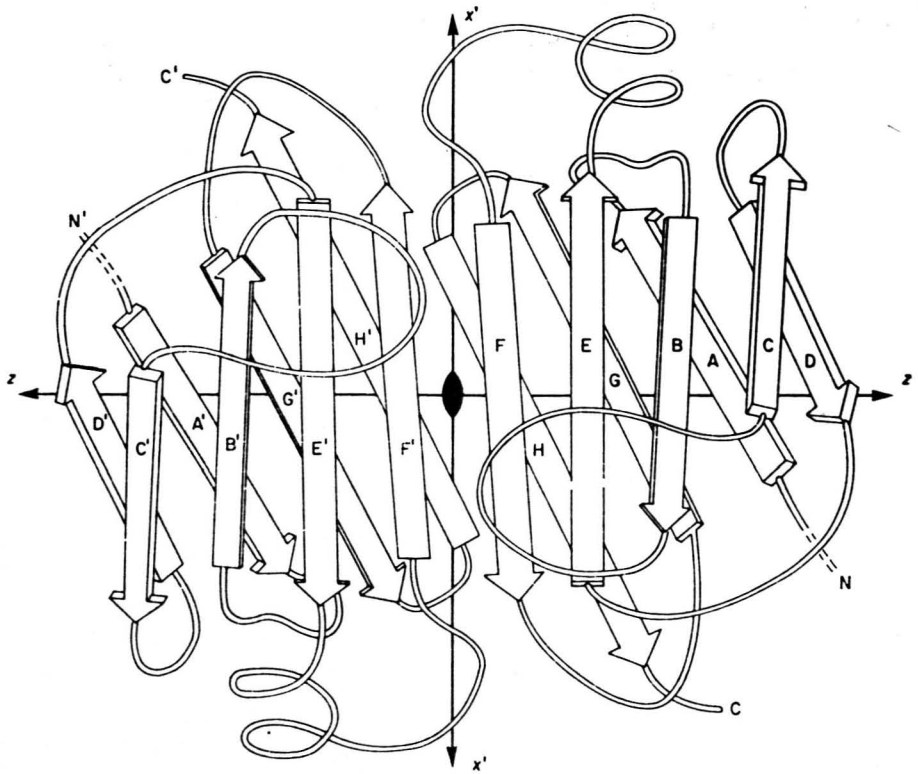
half the peptide residues of TBG were unordered, with the remainder being distributed approximately equally between α-helices and β-structures.

ii) TBPA

Although early studies suggested a molecular weight for TBPA of 60 - 70,000 (Robbins, 1976), the complete amino acid sequence of TBPA is now established (Kanda et al, 1974) and gives a molecular weight of 55,000. The higher values in early studies may have been due to inclusion of retinol-binding protein (M.Wt. 21,000, Kanai, Raz and Goodman, 1968) with the TBPA molecule.

TBPA is a tetramer, consisting of 4 identical subunits, and this has been beautifully confirmed using X-ray diffraction studies of purified crystalline TBPA (Blake et al, 1971, 1974). The original deductions concerning the tetrameric structure of TBPA were based on a study of TBPA polymorphism in the rhesus monkey (Alper, Robin and Refetoff, 1969; Bernstein, Robbins and Rall, 1970).

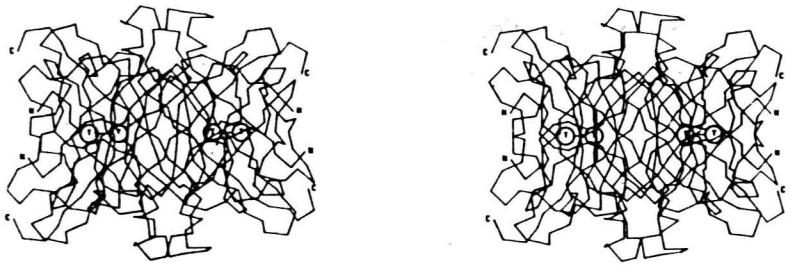
TBPA shows a two-fold symmetry, with the subunits arranged as two dimers, which are in turn arranged with two opposing pleated sheets of β-structures, so as to form a slot within the molecule, with the dimensions 25Å long by 8Å diameter (Blake et al, 1971, 1974; Blake and Oatley, 1977, Figs. 1.2 and 1.3). In addition to T_4-binding, TBPA also interacts strongly with retinol-binding protein (RBP) and normally circulates as a 1 - 1 molar complex of TBPA - RBP (Fig. 1.4) (Raz et al, 1970). Like albumin, TBPA does not contain carbohydrate, and it is also unusual in its extreme resistance to denaturation by heating, acid, alkali, urea, guanidine and detergents (Raz et al, 1970; Branch, Robbins and Edelhoch, 1971; Rask et al, 1971; Branch, Robbins and Edelhoch, 1972).



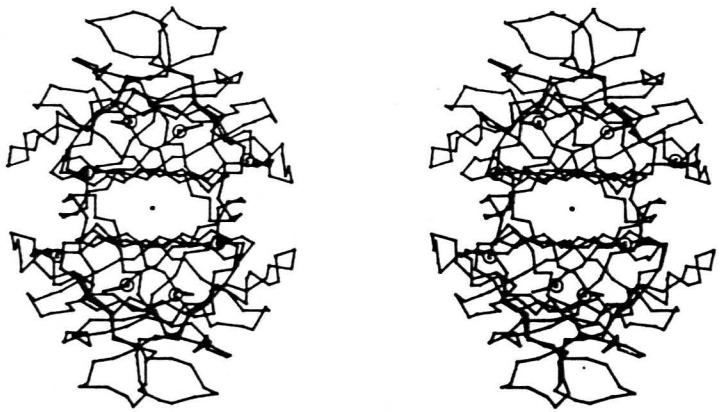
A schematic drawing of the main-chain conformation in the prealbumin dimer
The arrows indicate β-sheet strands, labelled A to H in one monomer and A′ to H′ in the other, in the order in which they occur in the polypeptide chain. The molecular *x*′ and *z*-axes are indicated. N indicates residue 10 and C indicates residue 126.

Figure 1.2

(From Blake et al, 1974)



A stereo-view of the prealbumin tetramer down the molecular y'-axis. N ≡ residue 10; C ≡ residue 126. T———T represents the two di-iodotyrosyl residues of thyroxine located on the molecular z-axis.



stereo-view of the prealbumin tetramer looking down the molecular z-axis, shown by the dot, with the molecular x' and y'-axis horizontal and vertical, respectively. The thyroxine binding sites are superposed along the z-axis.

Figure 1.3

(From Blake et al, 1974)

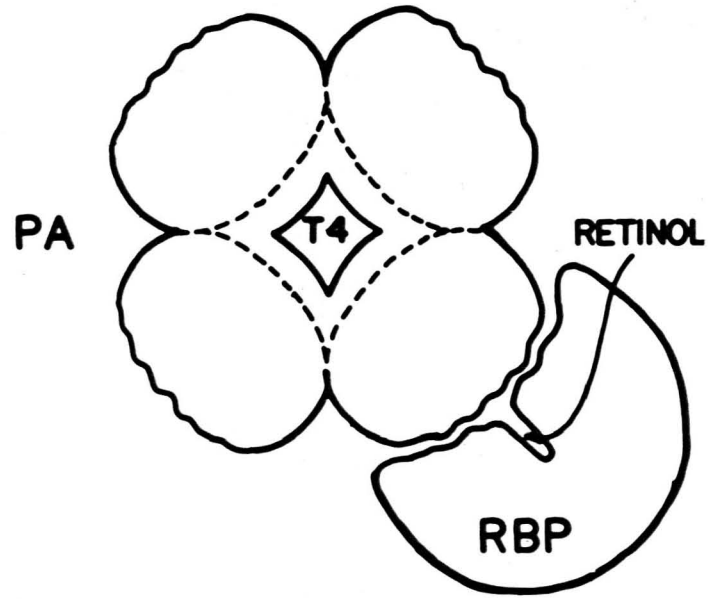


Figure 1.4

(From Goodman, 1976)

Cartoon model for the RBP-prealbumin complex. Prealbumin is shown as a tetramer containing a central channel which is the site of thyroxine (T4) binding. The locations of the different binding sites suggest the known relations between the several interactions: RBP and T4 bind at independent sites on prealbumin, whereas the binding of retinol to RBP is stabilized by formation of the protein-protein complex.

b) Thyroid hormone binding sites and hormone-protein interaction

T_4 is relatively tightly bound to TBG, and estimates of the affinity constant (Ka) for this reaction based on ultrafiltration and equilibrium dialysis measurements range from $1.6 \times 10^{9} M^{-1}$ to $2.3 \times 10^{10} M^{-1}$ (Green et al, 1972; Nilsson and Peterson, 1975; Korcek and Tabachnik , 1976, 1978; Snyder et al, 1976; Cheng et al, 1979). It is generally accepted that there is one TBG binding site for T_4 (Marshall and Pensky, 1971; Green et al, 1972; Gershengorn et al, 1977a).

The association of T_4 and TBG is pH-dependent (Marshall and Pensky, 1971; Korcek and Tabachnik, 1976, Gershengorn et al, 1977b), with maximal binding in the region of physiological pH, pH 6.8 - 7.7 and a 25% reduction at pH 8.6 (Korcek and Tabachnik, 1976). Binding diminishes progressively with temperature increase from 5°C to 37°C (Korcek and Tabachnik, 1976) and is also dependent on ionic strength and concentration of chloride and phosphate ions (Spaulding and Gregerman, 1972). Thermodynamic studies of T_4-TBG binding have shown a high positive entropy change, suggesting hydrophobic bonding (Green et al, 1972, Korcek and Tabachnik, 1976).

Removal of sialic acid residues does not greatly affect T_4 binding (Marshall et al, 1972, 1973), and even 86% deglycosylation resulted in only a 3-fold reduction in T_4 binding (Cheng et al, 1979).

The nature of the TBG binding site has been explored using derivatisation with a number of protein-modifying agents (Robbins et al, 1978; Siegel et al, 1979). These studies suggest the importance of the ϵ-amino group of a lysyl residue at or near the T_4-binding site. Other work has suggested that the binding site on TBG may be commodious, and able to accommodate thyroxyl peptides (Tabachnik, Hao and Korcek, 1971).

The binding affinity of TBG for T_3 is approximately ten times less than the affinity for T_4, and is also less than that of rT_3. Table 1.2 shows the relative potency of various T_4-analogues for binding to TBG, and also compares binding to TBPA, albumin and hepatic nuclei. Comparison of the binding potencies of various analogues has shown that most of the structural characteristics of T_4 are necessary for optimal binding, including an intact alanine side chain, iodine atoms at positions 3', 5', iodine or bromine at positions 3, 5, an intact phenolic hydroxyl group, and a diphenyl ether structure (or sulphur or methylene bridge)(Snyder et al, 1976; Tabachnik and Korcek, 1978).

Early studies of T_4-TBPA binding were thought to show only one binding site on the TBPA molecule (Raz and Goodman, 1969). This was difficult to reconcile with the fact that several thyroxine analogues and ANS appeared to occupy two equal sites on TBPA (Nilsson and Peterson, 1971; Pages, Robbins and Edelhoch, 1973). The problem was eventually resolved by two groups of workers. Firstly, the elegant work of Blake and co-workers (Blake et al, 1974; Blake and Oatley, 1977) showed clearly that there were two symmetrically equivalent binding sites for both T_4 and T_3, comprising the opposite ends of the slot which runs through the TBPA molecule (vide supra). Secondly, Robbins' group demonstrated that there were two T_4 binding sites on TBPA, but that they displayed the feature known as "negative cooperativity" whereby occupation of the first site by T_4 made binding to the second site more difficult. The apparent Ka's for the two sites differed by a factor of 100 (Ferguson et al, 1975).

The binding of T_4 to TBPA is lower than the binding to TBG by two orders of magnitude (Pages et al, 1973; Ferguson et al, 1975 and see Table 1.2). T_3 binding to TBPA was not observed in early studies (Ingbar, 1963) but has subsequently been shown to occur with an affinity ten times

Table 1.2*

	TBG[a]	TBPA[b]	HEPATIC NUCLEI[c]	ALBUMIN[d]
T_4	100($2\times10^{10}M^{-1}$)	100($2\times10^{8}M^{-1}$)	100($0.8\times10^{8}M^{-1}$)	100($2\times10^{6}M^{-1}$)
T_3	9	8	800	55
rT_3	38		0	100
3'-Isopropyl T_2	3.5		800	
T_4 Propionate	3.6	160		135
T_4 AC	1.7	160		110
T_3 AC	0.3		800	
D T_4	54			100
D T_3			560	

a. Snyder et al (1976). Affinity constant from Korcek and Tabachnik (1976).

b. Cheng et al (1977). Affinity constant for first site.

c. Oppenheimer et al (1976); Koerner et al (1975).

d. Tabachnik and Giorgio (1964). Affinity constant from Steiner et al (1966).

* from Robbins et al (1978).

THE RELATIVE AFFINITY OF THYROID HORMONES AND ANALOGUES FOR PLASMA AND NUCLEAR BINDING PROTEINS

less than that of T_4 (Davis, Handwerger and Gregerman, 1972; Cheng et al, 1977).

The concentrations and clearance rates of the TBP are given in Table 1.3. This also shows the amount of T_4 and T_3 carried by the different binding proteins, and brings out the fact that because the serum concentration of TBPA is much greater than that of TBG, less than 0.5% of TBPA molecules are bound to T_4, whereas 30% of TBG molecules carry T_4.

There are two other unique features of TBPA which make it of great physiological and theoretical interest. The first is the existence of a binding site for RBP on each TBPA subunit. TBPA may thus participate in vitamin A metabolism, especially as a means whereby RBP is retained within the circulation. T_4-binding may be purely incidental to this function (see Goodman 1976 for review). Secondly, Blake and Oatley (1977) have discovered that the surface of the TBPA tetramer has two symmetrical depressions which complement the structure of double-helical DNA. They propose that this may at least constitute a model of the thyroid hormone nuclear receptor, while there is no evidence for the other possibility of a direct DNA-TBPA interaction.

4) PHYSIOLOGY OF THYROXINE-BINDING PROTEINS

a) TBP and thyroid hormones in plasma

i) The concept of "free" hormone

In 1952, it was first proposed that thyroid hormones (T_4 and T_3) only exert their physiological activity when in the "free" form (i.e. unbound to plasma proteins) (Recant and Riggs, 1952). This proposal was developed and extended by the work of Robbins and Rall (1957, 1960) into a concept of thyroid hormones in plasma being part of a multi-equilibrium system,

Table 1.3.

	K		concentration	
	Fraction/ day	ml/hour	mg/l	nmol/l
TBG	0.14	40	15	220
TBPA	0.36	90	340	6,200
ALBUMIN	0.05	13	40,000	580,000
T_4	0.10	50	70×10^{-3}	90
T_4-TBG		10		67
T_4-TBPA		2×10^{-1}		13
T_4-ALB		2×10^{-4}		9
T_3	0.57	1,100	1.4×10^{-3}	2.1
T_3-TBG		2×10^{-1}		0.17
T_3-TBPA		3×10^{-3}		0.02
T_3-ALB		1×10^{-5}		0.02

(Adapted from Robbins, 1976

Robbins et al, 1978

Prince and Ramsden, 1977)

SERUM CONCENTRATIONS AND CLEARANCE OF MAJOR THYRONINES, BINDING PROTEINS AND HORMONE-PROTEIN COMPLEXES

obeying the laws of mass action. T_4 and T_3 bound to any protein binding site are considered to be readily exchangeable with hormones on other binding sites and with free hormone, the actual distribution within the system being governed by the concentration of hormones and the concentration and affinities of the various binding sites.

Robbins and Rall gathered together a considerable amount of clinical and theoretical evidence supporting the idea that the metabolic degradation of thyroxine was proportional to the amount of free hormone. Later work, in which free T_4 concentration was measured directly and found to correlate with T_4-half-time in non-thyroidal illness (Inada and Sterling, 1967a, Bellabarba et al, 1968) lent further support to the hypothesis. It followed that factors such as increased temperature and altered pH, which are known to diminish the affinity of TBG for T_4, had a pronounced effect on free T_4 and T_4 degradation (Oppenheimer, 1968).

Although the concept of free T_4 has proved useful over a number of years, and has generated considerable research work and served to emphasise the importance of the T_4-binding proteins, doubts have recently been expressed as to whether free T_4 should always be regarded as the sole determinant of physiologically active hormone. Ekins (1979) has drawn attention to the fact that the delivery of hormone to target cells is a dynamic process, whereas assessment of free hormone is made at equilibrium. If hormone delivery to the target tissue is viewed as a "flow" from protein-bound hormone $\longrightarrow$ free hormone in plasma $\longrightarrow$ across capillary membrane to extracellular fluid $\longrightarrow$ across cell membrane to target cell, then the rate of delivery of hormone can be shown in theory to depend on factors including the amount of bound hormone in plasma, and the dimensions of the capillary vessel. Ekins has suggested that an influence of protein-bound hormone on hormone delivery may explain the finding of low free hormone

concentrations in pregnancy (Ingbar et al, 1965; Sterling and Brenner, 1966).

An additional factor to consider in relation to the delivery of thyroid hormones to their site of action is the existence of cellular receptors (Oppenheimer, 1968; Oppenheimer et al, 1976). High-affinity, low-capacity T_3 binding sites have been demonstrated in the pituitary (Schadlow et al, 1972), and in liver and kidney nuclei (Oppenheimer et al, 1972). Alterations in such receptors could well influence the cellular uptake of thyroid hormone.

ii) Measurement of TBP

Measurement of both TBG and TBPA was originally accomplished by electrophoresis and capacitance techniques, whereby the amount of radio-labelled T_4 taken up by the protein at saturating concentrations of non-radioactive T_4 was measured. The binding capacity of TBPA was found to be between 216 and 342 μg T_4 (280 - 445 nmol) per 100 ml of serum (Oppenheimer, Martinez and Bernstein, 1966) and of TBG, 16 - 25 μg T_4 (21 - 33 nmol) per 100 ml (Robbins, 1956). The serum concentration of TBPA (Table 1.3) was sufficiently great to allow direct quantitation by densitometric techniques after electrophoretic separation, but the low serum concentration of TBG caused problems, and resulted in the development of a variety of indirect assay systems (see Chapter 3.3). The development of monospecific antisera to TBG (Levy, Marshall and Velayo, 1971; Bradwell et al, 1976) eventually enabled the use of radioimmunoassay and immunoelectrophoretic measurement of TBG.

The lack of a direct assay for TBG was probably also the stimulus for the development of a number of other indirect assays of T_4-protein binding. Most of those assays purport to measure the unoccupied protein binding sites of plasma samples by the uptake of ^{125}I or ^{131}I labelled thyronines (T_3 or T_4). These assays include the red-cell (Hamolsky, Stein and Freedberg, 1957) and resin (Mitchell, 1958; Sterling and Tabachnik, 1951) uptake tests, and Sephadex uptake tests (Clark and Brown, 1970). The main importance of these

tests lies in their wide clinical routine application as a measure of TBP, and in particular their combination with total T_4 measurements to derive a free T_4 index (FTI = T_4 x T_3 resin x 100, or FTI = $\frac{T_4}{T_3 \text{ uptake}}$ x 100) as an approximation of the free T_4 concentration (Clark and Horn, 1965; Wellby and O'Halloran, 1966).

The plasma concentrations of the T_4-transport proteins are shown in Table 1.3. The distribution of TBP in other fluids has been reviewed (Robbins and Rall, 1960; Ingbar and Freinkel, 1960). TBG and TBPA have been repeatedly demonstrated in lymph, and in pleural, peritoneal and synovial fluids. Both proteins have been identified in cerebrospinal fluid, where the proportion of TBPA relative to TBG and other plasma proteins was found to be ten times greater than in plasma (Alpers and Rall, 1965; Hagen and Elliot, 1973).

TBG in urine was initially recognised only in patients with nephrosis (Robbins, Rall and Petermann, 1957; Gavin et al, 1978). However, TBG has now been identified in normal urine, at a concentration of 1.7 µg/100 ml, where it apparently has minor conformational changes resulting in decreased affinity for T_4 (Gavin et al, 1979).

iii) Biosynthesis and metabolism

Synthesis of TBG by isolated monkey hepatocytes has been clearly demonstrated by Glinoer, Gershengorn and Robbins (1976), and the enhancement of synthesis by oestrogen-pretreatment of female monkeys has also been shown (Glinoer et al, 1977a). In vivo administration of β-oestradiol resulted in stepwise increase in TBG production within 24 hours (Glinoer et al, 1977b). In vitro work with monkey hepatocarcinoma cells also demonstrated that at

physiological concentrations or lower, T_4 itself stimulated TBG synthesis, while at higher concentrations, TBG synthesis was inhibited (Robbins et al, 1978, Glinoer et al, 1979). This effect was thought to be a manifestation of thyroid hormone action on protein synthesis in general, rather than an example of a hormone eliciting the production of its own transport protein (cf vitamin A causing acute release of RBP from hepatocytes, Smith et al, 1973).

As with other glycoproteins, TBG is protected from hepatic degradation by its sialic acid residues. Refetoff, Fang and Marshall (1975) showed that desialylated TBG was rapidly cleared from the circulation of normal subjects, while desialylated TBG has been shown to accumulate in the serum of cirrhotics (Marshall et al, 1972; Gartner et al, 1979). Asialo-TBG has been shown to bind in vitro to hepatic membranes, and asialoglycopeptides of TBG inhibit this binding, thus confirming the in vivo findings (Zinn et al, 1978b).

iv) Physiological significance of the thyroxine-binding proteins

In spite of an enormous amount of research into the nature and role of thyroxine-binding proteins since the discovery of TBG in 1952, their function remains obscure. Phylogenetic studies of TBP have been performed (Farer et al, 1962; Tanabe, Ishii and Tamaki, 1969; Refetoff, Robin and Fang, 1970), but are unfortunately of limited help. TBG can be identified in most mammals, but there are exceptions in the case of the rat and rabbit (Sutherland and Brandon, 1976). Prealbumin is more widely distributed than TBG among vertebrates, and is the major transport protein of several avian species.

Genetic studies have been more helpful, in demonstrating complete absence of TBG in perfectly healthy males (see Chapter 1.4.b.i.), thus showing

that TBG cannot have any vital rôle. The same cannot be said of TBPA, since there are no reliable reports of total absence of this protein, although levels may be very low in severe illness (see Chapter 4.5).

One function of thyroxine-binding proteins in general may be that of providing a means whereby a relatively insoluble, hydrophobic hormone may be held in quite large quantities in aqueous solution in the blood. In addition, it is believed that the TBP may limit the renal and possibly gastrointestinal loss of thyroid hormones, as well as controlling the amount of hormone available for degradation or the induction of metabolic effects. Short-term alterations in the concentration of TBP (especially TBPA with a short biological ½ life), may also be able to modulate the turnover of thyroid hormones.

The above hypothesis conceives the role of TBP as being rather passive, but more active roles, such as the delivery of hormone to specific sites of action by hormone-plasma protein complexes, have also been proposed (Robbins, 1976). However, the presence of binding protein does not seem to be necessary for cellular uptake of thyroid hormones, and kinetic studies suggest a barrier for TBG diffusion at the capillary wall (Irvine and Simpson-Morgan, 1974). Some plasma binding proteins (e.g. RBP) have been shown to play an active role in hormone delivery by binding to cell surfaces (Rask and Peterson, 1976). Evidence for this does not exist for the TBP, but the mechanism could operate for one or more of the protein-thyroid hormone complexes, perhaps for specific target sites.

TBPA would appear to be a more likely candidate than TBG for this rôle - being apparently essential for life, and having a rapid metabolic turnover. In this context, an interesting and unexplained phenomenon is the ten-fold increase in TBPA in CSF relative to other proteins (Tourtellotte, 1970;

Hagen and Elliot, 1973).

v) Consequences of altered thyroxine-binding protein concentration

As mentioned above (Chapter 1.4.a.i.) the concept of binding proteins and hormones in plasma as being an isolated multi-equilibrium system is probably an over-simplification since it ignores dynamic and cellular factors which may be concerned with hormone uptake by target tissues. However, in considering the effects of alterations in binding protein concentration, a model based on the law of mass action is of value. Several groups of workers have derived multi-ligand/multi-protein-binding site equations, usually requiring computer techniques, which may be used to predict the effects of binding protein changes on total and free T_4 concentrations (DiStefano and Chang, 1971; Wosilait and Nagy, 1976; Princé and Ramsden, 1977). The most comprehensive programme is that of Princé and Ramsden, which not only allows for the interaction of T_3 on T_4 binding and vice versa, but also takes into account the two negatively interacting binding sites of TBPA, and up to six secondary binding sites on albumin (Steiner, Roth and Robbins, 1966).

Using their model, Princé and Ramsden made a number of predictions of the effect of different thyroid states on the distribution of thyroid hormones among the binding proteins, and of the effect of changes in binding protein concentration on free and total concentrations of T_3 and T_4. Among the more important of these predictions was that the number of secondary binding sites on albumin had an appreciable influence on the distribution of bound ligand, and also that a slight elevation of serum T_4, with normal concentration of total T_3 (so-called T_4-toxicosis-Hadden et al, 1975) caused a profound increase in serum free T_3.

Table 1.4 summarises the effects of binding protein changes on free and

Table 1.4

	Normal	Low TBG	Low TBG normal free T_4	High TBG x 2 normal free T_4	Reduced TBPA(50%)	Reduced Alb(50%)
Total T_4 mol/l	1.09×10^{-7}	1.09×10^{-7}	3.5×10^{-8}	1.85×10^{-7}	1.09×10^{-7}	1.09×10^{-7}
% free T_4	0.0355	0.1118	0.111	0.0213	0.0408	0.0397
Free T_4 mol/l	3.87×10^{-11}	1.22×10^{-10}	3.89×10^{-11}	3.93×10^{-11}	4.45×10^{-11}	4.33×10^{-11}
Total TBG mol/l	1.70×10^{-7}	1.70×10^{-10}	1.70×10^{-10}	3.40×10^{-7}	1.70×10^{-7}	1.70×10^{-7}
$\%T_4$ bd. by TBG	68.08	0.11	0.21	80.91	73.42	72.36
Total TBPA mol/l	4.50×10^{-6}	4.50×10^{-6}	4.50×10^{-6}	4.50×10^{-6}	2.25×10^{-6}	4.50×10^{-6}
$\%T_4$ bd by 1st TBPA site	17.52	54.60	54.76	10.48	10.05	19.56
Total ALB mol/l	7.35×10^{-4}	7.35×10^{-4}	7.35×10^{-4}	7.35×10^{-4}	7.35×10^{-4}	3.68×10^{-4}
$\%T_4$ bd by 1st ALB site	10.45	32.86	32.66	6.25	11.99	5.84
$\%T_4$ bd by five secondary ALB sites	3.92	12.32	12.25	2.34	4.50	2.13

* From Prince and Ramsden, (1977).

EFFECT OF ALTERATIONS IN THYROXINE-BINDING PROTEIN CONCENTRATION ON THE DISTRIBUTION OF T_4

total hormone concentrations. The most important changes to note with respect to work to be presented in this thesis are that in the absence of TBG, with normal free T_4, a total T_4 concentration of 35 nmol/l is predicted and that a 50% reduction in TBPA causes an increase in free T_4 from 3.87×10^{-11} mol/l to 4.45×10^{-11} mol/l.

b) Genetic variations of thyroxine-binding proteins

i) TBG concentration

The phenomenon of genetically-determined variations in TBG concentration has been thoroughly investigated and reviewed (Refetoff et al, 1972; Robbins, 1973). The first family study of TBG elevation was thought to show an autosomal dominant inheritance (Beierwaltes and Robbins, 1959), but was also compatible with an X-linked dominant inheritance which was suggested by later studies (Nikolai and Seal, 1966). It is now known that there are three types of inherited TBG abnormality, resulting in TBG elevation, reduction or absence. As with other X-linked abnormalities, the males are most severely affected, possessing only an abnormal X-chromosome, while females are heterozygous and have intermediate TBG concentrations.

The abnormalities of TBG have been shown to result from altered concentration of an immunologically normal protein, and not due to defective T_4-binding (Hansen and Siersbæk-Nielsen, 1972; Levy et al, 1971; Refetoff et al, 1972). The defect has been shown to be an abnormality of TBG synthesis, and it has been suggested that the abnormalities result from mutations at a single genetic locus controlling TBG synthesis (Refetoff et al, 1976) (see Chapter 5). Robbins (1973) has produced alternative explanations, in particular that a somatic point mutation of the protein may occur and lead indirectly to alterations of synthetic rate; this has been shown to occur with glucose-6-phosphate dehydrogenase-Hektoen, which is one of the few proteins apart from TBG to undergo a genetic increase

(Yoshida, 1970).

The main physiological importance of the genetic absence and excess of TBG lies in the fact that affected individuals are clinically euthyroid, and, despite some reports to the contrary, free T_4 concentrations are probably normal (Robbins, 1973). In support of this, the TSH response to TRH injection has been found to be normal in TBG deficiency (Hansen et al, 1975, Konno, 1976). The normality of individuals with highly abnormal TBG concentrations, and the normal T_4 degradation rate in hereditary TBG decrease (Ingbar, 1961) and increase (Jones and Seal, 1967) constitute powerful evidence against any rôle of the T_4-TBG complex in thyroid hormone action.

ii) Microheterogeneity of TBG

Microheterogeneity of TBG was first elegantly demonstrated by Marshall and co-workers (1973), who described 9 bands in purified TBG subjected to isoelectric focusing (I.E.F). All bands bound $^{125}IT^4$, and persisted after desialylation. A difference in the number of bands was observed between TBG from pooled serum and from a single donor. The authors therefore felt that TBG exists in serum as a heterogeneous collection of proteins differing by subtle changes in amino acid composition.

The findings of Henze, Gartner and Horn (1979) are in complete contrast. They identified four TBG bands during IEF, and these were reduced to one by complete desialylation. Quantitative analysis revealed that the bands differed in their content of N-acetyl neuraminic (sialic) acid. Ramsden and Burnett (1980) have carried out IEF for TBG on microlitre quantities of serum from different donors, and have found multiple bands, which are reduced in number, but not abolished by desialylation. They feel that the persisting bands may represent true polypeptide microheterogeneity of TBG,

although incomplete desialylation would also explain the findings.

iii) Genetic polymorphism of TBPA

Genetic polymorphism of rhesus monkey prealbumin has already been mentioned (v.s. Chapter 1.4.a), because the five components of the intermediate variety of rhesus TBPA provided the first indication of the tetrameric structure of TBPA (Alper et al, 1969; Bernstein et al, 1970). The inheritance of the polymorphic forms of rhesus TBPA is autosomal codominant, and the two homozygous forms have been shown to at least differ by one amino acid in the 5 position from the N-terminal (Van Jaarsveld et al, 1973b). The different polymorphic forms of TBPA resulted in differing TBPA concentrations (Van Jaarsveld et al, 1973c), which is an interesting finding in view of the genetic variations of TBG concentration.

Polymorphism of TBPA has not been demonstrated in man (see Robbins, 1976 for review).

5) THE THYROXINE-BINDING PROTEINS IN DIFFERENT PATHOLOGICAL STATES

a) Thyroid disease

An increase in TBG concentration in myxoedema and a decrease in thyrotoxicosis was noted in early studies using capacitance methods (Inada and Sterling, 1967b). Direct measurements have tended to confirm these observations, when appropriate control populations are studied (see Chapter 3.1.e and 3.3 for detailed discussion).

The changes in TBPA capacity in thyroid disease were also studied by Inada and Sterling (1967b). No consistent change was found in myxoedema, but in active thyrotoxicosis, TBPA was invariably reduced, by a mean of approximately 40%.

B) Non-thyroidal illness

i) Acute and chronic illness

Studies carried out in a wide variety of different diseases, characterised mainly by weight loss, fever and debilitation (including leukaemias, carcinomatosis, tuberculosis and a miscellany of acute and chronic illness), showed diminished protein binding of thyroxine, evidenced by an increased dialysable fraction (Richards, Dowling and Ingbar, 1959; Oppenheimer et al, 1963; Ingbar et al, 1965). This was initially attributed to a reduced concentration of TBPA (Oppenheimer et al, 1963) although subsequent studies showed that TBG also was reduced, especially in chronic, severe illness (Bellabarba et al, 1968; Harvey, 1971). Kinetic studies with purified TBPA demonstrated that it had a very short half life of only 2 - 3 days, and that serum TBPA underwent a rapid fall in a variety of stressful situations as a result of reduced synthesis (Socolow et al, 1965; Oppenheimer et al, 1965). Since T_4 disposal rate was shown to be increased five-fold during fever (Gregerman and Solomon, 1967) and T_4 disposal correlated strongly with measured free T_4 (Bellabarba et al, 1968), the above was taken to be strong evidence in favour of the control of T_4 metabolism by changes in TBPA, and to a lesser extent TBG. The sequential studies of Lutz et al (1972), during experimental acute infections demonstrated, however, that changes in % FT_4 and free T_4 concentration were not related in time to acute alterations of TBPA and TBG concentration. These authors suggested instead the appearance of inhibitors of T_4 binding during infection.

More recent studies of the effect of non-thyroidal illness on thyroid function have focussed on the peripheral metabolism of T_4. Following the initial report of Chopra et al (1974) of low serum T_3 concentrations in hepatic cirrhosis, this finding has been noted in a wide variety of acute and chronic illnesses (Carter et al, 1974; Bermudez, Surks and Oppenheimer,

1975; McLarty et al, 1975). It has further been demonstrated that the monodeiodination of T_4 may be either from the phenolic ring to yield 3,3',5,-triiodothyronine (T_3) or from the inner tyrosyl ring to yield 3,3',5'-triiodothyronine (reverse T_3,rT_3), which is thought to be metabolically inactive (Pittman, Brown and Register, 1962). Illnesses and acute stresses causing low T_3 have almost always been shown to result in reciprocal elevation of rT_3 (Chopra et al, 1975a, Burr et al, 1975; 1976; Burger et al, 1976).

Kinetic studies are consistent with the idea that the changes result from inhibition of thyronine 5'-deiodinase (Chopra, Sack and Fisher, 1975; Vagenakis et al, 1977; Visser, 1978), which leads to reduced 3,3',5-triiodothyronine (T_3) production and 3,3',5'-triiodothyronine (rT_3) degradation.

It is not known whether the thyroxine-binding proteins have any function in mediating the changes described above, but current thinking would hold that they do not have any rôle and that the changes are more likely to result from subtle changes of the redox state and availability of -SH groups within cells (Visser, 1978; Harris et al, 1979).

The changes in thyroid hormones and binding proteins attributable to non-thyroidal illness are summarised in Table 1.5.

ii) Surgery

Surgery results in a decrease in TBPA binding capacity (Surks and Oppenheimer, 1964; Kirby, Clark and Johnston, 1973) and concentration (Minchin-Clarke et al, 1971; Aronsen et al, 1972). The fall is apparent within a few hours of surgery (Surks and Oppenheimer, 1964) and is maximal on about the third postoperative day, when concentrations of 50% of the preoperative concentration have been noted after uncomplicated abdominal

Table 1.5

	Acute and chronic illness	Febrile illness	Surgery
T_4	↓ (10-15) ↓↓ in severe illness	→	Variable - see text
% Free T_4	↑ (80)	↑ (25-100)	↑ (50-60)
Abs. Free T_4	↑ (40-50)	↑ (50)	↑ (40-60)
T_3	↓ (15-80)	↓ (30-50)	↓ (30)
% Free T_3	↑ (40)	↑ (80)	↑ (20)
Abs. free T_3	→ ↓ (0-50)	↓ (30-50)	↓ (50)
rT_3	↑ (180)	↑ (80)	↑ (70-140)
TBG	↓ (15-25)	→	→
TBPA	↓ (50)	↓ (50)	↓ (50-70)
Alb.	↓ (20)	↓	↓ (15-20)
T_4 Production rate	→	↑ (500)	↑ (20)
Urine T_4	-	-	↑ (100)
Urine T_3	-	-	
TSH	→ or ↓	→	→ or ↓

See Appendix A for references on which this table was based.

THE EFFECTS OF ILLNESS AND SURGERY ON THYROID HORMONES AND BINDING PROTEINS

(Figures in parentheses refer to approximate percentage change)

operations (Surks and Oppenheimer, 1964; Minchin-Clarke et al, 1971; Aronsen et al, 1972). As in other types of illness, the fall in TBPA is thought to be caused by reduced synthesis (Socolow et al, 1965; Oppenheimer et al, 1965). A postoperative increase in free T_4 (Surks and Oppenheimer, 1964) and percentage free T_3 (Kirby et al, 1973) has been attributed to decreased TBPA concentration.

Serum TBG concentrations have not been found to alter after uncomplicated surgery (Surks and Oppenheimer, 1964; Kirby et al, 1973).

Thyroid hormone changes after surgery are basically similar to those occurring after other types of illness (Table 1.5). There is a reduction of serum TBPA, which may or may not be enough to account for increases in the percentages of free T_4 and free T_3. Serum total T_4 concentrations have been variously reported to be unchanged (Kirby et al, 1973; Ramsden et al, 1978), slightly reduced (Burr et al, 1975; Chan, Wang and Yeung, 1978; Kehlet, Klauber and Weeke, 1979) or even increased (Prescott et al, 1979). The differences may relate to differences in anaesthetic agent, or to postoperative fluid replacement. Peroperative T_4 changes have certainly been highly variable, perhaps as a result of T_4 displacement by some anaesthetic agents (Oyama, 1973).

After operation, T_4 metabolism is altered as in other types of illness, so that total T_3 falls, and free T_3 is reduced in spite of increased percentage free T_3. Serum rT_3 increases, suggesting altered peripheral monodeiodination of T_4 (Burr et al, 1975; Chan et al, 1978; Kehlet et al, 1979; Prescott et al, 1979).

iii) Malnutrition and fasting

Both TBPA and TBG are reduced in protein-calorie malnutrition

(Ingenbleek, deVisscher and deNayer, 1972; Ingenbleek, deNayer and deVisscher, 1974). The concentration of TBPA falls rapidly during fasting (~ 30% fall in one week) (Frey et al, 1979; Shetty et al, 1979) - perhaps because of its high tryptophan content and short biological half-life (Ingenbleek et al, 1975). It has been suggested that TBPA (and RBP) measurement may be useful in the assessment of protein-calorie malnutrition (Ingenbleek et al, 1975) and of sub-clinical malnutrition (Shetty et al, 1979), although such proposals disregard the non-specific nature of changes in TBPA concentration (Minchin-Clarke, Freeman and Pryse-Phillips, 1971; Johansson et al, 1972). TBG undergoes a gradual fall during total fasting for 7 - 15 days (Scriba et al, 1979) and after calorie restriction for two weeks (Moreira-Andres et al, 1980).

It should also be mentioned at this point that fasting induces similar changes in peripheral T_4 monodeiodination to those mentioned in section 1.5.b.i., with low T_3 and increased rT_3 concentration (Rothenbuchner et al, 1973; Vagenakis et al, 1975; Danforth et al, 1975).

iv) Hepatic disease

Abnormalities of PBI measurements were observed many years ago in patients with a variety of hepatic diseases. TBG-capacity measurements were subsequently shown to be very variable - either low, normal or raised, thus explaining the variation of PBI (Kydd and Man, 1951, Vanotti and Béraud, 1959; Inada and Sterling, 1967a). TBG becomes elevated early in the course of viral hepatitis (Vanotti and Béraud, 1959), and high concentrations have also been noted in chronic active hepatitis and primary biliary cirrhosis (Schussler, Schaffner and Korn, 1978). In cirrhosis of the liver TBG capacity was found to be reduced (Inada and Sterling, 1967a), while a relative increase in desialylated TBG has been observed and attributed to impaired removal of partially-denatured TBG (Marshall et al,

1972; Gartner et al, 1979). The alterations in TBG concentration in hepatic disease have not been fully explained, but the variable levels are presumably due to different combinations of impaired synthesis, impaired degradation, and possibly increased synthesis secondary to high oestrogen levels.

TBPA capacity in hepatic disease, as in other chronic illness, has been found to be low (Inada and Sterling, 1967a).

v) Renal disease

Abnormalities of the thyroxine-binding proteins in nephrosis were first demonstrated by Robbins and co-workers (Robbins et al, 1957), who found low TBG-capacity in 5 of 7 patients with the nephrotic syndrome. However, most of these patients were receiving adrenal corticosteroids, which are now known to lower serum TBG (vide infra, Chapter 1.5.c). Other workers have reported normal TBG levels in nephrosis (Musa, Seal and Doe, 1967), and recently Gavin et al (1978) clearly showed that serum TBG concentrations in the nephrotic syndrome are normal in all but the most extreme cases, in which there is gross proteinuria.

In renal failure without nephrosis, TBG concentrations have only been assessed by capacitance measurements, or by indirect methods such as resin uptake tests. These assays might in theory be unreliable in renal failure, because of the presence of indoles and phenols which could interfere with T_4 binding (Spaulding and Gregerman, 1972). In fact, most reports suggest that TBG concentration is normal in renal failure (Oddie et al, 1970; Neuhaus et al, 1975; Ramirez et al, 1976; Hershman et al, 1978), although in two studies, a low concentration was found (Joasoo et al, 1974; Lim et al, 1977). It would appear that reduction of TBG concentration in uraemia is not sufficient to explain the low T_4 concentrations reported by many investigators (Musa et al, 1967; Silverberg et al, 1973; Joasoo et al, 1974,

Ramirez et al, 1976; Spector et al, 1976; Lim et al, 1977, Hershman et al, 1973; Gomez-Pan et al, 1979). An unexplained feature of renal failure is that patients remain clinically euthyroid in spite of apparently low total and free thyroid hormone levels (Spector et al, 1976).

There have been very few studies of TBPA metabolism in renal failure, but Vahlquist, Peterson and Wibell (1973) reported that serum TBPA concentration, metabolic clearance and production rates were all normal in chronic renal failure.

c) Drugs and hormones

The increase in TBG concentration following oestrogen administration is now well-established (Dowling, Freinkel and Ingbar, 1956a; Engbring and Engstrom, 1959) and oestrogen is thought to be responsible for the equally well-established increase of TBG concentration in pregnancy (Dowling, Freinkel and Ingbar, 1956b). These observations have been recently given a sound physiological basis by the work of Glinoer et al (1977a, b) showing increased hepatic synthesis of TBG in response to oestrogen. Oestrogens and pregnancy cause a slight reduction in TBPA concentration (Oppenheimer et al, 1963; Sakurada et al, 1967). Androgens and anabolic steroids have the effect of decreasing TBG concentration, and increasing TBPA concentration (Federman, Robbins and Rall, 1958; Braverman and Ingbar, 1967), and adrenal corticosteroids produce similar effects (Oppenheimer and Werner, 1966) which are apparently mediated via decreased TBG synthesis and increased TBPA synthesis (Braverman and Ingbar, 1967; Braverman et al, 1968).

Other conditions and drugs associated with TBP changes are shown in Table 1.6. The most recent, and intriguing addition to this list is L-asparaginase (Garnick and Larsen, 1979), the anti-leukaemic agent, which apparently inhibits TBG synthesis almost completely within a few hours of

Table 1.6[1]

	INCREASE	DECREASE
TBG	PREGNANCY	PROTEIN-CALORIE MALNUTRITION and FASTING
	HEPATIC DISEASE: Viral hepatitis	MAJOR ILLNESS
	Chronic active hepatitis	CIRRHOSIS
	Primary biliary cirrhosis	NEPHROTIC SYNDROME
	MYXOEDEMA	ENDOCRINE DISEASE: Thyrotoxicosis
	DRUGS: Oestrogens	Acromegaly
	Phenothiazines	Cushing's syndrome
	Clofibrate	DRUGS: Androgens
	Methadone[2]	Anabolic steroids
	ACUTE PORPHYRIA	L-Asparaginase
	ANALBUMINAEMIA	{Phenytoin, Salicylates}[3]
	GENETIC	MALES
	AGE < 5 years	
	FEMALES	
TBPA	ENDOCRINE DISEASE: Acromegaly	ACUTE OR CHRONIC ILLNESS
	Cushing's syndrome	SURGERY
	DRUGS: Corticosteroids	PROTEIN-CALORIE MALNUTRITION and FASTING
	Androgens	THYROTOXICOSIS
	Anabolic steroids	

1. Based on Oppenheimer (1968), additions referenced in text.
2. TBG increase in drug addicts may be due to liver dysfunction.
3. Low T_4 and TBG-capacity, probably due to competition for T_4-binding site. TBG concentration normal.

FACTORS CAUSING ALTERATIONS IN THYROXINE-BINDING PROTEIN CONCENTRATION

administration.

6) INDICATIONS FOR THE PRESENT STUDY

a) Assay of TBG and TBPA

Early attempts to quantitate the thyroxine binding proteins relied upon binding-capacitance assays (v.s. Chapter 1.4.a.ii.). Not only were these assays time-consuming and cumbersome to perform - so that they were not suited to the processing of large numbers of samples - but they were also open to the criticism that they were not a direct measure of protein concentration and could be subject to interference by inhibitors of T_4 binding. Thus, although pregnancy was known to increase TBG-capacity, it was not known for certain whether this was due to a quantitative increase in protein, or to an alteration of the protein, causing increased T_4-binding affinity, or an increased number of binding sites.

To circumvent these problems, an assay was required for TBG which was direct, simple, rapid and reproducible. When the present study commenced, several direct assays for TBG had been described (Freeman and Pearson, 1969; Nielsen et al, 1972), but these were cumbersome, time-consuming and involved handling relatively large quantities of radioactivity. A radioimmunoassay for TBG had been described (Levy et al, 1971), but because of the difficulties involved in purifying TBG (v.s. Chapter 1.2) it was not known to what extent this assay might be subject to errors due to impurity or denaturation of TBG.

A major aim of this project was therefore to develop suitable assays for TBG and TBPA.

b) Rôle of TBG and TBPA

The second aim of this study was to gain further information concerning

the function of thyroxine-binding proteins, particularly in relation to changes in thyroid hormone metabolism and alterations in free hormone concentration. The thyroid hormones and binding proteins were therefore measured in patients before, during and after non-thyroidal surgery. Earlier studies had shown that this stress would result in a reduction in serum TBPA (see chapter 1.5.b.ii).

Profound alterations of TBPA were observed, together with lesser changes of TBG. Serum T_3 was markedly reduced, and there were also changes in free hormone concentration. The study was therefore extended, in order to discover whether the findings were specific to surgery, or were applicable in general to patients with non-thyroidal illness. A group of patients with recent myocardial infarction were selected, and, as a final control group, a series of obese patients undergoing therapeutic starvation were studied.

c) Use of TBG assay in the assessment of thyroid function

With increasing use of the TBG assay a considerable number of patients were identified with absent, low or increased TBG concentration. In general, these patients were fit, euthyroid and on no drug therapy. They were shown to have hereditary abnormalities of TBG concentration. Routine thyroid function tests in these people were frequently abnormal, predicting either myxoedema or thyrotoxicosis when individuals had low and high TBG respectively.

In the final part of this study, the $^{125}IT_3$-uptake test was tested for its ability to measure low and high concentrations of TBG. Certain limitations were identified, and the direct measurement of TBG was evaluated as a replacement for T_3-uptake tests in the assessment of thyroid function.

Chapter 2 MATERIALS AND METHODS

1). Materials

2). Methods

A Qualitative

i) Two-dimensional immunoelectrophoresis

ii) Autoradiography

iii) Analytical polyacrylamide gel electrophoresis

B Preparative

i) Fractionation of TBG and TBPA from human serum

ii) Preparation of antisera

a) anti-TBG

b) anti-TBPA

C Development of assays

i) Autoradiographic assay for TBG

ii) Monospecific anti-TBG assay for TBG

iii) TBPA assay

iv) Albumin assay

D Hormone assays

i) T_4 and T_3

ii) Reverse T_3

iii) TSH

iv) Free T_3 and T_4

v) T_3 uptake

Chapter 2 MATERIALS AND METHODS

1) MATERIALS

Sephadex G100 and G150 was obtained from Pharmacia (Great Britain) Ltd., London.

Diethyl aminoethyl cellulose (DEAE cellulose) grade DE32 from Whatman Biochemical Ltd., Maidstone, U.K., prepared according to the manufacturers instructions.

Agarose was obtained from British Drug Houses (BDH), Poole, U.K. or L'Industrie Biologique Francaise, Gennevilliers, France.

Radioiodine and radioiodinated T_4 from the Radiochemical Centre, Amersham, U.K.; labelled T_4 and T_3 was also prepared in the laboratory according to the method of Weeke and Örskow (1973), and high specific activity $I^{125}T_3$ was obtained from Abbott Laboratories, Queensborough, Kent.

Coomassie Blue and Triethyl-aminoethyl cellulose (TEAE) were obtained from Sigma Chemical Company Ltd., Kingston-upon-Thames, U.K.

The Department of Experimental Pathology, Birmingham University, kindly provided Freund's complete adjuvant and sheep anti-whole human serum (Dr. A. R. Bradwell).

Other antisera were obtained from Wellcome Diagnostic Reagents Ltd., London (sheep anti-whole human serum) and Dakopatts (rabbit anti-human prealbumin), British agents for Dakopatts are Mercia Diagnostics Ltd., Watford, U.K.

X-ray film was obtained from Kodak U.K. Ltd., London (RP/LX-Omat) or Ilford Ltd., Ilford, U.K. (Rapid R, type S), and was developed in the X-ray Department, Queen Elizabeth Hospital, Birmingham, using an X-Omat Processor Model M 6-N (Kodak U.K. Ltd.) by courtesy of Dr. O. E. Smith.

Visking dialysis tubing was obtained from Scientific Supplies Company Ltd., London, ultrafiltration membranes, cells and concentrators ("Minicon") from Amicon Ltd., High Wycombe, U.K. and Millex disposable filters from Millipore (U.K.) Ltd., London.

A heated levelling plate was manufactured in the workshops of the Department of Medicine, Birmingham University, and a sample dispenser for use with a Hamilton 700 Series microlitre syringe and Chaney Adaptor (Hamilton Micromesure N.V. - U.K. Agents V. A. Howe and Co. Ltd., London) was purchased from the Department of Experimental Pathology, University of Birmingham.

Glass electrophoresis plates (24 x 8 cms) were obtained from Pearce and Cutler Ltd., Birmingham, U.K. and from A. Gallenkamp Co. Ltd., Birmingham, U. K. (76 x 50 mm).

Direct current power equipment, type BES-2 was manufactured by PCD Ltd., Farnborough, U.K. and electrophoresis tanks were obtained from Chem Lab Instruments Ltd., Ilford, U.K., or were constructed in the Department of Medicine, Birmingham University.

Glass-fibre paper (Whatman GF/A) and ashless tablets (Whatman) were obtained from A. Gallenkamp Co. Ltd., Birmingham, U. K.

All other chemicals used were obtained either from BDH Ltd., Poole, U.K. or Hopkins and Williams, Chadwell Heath, U.K.

2) METHODS

Although TBG measurements were initially made with an immunoelectrophoretic technique utilising antiserum to whole human serum and radioautography (see Chapter 2.2.c.i.) assays for both TBG and TBPA were eventually based on monospecific antisera. These antisera were prepared in the Department of Medicine, and their production required preliminary purification of TBPA and TBG. The qualitative and quantitative methods to be described were carried out in collaboration with Dr. H. P. Prince.

A) QUALITATIVE METHODS

i) Two-Dimensional immunoelectrophoresis (2-DIEP)

This technique was first described by Laurell (Laurell, 1965) and is frequently referred to by his name. In the first dimension, the proteins are separated according to their electrophoretic mobilities by electrophoresis at relatively high voltages in agarose gel. A second electrophoresis, carried out at 90° to the first, and at lower voltage, forces the proteins into a bed of agarose gel containing antiserum. Immunoprecipitation occurs when protein and antiserum are present in equivalent amounts. The initial precipitate is soluble in antigen excess, and a precipitation line is formed which advances into the antiserum gel until the supply of antigen is exhausted. Gaussian, or near-Gaussian peaks are formed (Fig. 2.1), whose area is directly proportional to the amount of antigen in the sample.

The proteins within a sample may be identified by their electrophoretic mobility, specific staining characteristics, by their ability to form precipitation lines with known added antigens, and in the case of transport proteins, by their ability to bind radiolabelled ligands.

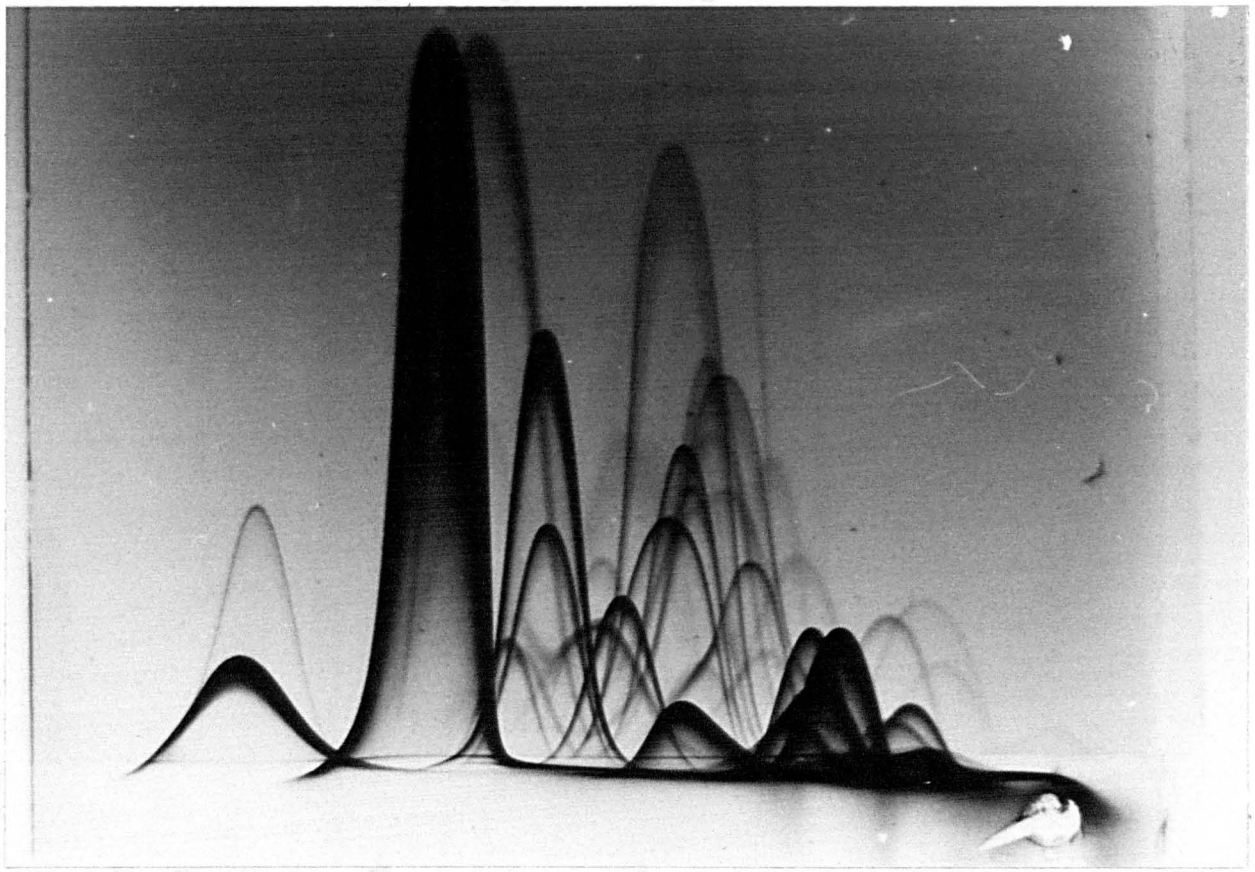


Figure 2.1

Two dimensional immuno-electrophoresis of whole human serum into antiserum to whole human serum, plate stained with Coomassie Brilliant Blue to show near-Gaussian peaks of individual proteins separated according to electrophoretic mobility.

The first dimension electrophoresis was performed under refrigeration (+5°C) at 10 V/cm in 60 mM barbitone buffer, pH 8.6, containing 3mM sodium azide as preservative. The support consisted of a 1% agarose gel strip, 1 mm x 1 cm x 5 cm, in which a 2 mm origin well had been cut with a sharp-edged metal punch. The progress of the electrophoresis was monitored by using 1% bromophenol blue in water as a marker.

After completion of the first electrophoresis, the agarose strip was carefully aligned at the base of a 76 x 50 mm slide, onto which was then poured a 1 mm layer of 1% agarose containing antiserum. The second dimension electrophoresis was then carried out overnight, at room temperature at 2 V/cm. The plates were then dried with filter paper and paper towels, under pressure and without washing. They were stained with 0.5% Coomassie Brilliant blue in a mixture of glacial acetic acid:methanol: water (1 Vol:5 Vols:5 Vols).

ii) Autoradiography

For experiments involving the purification and quantitation of TBG and TBPA, the protein peaks were identified by autoradiography following labelling with ^{125}I-T_4 (Freeman and Pearson, 1969). Radiolabelled T_4 was added prior to electrophoresis (Nielsen, Buus and Weeke, 1972), but problems were then encountered due to stripping of T_4 from the binding proteins when barbital buffer was used (Drysdale et al, 1975). The problems were overcome by using 0.05M phosphate buffer at pH 7.4, which improved labelling of TBPA and TBG. However, this was achieved at the expense of a considerable increase in electroendosmosis due to negatively charged agaropectins contained in the agarose gel (Grubb, 1973). Electroendosmosis was reduced by removal of these negatively charged molecules using TEAE-cellulose as an insoluble adsorbent.

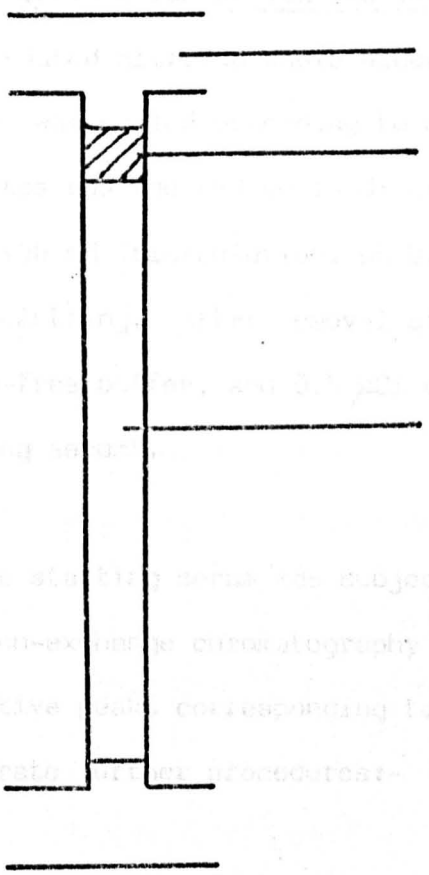
A 2% solution of agarose in distilled water was made up and placed on a heated stirrer. An aqueous slurry of Triethylaminoethyl cellulose (TEAE-containing sufficient to make a final concentration of 10 g/l) was added, and the mixture stirred rapidly. TEAE-cellulose and adsorbed materials were removed by hot vacuum filtration through glass-fibre paper (Whatman GF/A) and a suspension of ashless cellulose filter tablets (Whatman). This procedure was found to reduce electroendosmosis (measured by migration of Dextran 70) from 1.2 mm/V/hr to 0.6 mm/V/hr.

Autoradiography was carried out before or after drying of the electrophoresis plates according to requirements. In the former case, the gel was covered with a polythene film and placed in contact with the X-ray film. Dry plates were placed with gel facing the film, and were then placed in an ordinary X-ray cassette. Autoradiography was carried out at -70°C to increase the sensitivity of the X-ray film, and exposure lasted from 1 - 7 days, after which the film was automatically developed in the X-O mat M-6N Processor.

iii) Analytical polyacrylamide gel electrophoresis

This was carried out in a Labora serial disc electrophoresis apparatus (Linton Instrumentation, Harlow, U.K.), as shown in figure 2.2, which also shows the buffer compositions. Gels consisted of acrylamide together with 5% NN'-methylenebisacrylamide as a crosslinking agent. 25 µl of a 0.5 mg/ml solution of protein(s) was applied to the starter gel and electrophoresed for 45 minutes at a constant electrical potential of 120 V. Gels were stained in a 0.1% solution of amido black in 1.2 M acetic acid, and washed overnight with 1.2 M acetic acid in which they were also stored. Autoradiography of gels was carried out after prior labelling with $[^{131}I]T_4$.

Figure 2.2



UPPER BUFFER = LOWER BUFFER

Glycerine (384 mM)/Tris, pH 8.2

Upper gel buffer:

Tris (56 mM)/phosphate pH, 7.0

Lower gel buffer:

Tris (375 mM)/Chloride, pH 9.5

Running pH 9.2 (after equilibration)

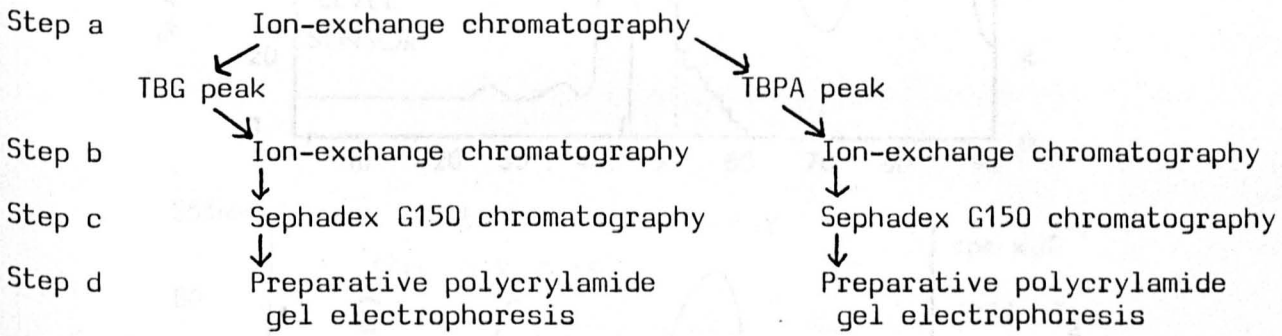
LABORA DISC-GEL APPARATUS

B) PREPARATIVE METHODS

i) Fractionation of TBG and TBPA from human serum

Outdated citrated whole blood, obtained from the Blood Transfusion Service, was pooled according to blood group, centrifuged at 1000 G for 15 minutes and the red cells discarded. The plasma was dialysed against buffer (50 mM Tris/chloride, pH 8.0) containing 10 mM calcium chloride to induce clotting. After removal of the clot, serum was dialysed against calcium-free buffer, and 0.5 μCi of $^{125}I\text{-}T_4$ was added. This constituted "starting serum".

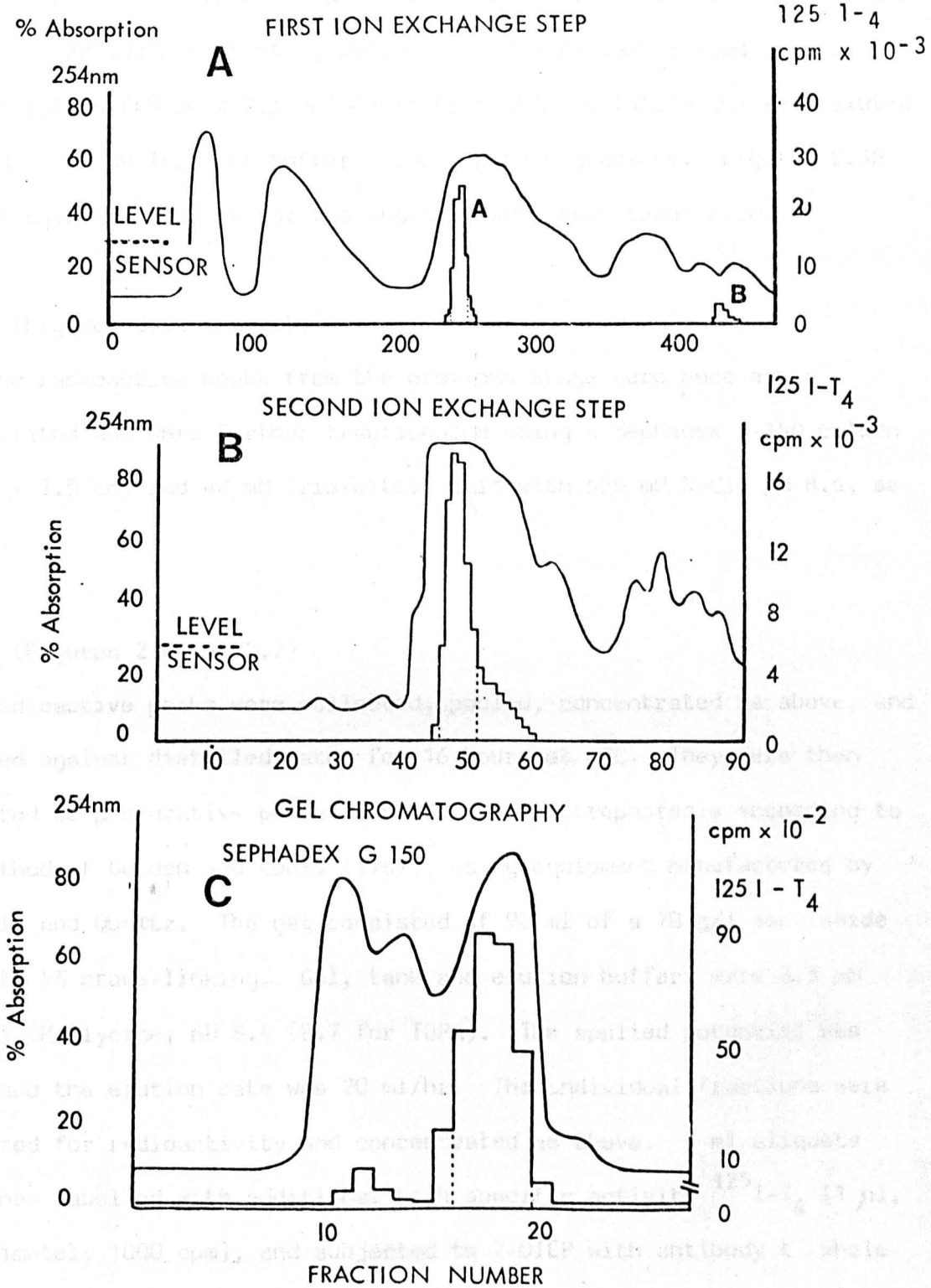
The starting serum was subjected to a preliminary fractionation using ion-exchange chromatography (step a). This produced separate radioactive peaks corresponding to TBG and TBPA and these were subjected to separate further procedures:-



Step a (Figure 2.3A)

The first ion-exchange chromatography was carried out on a DEAE cellulose column (60 cm x 5 cm) using increasing concentrations of 50 mM - 500 mM Tris/HCL buffer, pH 8.0, for elution. A linear concentration gradient generated by an LKB Ultrograd (LKB Instruments Ltd., South Croydon, U.K.) formed the basic elution gradient but this gradient was varied using an LKB Level Sensor, preset to hold the buffer concentration when effluent transmittance was $<$ 65% of starting buffer transmittance.

Figures 2.3.A,B,C.



CHROMATOGRAPHIC PURIFICATION OF TBG

see text for details

Step b

The TBG and TBPA peaks ("A" and "B" respectively in figure 2.3A) were identified by radioactivity and were separately pooled. They were concentrated in an Amicon ultrafiltration cell with a UM-10 membrane, and were dialysed against starting buffer. They were then loaded onto a smaller column (40 cm x 2.5 cm) containing DEAE cellulose and were eluted with 50 - 500 mM Tris/HCL buffer using a preset gradient. Figures 2.3B and 2.4 show the results for the TBG and TBPA peaks respectively.

Step c (Figures 2.3C and 2.5)

The radioactive peaks from the previous stage were once again concentrated and were further fractionated using a Sephadex G-150 column (90 cm x 2.5 cm) and 40 mM Tris/citric acid with 550 mM NaCl, pH 8.6, as eluant.

Step d (Figures 2.6 and 2.7)

Radioactive peaks were collected, pooled, concentrated as above, and dialysed against distilled water for 16 hours at 4°C. They were then subjected to preparative polyacrylamide gel electrophoresis according to the method of Gordon and Louis (1967), using equipment manufactured by Quickfit and Quartz. The gel consisted of 90 ml of a 70 g/l acrylamide gel with 5% cross-linking. Gel, tank and elution buffers were 8.3 mM Tris/25 mM glycine, pH 8.4 (8.7 for TBPA). The applied potential was 250 V and the elution rate was 20 ml/hr. The individual fractions were monitored for radioactivity and concentrated as above. 5 ml aliquots were then labelled with additional high specific activity ^{125}I-T_4 (1 μl, approximately 1000 cpm), and subjected to 2-DIEP with antibody to whole human serum (TBPA) or antibody to the α_1 fraction of human serum (TBG - obtained from the Department of Experimental Pathology, University of Birmingham). The plates were subjected to autoradiography while still wet,

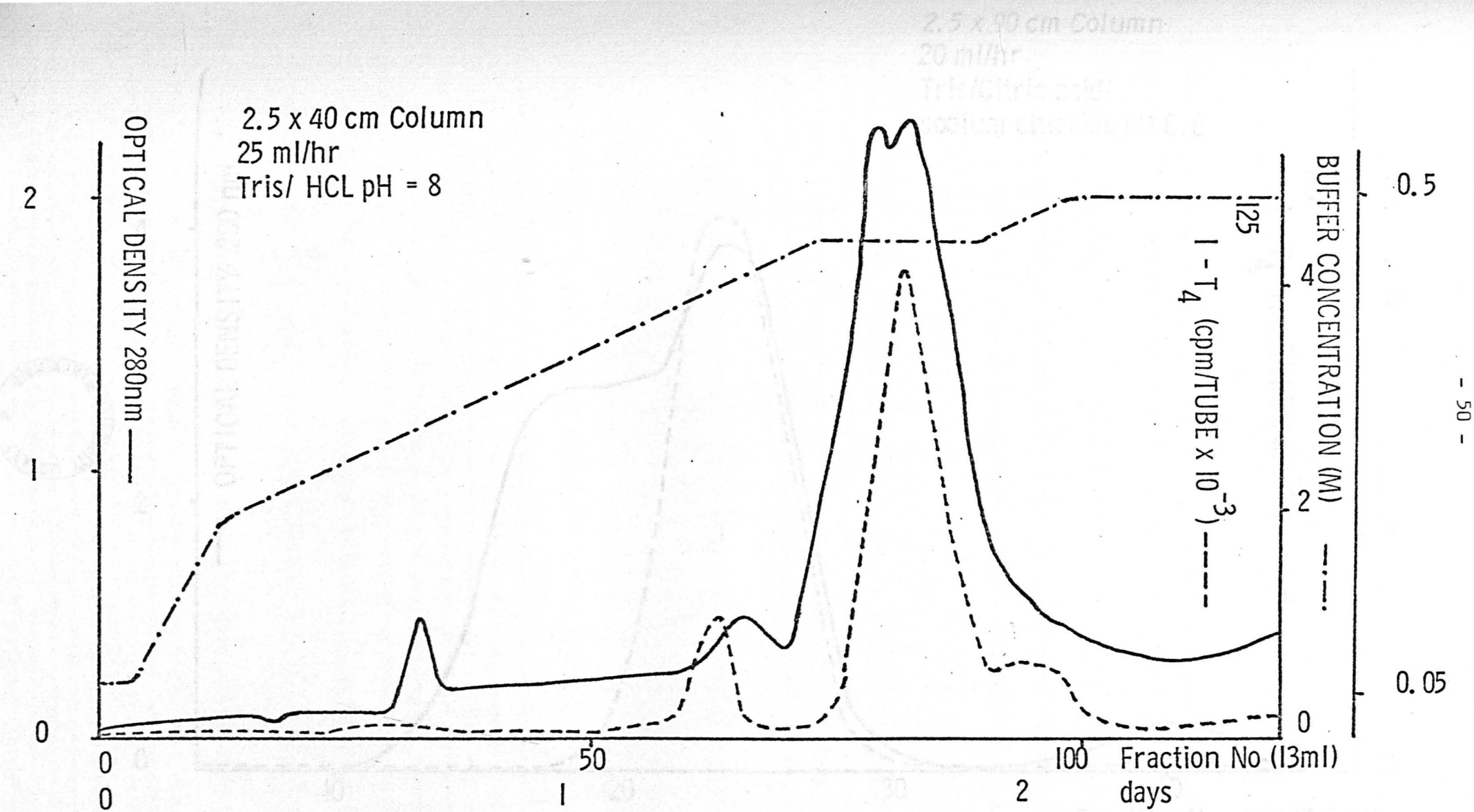


Figure 2.4 PURIFICATION OF TBPA BY ION-EXCHANGE CHROMATOGRAPHY WITH DEAE-CELLULOSE

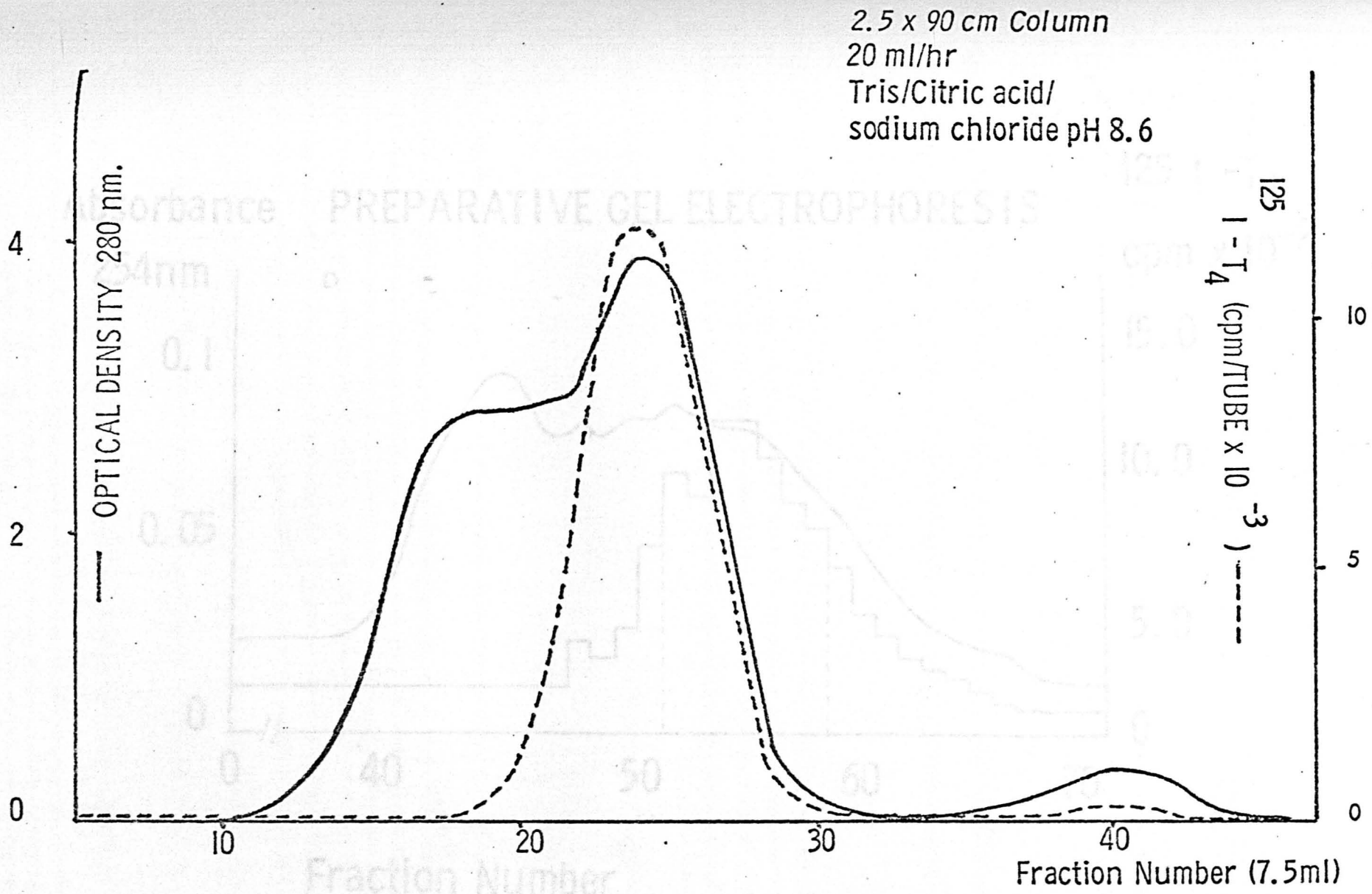


Figure 2.5 PURIFICATION OF TBPA BY GEL-FILTRATION WITH SEPHADEX G-150

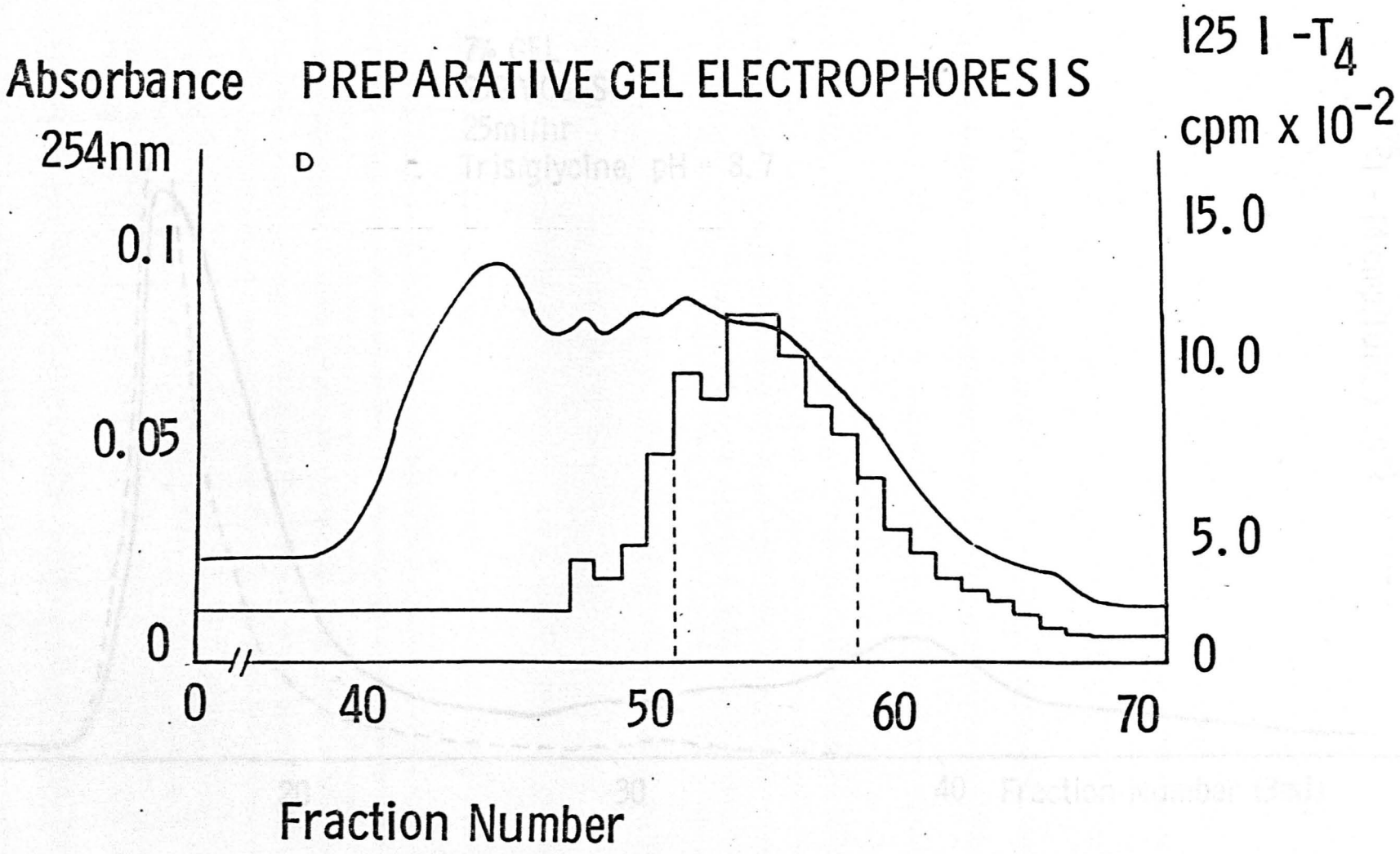


Figure 2.6 TBG PURIFICATION:- ABSORBANCE = CONTINUOUS LINE

RADIOACTIVITY = HISTOGRAM

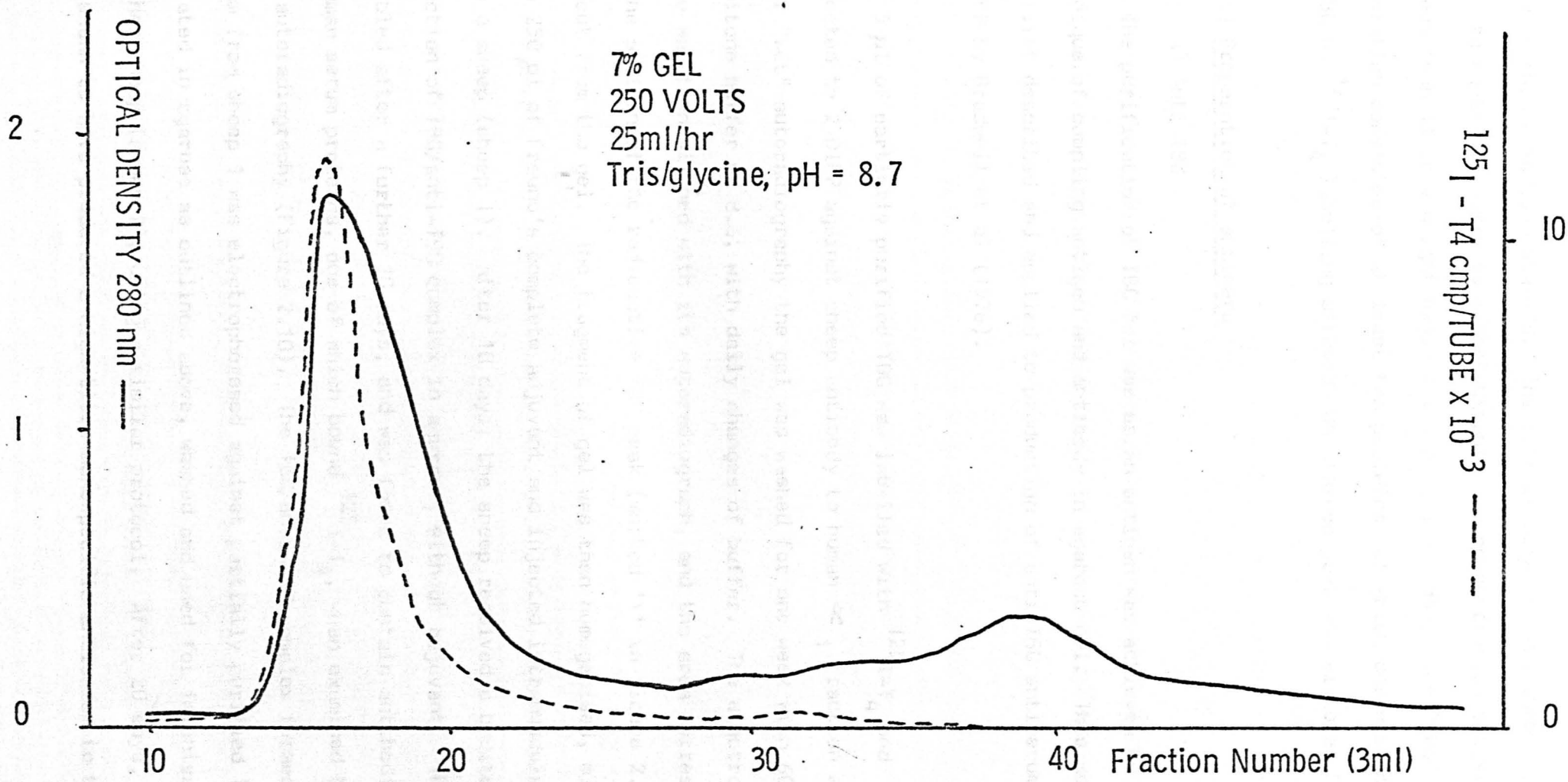


Figure 2.7 TBPA PURIFICATION:- PREPARATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS

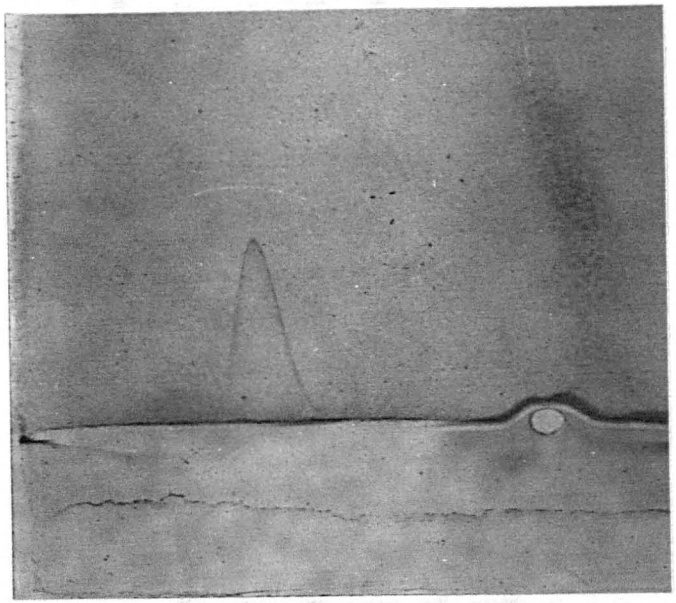
and were then dried and stained. The TBPA preparation was shown by these means to contain albumin as a contaminant in a concentration estimated to be less than 1% on a weight basis (Fig. 2.8a and 2.8b). The "TBG" preparation consisted of at least ten proteins, of which one was shown to be TBG by $^{125}I\text{-}T_4$ labelling although no stained peak was visible (Fig. 2.9).

ii) Preparation of antisera

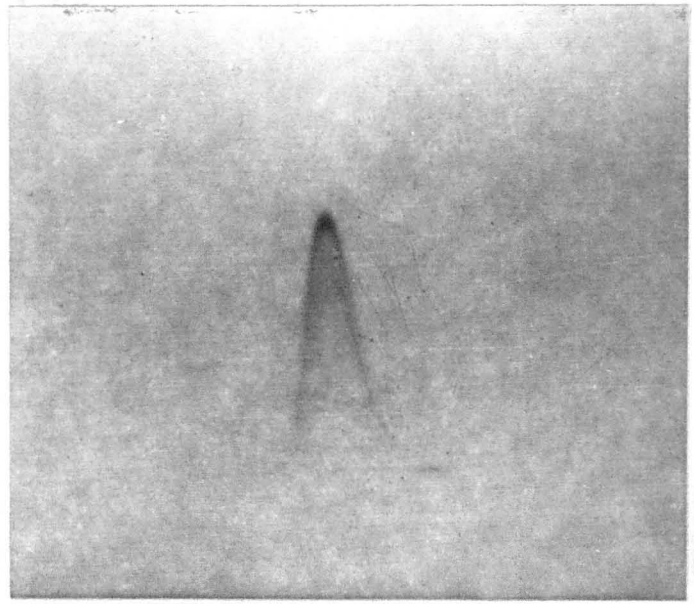
a) Anti-TBG

The purification of TBG for use as an antigen was achieved by the technique of coupling antigen and antibody in agarose gel. This method was first described and applied to production of anti-TBG antiserum using 2-DIEP by Bradwell et al (1976).

5 µl of partially purified TBG was labelled with $^{125}I\text{-}T_4$ and subjected to 2-DIEP against sheep antibody to human α_1 fraction as above. After "wet" autoradiography the gel was washed for one week with 60 mM barbitone buffer pH 8.6, with daily changes of buffer. The electrophoresis plate was then aligned with its autoradiograph, and the area corresponding to the positon of the radioactive TBG peak (marked 'Y' in figure 2.9A) was cut from the gel. The fragment of gel was then homogenised, mixed with 250 µl of Freund's complete adjuvant and injected intramuscularly into a sheep (sheep 1). After 10 days, the sheep received a booster injection of TBG/anti-TBG complex in agarose, without adjuvant. The sheep was bled after a further 10 days, and was found to contain antibodies to 2 human serum proteins, one of which bound $^{125}I\text{-}T_4$, when examined by 2 DIEP and autoradiography (Figure 2.10). The TBG/anti-TBG complex formed when serum from sheep 1 was electrophoresed against partially purified TBG was isolated in agarose as outlined above, washed and used for immunisation of another sheep (sheep 2), using a similar protocol. After 20 days, sheep 2 was shown to have produced a high-titre monospecific antiserum to human



a



b

Figure 2.8.a. & b.

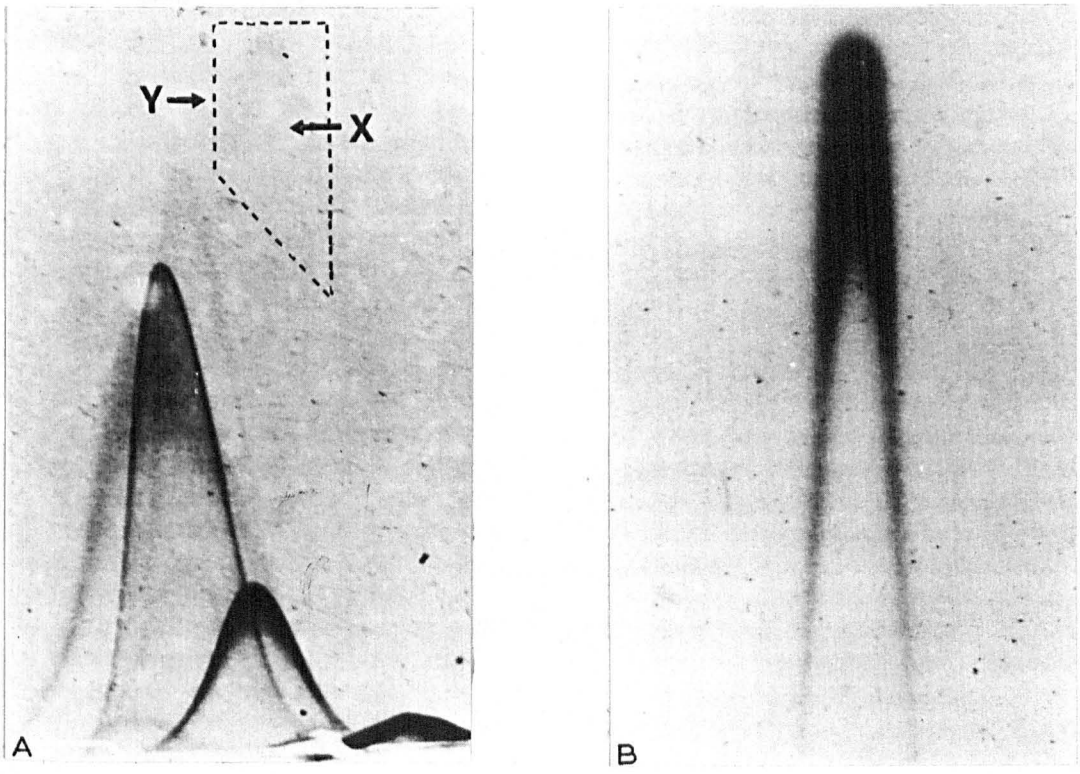
2 DIEP plate of purified TBPA against antibody to whole human serum:

a) stained plate

b) autoradiograph.

Albumin contaminant is not visible on stained plate, but can just be seen on the autoradiograph.

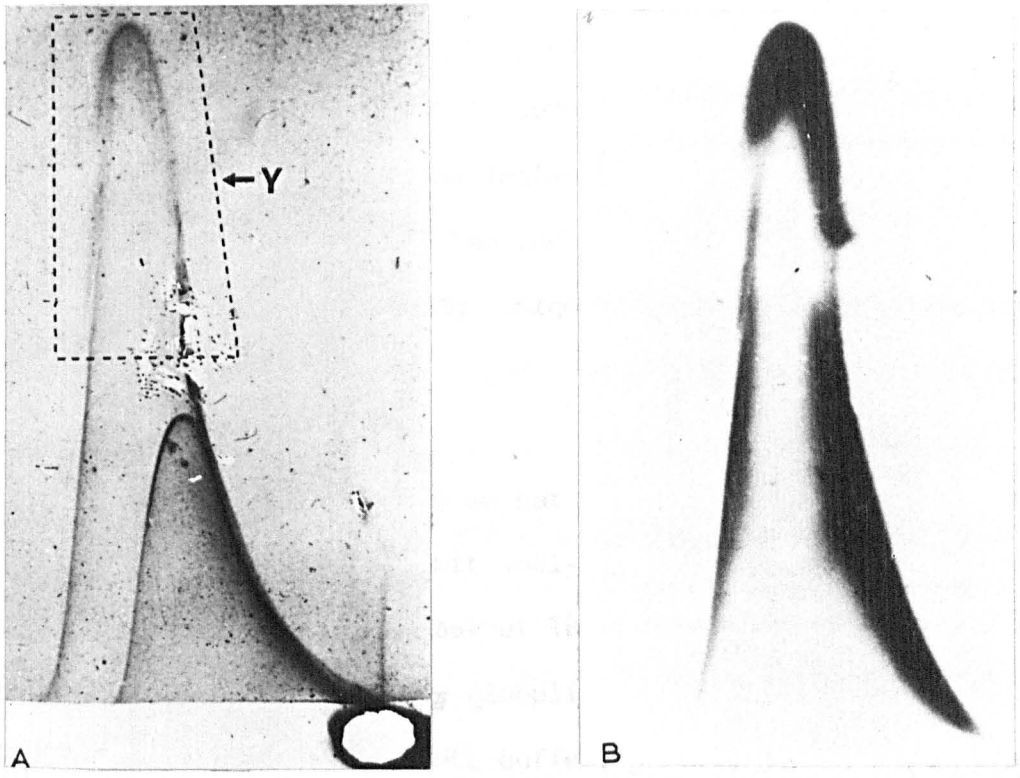
Figure 2.9



TBG PREPARATION

2 DIEP of sample obtained after step d (preparative polyacrylamide gel electrophoresis) electrophoresed into antibody to human ∝-1 fraction. At least 9 protein bands are visible on the stained plate (A). Autoradiography (B) reveals the position of the TBG peak (marked X) although no stained peak is visible. Area 'Y' was removed from the gel for sheep immunisation - see text.

Figure 2.10



TBG PREPARATION

2 DIEP of partially purified TBG (obtained after polyacrylamide gel electrophoresis), run into antiserum from sheep '1'. Serum contains antibodies to 2 proteins visible on stained plate (A). TBG peak was identified by autoradiography (B), and was removed from the gel (area 'Y') and used for further immunisation.

TBG (Figure 2.11).

b) anti-TBPA

Purified TBPA obtained after step 'd' of fractionation was used as antigen. Approximately 200 µg of protein in 0.5 ml of 154 mM NaCl was emulsified with 0.5 ml of Freund's complete adjuvant, and injected intradermally (~10 sites) into a New Zealand white rabbit. After one month, the procedure was repeated and two weeks after the second injection the rabbit was found to be producing a high-titre monospecific antiserum to human TBPA (Figure 2.12).

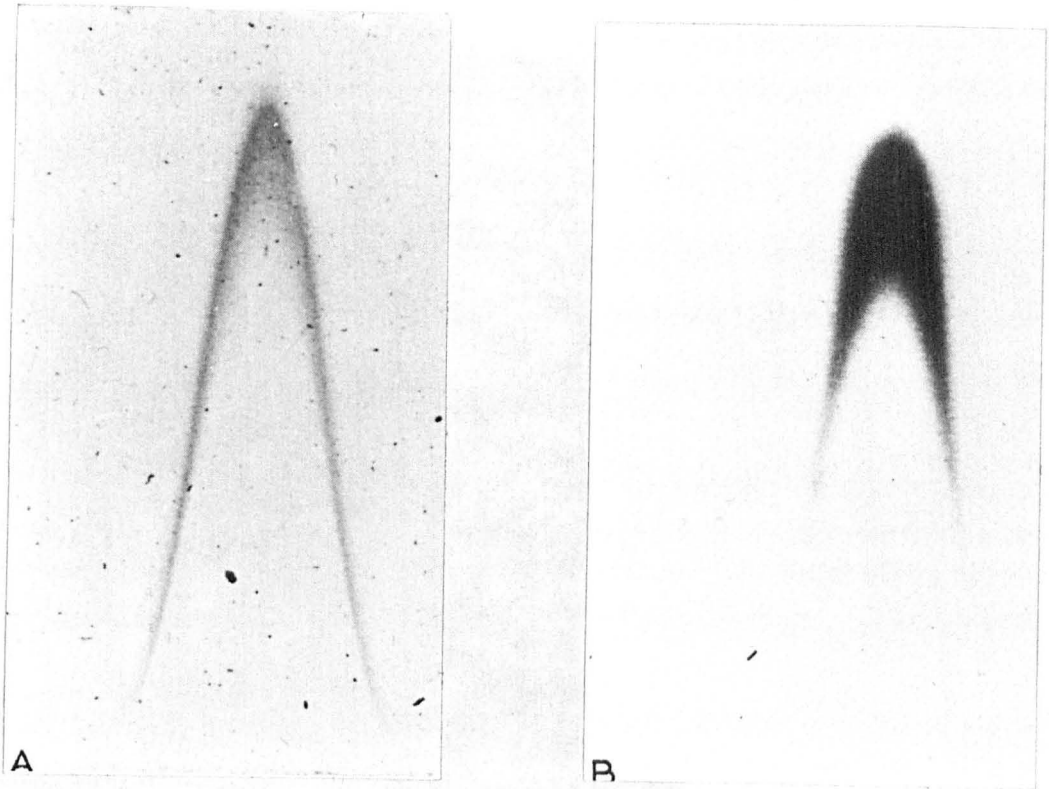
The rabbits were bled from an ear vein, the blood was allowed to clot and centrifuged. Suitable rabbit anti-TBPA antisera were used without further purification in the assay of TBPA, but the sheep anti-TBG was further purified to obtain a γ globulin preparation by fractionation on a DEAE cellulose column (Tris/HCL buffer, pH 8.0, 40 mM) after dialysis for 24 hours against the same buffer.

C) Development of assays

The assays of TBG, TBPA and albumin were established using the monorocket immunoelectrophoretic technique described by Laurell (1966). The method utilises a 60 mM barbitone/sodium barbitone buffer, pH 8.6 and 1% agarose gel, and resembles the 2nd dimension of the 2-DIEP described earlier in this chapter (Chapter 2.2.A.i.).

Glass plates measuring either 8 x 15 cm or 8 x 24 cm were used, and were layered with a 1 mm thickness of agarose, containing a suitable concentration of antibodies. The plates were placed on a levelling table prior to pouring; a high degree of precision was necessary in using the 24 cm plates because of the magnification of any errors in levelling.

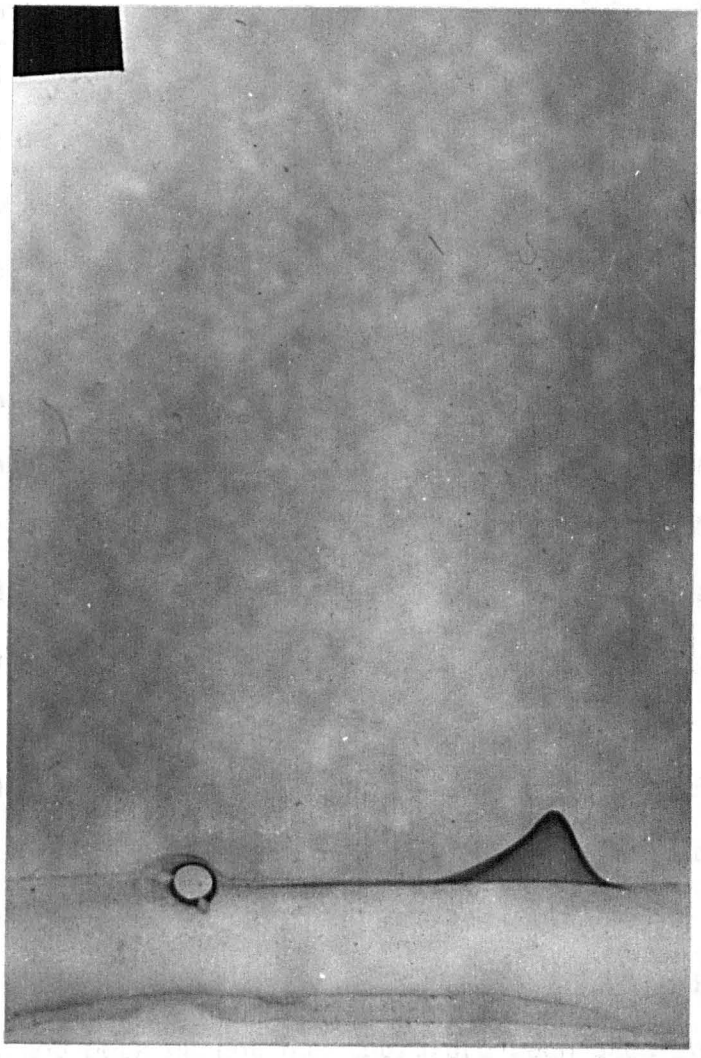
Figure 2.11



TBG PREPARATION

2-DIEP of human serum against antiserum from sheep 2. Monospecific antiserum to TBG is revealed on the stained plate (A) and by auto-radiography (B).

Figure 2.12



TBPA PREPARATION

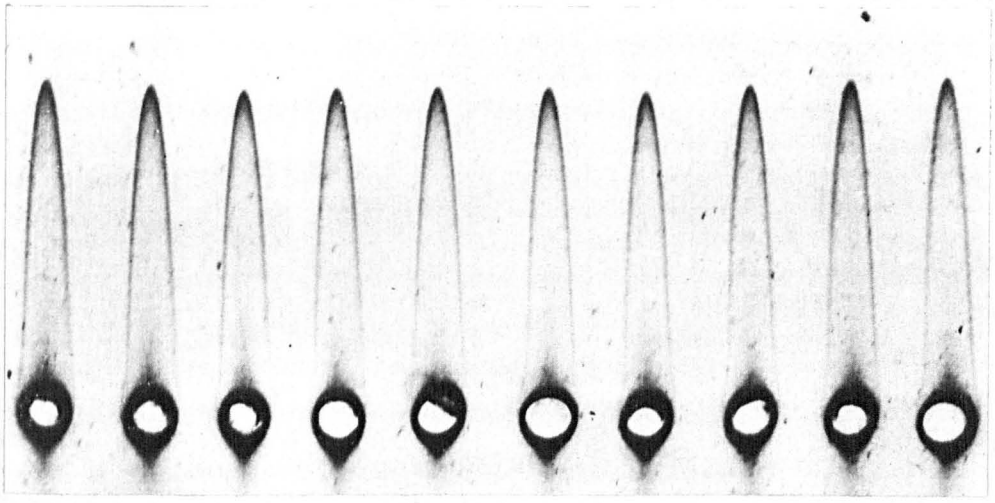
2 DIEP of whole human serum against monospecific anti-TBPA. Monospecificity and T_4-binding were confirmed by autoradiography after labelling with ^{125}I-T_4 (not illustrated).

It was also found necessary to warm the larger plates to ensure smooth layering of the agarose, and for this purpose a heated levelling plate was used, the level of which was checked daily.

A series of wells 2 mm in diameter were punched in the agarose, approximately 1 cm from a long edge of the plate. A carefully measured amount (1 - 3 μl) of sample or standard was dispensed into each well, using a 10 μl Hamilton syringe and Chaney adaptor in a specially developed holder (Department of Experimental Pathology, University of Birmingham). Standards usually consisted of 100%, 75%, 50% and 25% dilutions of a standard serum, and were repeated at each end of the 15 cm plates (which accommodated 30 wells) and at the ends and middle of the 24 cm plates (which accommodated 50 wells). A potential of 2 V/cm was applied overnight across the plate by means of lint wicks moistened with buffer. The plates were dried and stained as described for the 2-DIEP plates with the results shown in figure 2.13.

As described for the second dimension of 2-DIEP, peaks were formed on the plate when the antigen/antibody complexes were at equivalence. The area under the peak is directly proportional to the concentration of antigen. If the peaks are narrow, this area approximates to the height of the peak, and peak height has therefore been used in the past for protein quantitation by the monorocket method. However, slow-migrating proteins may undergo considerable lateral diffusion during the electrophoresis which alters the relationship between peak height and antigen concentration. Princé, Burnett and Ramsden (1977) showed that peak height (Y) was related to antigen concentration (X) by the formula: $Y = a X^{b} + k$. The constants a, b and k may be determined statistically using a programmable desk calculator (Wang 600), and dilutions of standard serum. The programme devised by Princé et al (1977) was used for calculation of TBG, TBPA and

Figure 2.13



STAINED PLATE SHOWING MONOROCKET ELECTROPHORESIS

FOR TBG ESTIMATION

albumin concentrations.

i) Autoradiographic assay for TBG

Prior to the development of monospecific antiserum to TBG, the visualisation of the TBG peak during Laurell immunoelectrophoresis necessitated the use of autoradiographic techniques. The TBG peak had no special staining characteristics, and was thus "lost" among many other proteins migrating in the α_1 position (Freeman and Pearson, 1969). Nielsen et al (1972) described a monorocket IEP technique for TBG measurement using anti-whole human serum and $^{125}I\text{-}T_4$ labelling prior to electrophoresis, followed by autoradiography. This method was improved by Drysdale et al (1975), who used an antibody-free 'starter' gel, and phosphate buffer (pH 7.4, 50 mM) to improve radiolabelling. These methods had the disadvantage of requiring individual radiolabelling of samples, by addition of $^{125}I\text{-}T_4$ to each well. It was found that this could be avoided by incorporating $^{125}I\text{-}T_4$ into the starter gel, and allowing radiolabelling of samples to occur during electrophoresis (Burr and Ramsden, 1977).

Monorocket IEP was performed for 12 hours at 2 V/cm using 50 mM phosphate buffer, pH 7.4. A 'starter' gel (Figure 2.14) consisting of 1% agarose and 500 nCi/ml of high specific activity $^{125}I\text{-}T_4$, was poured in a 1.5 cm x 1 mm strip along a long edge of the electrophoresis plate. Antibody-containing agarose filled the remainder of the plate. Antibodies were either sheep anti-whole human serum (Wellcome Reagents Ltd.) or sheep anti-human α_1 fraction.

It was found that $^{125}I\text{-}T_4$ had a greater electrophoretic mobility than TBG in the buffer system used. Antigen wells (2 mm diameter) were therefore punched approximately 5 mm from the starter gel/antibody gel interface, so that $^{125}I\text{-}T_4$ would flow past the samples during an electrophoretic run.

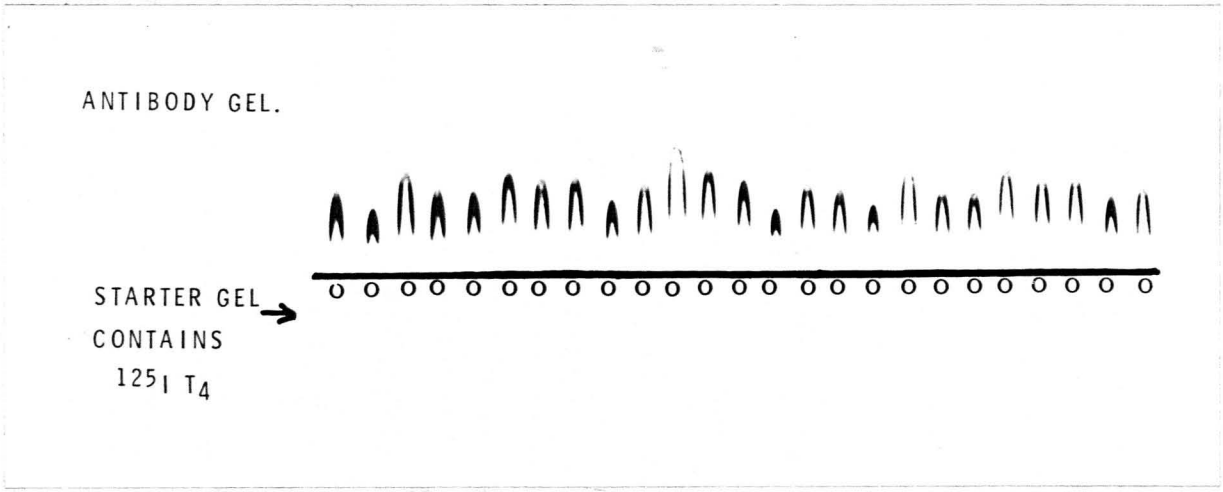


Figure 2.14

AUTORADIOGRAPHIC ASSAY OF TBG

Monorocket plate in which serum samples contained in wells punched into antibody-free 'starter gel', containing $^{125}IT_4$, have been electrophoresed into antiserum to whole human serum. Exposure of X-ray film for 12 hours allows visualisation of the TBG peak only.

Dried plates were subjected to autoradiography for 12 hours before rapid processing of the X-ray film.

Measurement of a single standard in 13 consecutive assays gave an interassay precision of 5.6%. The sensitivity of this technique was approximately 500 ng/l under routine conditions, although this could be increased by increasing the sample size.

ii) Monospecific anti-TBG assay for TBG

Development of the monospecific anti-TBG antiserum allowed direct visualisation of the TBG/anti-TBG complex on a stained electrophoresis plate (Figure 2.11). The assay was normally carried out on a 15 x 8 cm plate using a 1 µl sample as described previously. Precison with this method under routine conditions (Whitehead, 1977) was as follows:

TBG CONCENTRATION (mg/l)	PRECISION %
6.4	13.1
9.8	7.2
14.4	6.3

Sensitivity of the routine assay was 50 µg/l.

Development of the TBG assay had been accomplished without the isolation of pure TBG, and thus standardisation of the assay was required. There is no international standard for TBG, and the TBG assay was therefore standardised against that of Dr. M. C. Gershengorn of the Arthritis, Metabolism and Digestive Diseases Unit, National Institutes of Health, Bethesda, Maryland, U.S.A. (Gershengorn, Larsen and Robbins, 1976a). Dr. Gershengorn kindly measured a working standard serum in duplicate, obtaining results of 10.8 and 10.4 mg/l, a value of 10.6 mg/l was therefore

assigned to the standard.

iii) TBPA assay

This was initially established using commercial rabbit anti-human TBPA (Dakopatts), but owing to the prohibitive cost of this, a monospecific antiserum was produced as described above (Chapter 2.2.B.ii).

Details of the assay are similar to those just described for the assay of TBG using monospecific antiserum. The assay was standardised against a standard serum (ORDT 03) obtained from Behring Diagnostic Reagents.

Interassay precision was approximately 5%, using a 2 μl sample size.

iv) Albumin assay

Because of the characteristic staining of the albumin peak and the enormous excess of albumin relative to other serum proteins, a monospecific antiserum was not essential for albumin measurement by monorocket IEP. Instead, suitable dilution of serum samples and use of a sheep antiserum to whole human serum (Department of Experimental Pathology, University of Birmingham) produced peaks in which albumin was almost always the dominant protein and easily measured. A serum dilution of 1 in 31 was found to be suitable for a sample of 2 μl. Interassay precision was approximately 4%.

D) HORMONE ASSAYS

i) $T_4 + T_3$

Serum T_4 and T_3 radioimmunoassays were carried out in the Department of Medicine (Miss E. G. Black). Unextracted serum was used and 8-anilino-1-naphthalene sulphonic acid was added to inhibit hormone binding by the thyroxine-binding proteins. Specific rabbit antiserum to T_3 or T_4 and ^{125}I-T_3 or ^{125}I-T_4 were added. Polyethylene glycol (M.W. 6000) was used

to precipitate the immunoglobulins. The mixture was centrifuged (2500 x G) for 30 minutes, the supernate was removed, and the precipitate assayed for radioactivity in an automatic gamma-counter (Tracerlab Gammaset 500). Interassay precision was 7% for T_3 (10 replicates) and 4% for T_4 (8 replicates (Black et al, 1975).

Serum T_4 was also assayed in the Department of Clinical Chemistry, Queen Elizabeth Hospital, Birmingham (Dr. S. E. Evans). This assay differed mainly in the use of an anion exchange resin (Dowex-1, mesh 200-400, 8% cross-linked, Sigma Chemical Co., London) to separate free hormone from antibody-bound (Evans, Burr and Hogan, 1977).

ii) Reverse-T_3

Two assays for rT_3 were used in different phases of the work to be described.

At the University of Berlin (Klinikum Steglitz der Freien Universität Berlin - Dr. H. Meinhold), the assay involved extraction of serum with absolute ethanol and a double antibody technique to separate bound and free rT_3. Relative cross-reactivity to T_4 ranged from 0.025 to 0.1 %, and interassay precision (6 samples, 3 replicates) was 11% (Meinhold, Wenzel and Schürnbrand, 1975).

In the Department of Medicine, University of Birmingham, the rT_3 assay (Griffiths, Black and Hoffenberg, 1976) utilised non-extracted serum and a double-antibody separation. 50% displacement of rT_3 occurred with addition of 4000 nmol/l of T_4, while 10% displacement occurred with 100 nmol/l of T_4. The interassay precision was 9% (4 samples, 6 replicates).

iii) Serum TSH

TSH was measured in a radioimmunoassay, using method and reagents supplied by the Arthritis, Metabolism and Digestive Diseases Unit, National Institutes of Health, Bethesda, Maryland, U.S.A. The method utilised a rabbit anti-human TSH, and sheep anti-rabbit IgG to separate bound from free TSH. Interassay precision was approximately 7%.

iv) Free T_3 and T_4

Percentage free T_3 and T_4 was measured in a method based on that of Lee et al (1964), in which free hormone is taken up on a column of Sephadex gel (G25). Small disposable columns were used, consisting of the barrel of an insulin syringe (Steriseal Ltd., Redditch, Worcester). A double isotope method with ^{125}I-T_3 and ^{131}I-T_4 labelling allowed simultaneous measurement of percentage free T_3 and free T_4. Interassay precision (1 sample, 10 replicates) was 9% for free T_4 and 7% for free T_3 (Finucane and Griffiths, 1976).

v) T_3 uptake

This was measured in the Department of Clinical Chemistry, Queen Elizabeth Hospital, Birmingham (Dr. S. E. Evans) using a commercial kit method (Thyopac 3, The Radiochemical Centre, Amersham, U.K.). The test is based on the partition of ^{125}I-T_3 between serum thyroxine-binding sites and an adsorbent (Sephadex G25) at equilibrium. Results are expressed as a percentage of a standard serum (supplied with each kit). Precision was approximately 4% (Bold and Browning, 1975).

Chapter 3 TBG IN EUTHYROID PERSONS AND THOSE WITH THYROID DISEASE

1) Individuals studied
   a) Euthyroid volunteers
   b) Euthyroid hospital in-patients
   c) Pregnant women
   d) Contraceptive pill users
   e) Dysthyroid patients : thyrotoxicosis and myxoedema
2) Results
   a) Healthy volunteers and in-patients
   b) Effect of age on TBG concentration
   c) Effect of pregnancy and contraceptive pill treatment
   d) Effect of thyroid disease
3) Discussion

Chapter 3 TBG IN EUTHYROID PERSONS AND THE EFFECT OF AGE, OESTROGENS, PREGNANCY AND THYROID DISEASE

1) INDIVIDUALS STUDIED

TBG was measured in the following people:-

a) Euthyroid volunteers

116 healthy, euthyroid persons (70 male, 46 female), comprising laboratory staff, medical students and Civil Servants (subjected to an annual biochemical profile as part of a health screen).

b) Euthyroid hospital in-patients

180 hospital in-patients (89 male, 91 female), who were euthyroid, and had a normal biochemical profile (serum creatinine, urea, sodium, potassium, bilirubin, aspartate aminotransferase, alkaline phosphatase, albumin, globulin, calcium, urate, cholesterol and glucose).

c) Pregnant women

18 pregnant women in the second and third trimesters of pregnancy.

d) Contraceptive pill-users

12 women who were taking oestrogen/progestogen contraceptive pills. A further 5 women who were taking oral contraceptives and one regularly-menstruating women who was not taking the contraceptive pill were followed sequentially by blood samples taken every 2 - 4 days for 40 - 50 days.

e) Dysthyroid patients - thyrotoxicosis and myxoedema

101 patients with thyrotoxicosis and 36 patients with myxoedema, selected from patients attending the Thyroid Clinic, Birmingham General

Hospital, or in-patients at the Queen Elizabeth Hospital, Birmingham. Patients were all seen by the author. Diagnosis was made by clinical criteria and measurement of T_4 and T_3 uptake. Where necessary serum T_3 and TSH were measured, and occasionally the TSH response to TRH injection (200 μg i.v.), and the suppression of thyroidal ^{131}I uptake by T_3 administration (40 μg, 3 times daily for one week) were assessed.

A sub-group of 30 newly-diagnosed thyrotoxic patients were re-examined and blood taken after 3 months and 6 months of treatment with Carbimazole or Propylthiouracil.

2) RESULTS

a) Healthy volunteers and euthyroid in-patients

TBG concentrations in the different groups studied are shown in Table 3.1. TBG concentration in healthy persons did not differ from the concentration in euthyroid in-patients.

The TBG concentration in euthyroid females was 9% greater than the concentration in males, the difference being significant ($P < 0.001$ by Student's t-test, pregnant and oestrogen-taking individuals having been excluded).

The distribution of TBG concentrations in males was found to be normal by testing for kurtosis and skewness (Table 3.1), but the female population was shown to be skewed towards higher values of TBG, giving a logarithmic distribution. The combined population of males and females was also best described by a logarithmic distribution (Table 3.1 and Fig. 3.1).

b) Effect of age

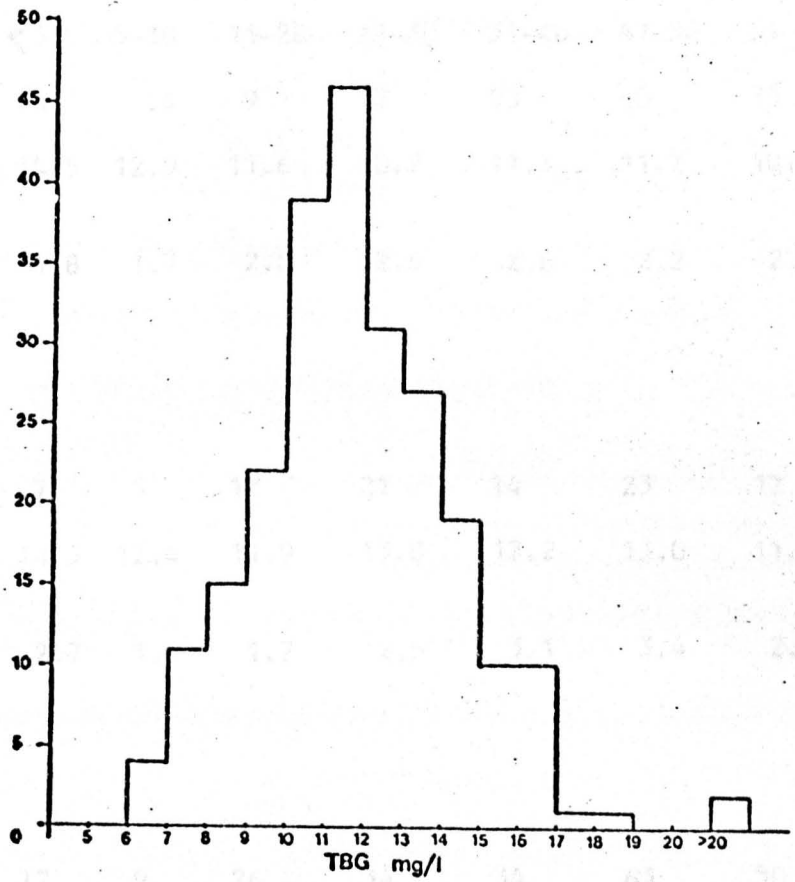
The effect of age on TBG concentration is shown in Table 3.2. The

Table 3.1

	n	Mean	S.D.	Kurtosis	Skewness
EUTHYROID ♂+ ♀	296	12.1	2.6	0.54	0.46
log TBG ♂+ ♀				0.05	-0.22
EUTHYROID ♂	159	11.6	2.6	-0.68	0.11
log TBG ♂				-0.51	-0.35
EUTHYROID ♀	137	12.7	2.6	1.22	0.87
log TBG ♀				0.37	0.16
ORAL CONTRACEPTIVE ♀	12	16.7	2.8		
PREGNANT	18	24.9	3.9		
THYROTOXIC ♂+ ♀	101	10.8	2.4		
MYXOEDEMA ♂+ ♀	36	14.7	2.4		

TBG CONCENTRATIONS (mg/l)

Figure 3.1



DISTRIBUTION OF TBG CONCENTRATION IN EUTHYROID HEALTHY MALES AND FEMALES

Table 3.2

AGE (years)

	0	<5	5-10	11-20	21-30	31-40	41-50	51-60	61-70	71+
MALE										
Number	6	10	.4	9	13	20	40	33	16	8
Mean TBG (mg/l)	14.5	14.5	12.9	11.6	10.7	11.3	11.2	10.8	11.9	12.6
S.D.	2.2	1.8	1.7	2.8	2.6	2.6	2.2	2.0	2.9	3.3
FEMALE										
Number	6	7	5	17	21	14	23	17	14	13
Mean TBG (mg/l)	16.4	14.3	12.4	11.9	13.0	12.2	13.0	11.8	12.5	11.8
S.D.	1.2	2.7	1.9	1.7	2.5	3.1	3.4	2.5	1.9	2.0
MALE + FEMALE										
Number	12	17	9	26	34	34	63	50	30	21
Mean TBG (mg/l)	15.5	14.4	12.6	11.8	12.1	11.7	11.9	11.2	12.2	12.1
S.D.	2.0	2.1	1.7	2.1	2.8	2.8	2.8	2.2	2.5	2.5

AGE AND TBG CONCENTRATION IN 296 EUTHYROID INDIVIDUALS

population of 296 euthyroid individuals was divided into twenty groups, according to age and sex, and the TBG results subjected to analysis of variance. This showed that significant differences existed between different age groups, whether these were processed with regard to sex or irrespective of sex ($F < 0.0001$).

TBG was elevated in neonates and in children under the age of 5 years. In males, adult TBG concentrations were fairly constant from age 11 years until age 60 years, with a tendency towards higher concentrations in the over-sixties. In females a slight increase in TBG concentration occurred in the 21 - 30 year group, with a slight decrease after the age of 50 years (Table 3.2).

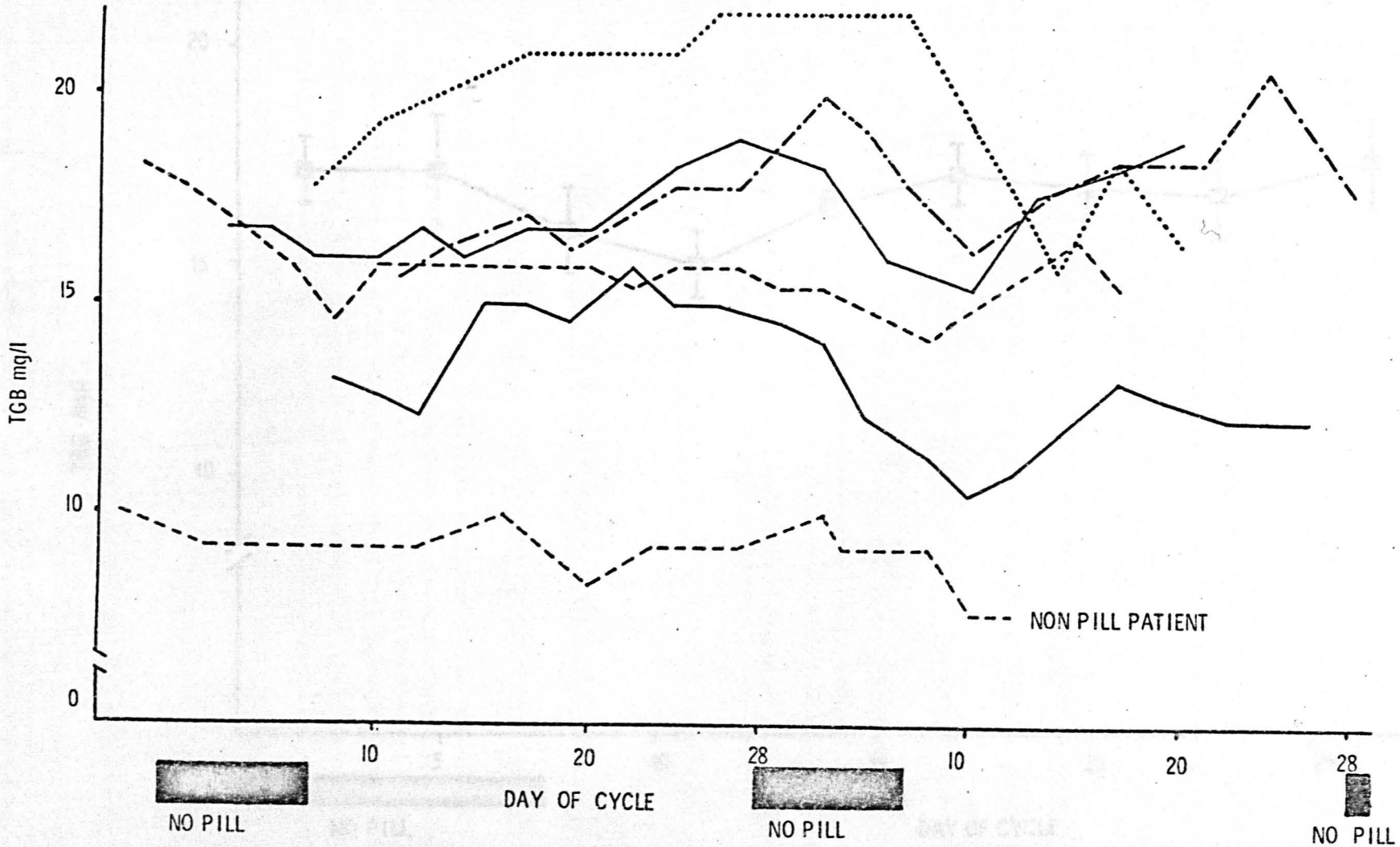
c) Effect of pregnancy and oral contraceptive therapy

When compared with untreated euthyroid females, TBG was increased by a mean of 31% in oral contraceptive users and 96% in pregnant women (Table 3.1). These increases were both significant ($P < 0.0005$ by Student's t-test).

Sequential TBG concentrations of five oral contraceptive users and one regularly-menstruating woman are shown in figure 3.2, and the mean values at 3-day time intervals through the menstrual cycle are shown in figure 3.3. TBG concentrations at various times in the cycle were compared with the concentration for the same individual at other times by paired t-testing. The results are shown in table 3.3.a. TBG concentration was significantly lower on days 10 - 12 of the cycle than on days 1 - 3 or 25 - 28.

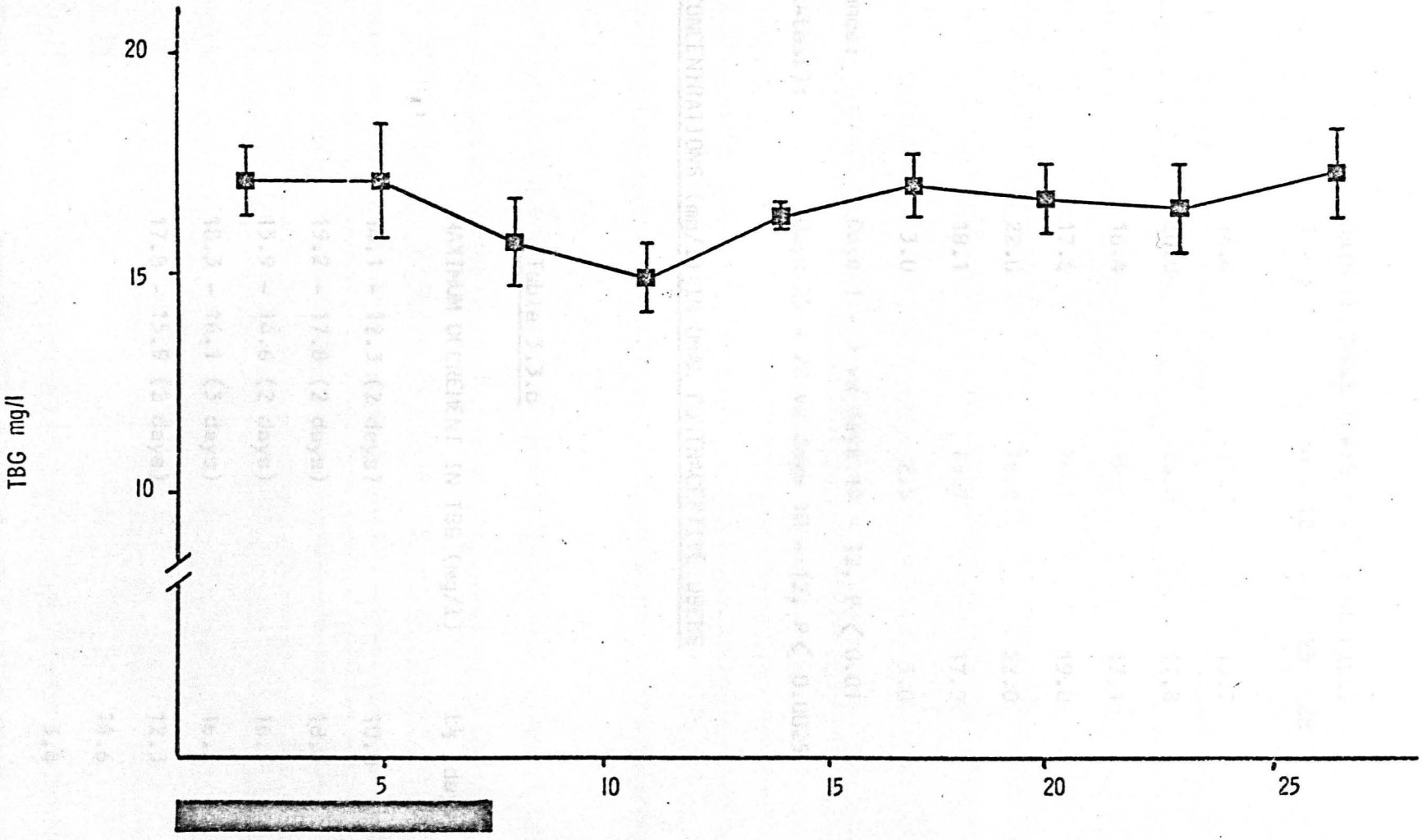
The ½-time of TBG in serum during the decrease in concentration after withdrawal of oral contraceptives was calculated from the slope of TBG concentration when the rate of fall was maximal (Table 3.3.b.). The mean

Figure 3.2



SEQUENTIAL TBG CONCENTRATIONS DURING THE MENSTRUAL CYCLE IN FIVE WOMEN TREATED WITH ORAL CONTRACEPTIVES AND ONE UNTREATED WOMAN

Figure 3.3



MEAN TBG CONCENTRATIONS (±SD) DURING THE MENSTRUAL CYCLE IN FIVE WOMEN TREATED WITH ORAL CONTRACEPTIVES

Table 3.3.a

SUBJECT	DAYS OF CYCLE (DAYS 1 - 7 = NO PILL)		
	1 - 3	10 - 12	25 - 28
F.B.	14.4	11.7	13.7
D.H.	20.0	16.0	17.8
F.A.	16.4	15.9	17.1
C.I.	17.6	16.1	19.0
J.K.	22.0	18.8	22.0
Mean	18.1	15.7	17.9
S.D.	3.0	2.5	3.0
Significance:	Days 1 - 3 vs days 10 - 12, $P < 0.01$		
(Paired t-test):	Days 25 - 28 vs days 10 - 12, $P < 0.0025$		

TBG CONCENTRATIONS (mg/l) IN ORAL CONTRACEPTIVE USERS

Table 3.3.b

SUBJECT	MAXIMUM DECREMENT IN TBG (mg/l)	$t_{\frac{1}{2}}$ (days)
F.B.	14.1 - 12.3 (2 days)	10.0
D.H.	19.2 - 17.8 (2 days)	18.3
F.A.	15.9 - 14.6 (2 days)	16.3
C.I.	18.3 - 16.1 (3 days)	16.2
J.K.	17.8 - 15.9 (2 days)	12.3
Mean		14.6
S.D.		3.4

TBG $\frac{1}{2}$-TIME IN SERUM AFTER INTERRUPTION OF ORAL CONTRACEPTIVE THERAPY

½-time was 14.6 days, with a range of 10 - 18.3 days.

No alteration of TBG concentration occurred during the menstrual cycle of the woman who was not taking oral contraceptives (Figure 3.2).

d) Effect of thyroid disease

In myxoedematous patients, TBG was elevated by 26% compared to the euthyroid population (Table 3.1). This was a highly significant elevation ($P < 0.0005$) whether compared with the whole euthyroid population or with females only. In thyrotoxicosis, the TBG concentration did not differ significantly from that of the whole euthyroid population, but when compared with the euthyroid female population there was a mean reduction of 12% ($P < 0.0005$).

Figure 3.4 shows the effects of antithyroid therapy on TBG and T_4 concentrations in thyrotoxicosis. There was a mean rise in TBG concentration of 2.7 mg/l (24%) to a mean of 13.8 $\pm$ 3.4 mg/l during the first three months of treatment, while serum T_4 concentration fell from 234 $\pm$ 73 nmol/l to 109 $\pm$ 53 nmol/l. After 6 months, when all subjects were euthyroid, TBG concentration was 14.2 $\pm$ 3.9 mg/l ($P < 0.0005$ for TBG at 3 and 6 months compared with pre-treatment TBG by paired t-test). The greatest increases in TBG concentration [6.7 mg/l (60%), 7.2 mg/l (51%) and 5.1 mg% (40%)] were observed in three thyrotoxic women who were on treatment with oral contraceptives.

3) DISCUSSION

A number of direct assays for TBG have been described in recent years (Table 3.4), and almost as many different normal concentrations for TBG have been cited, with values ranging from 7.4 mg/l (Wagner et al, 1975) to

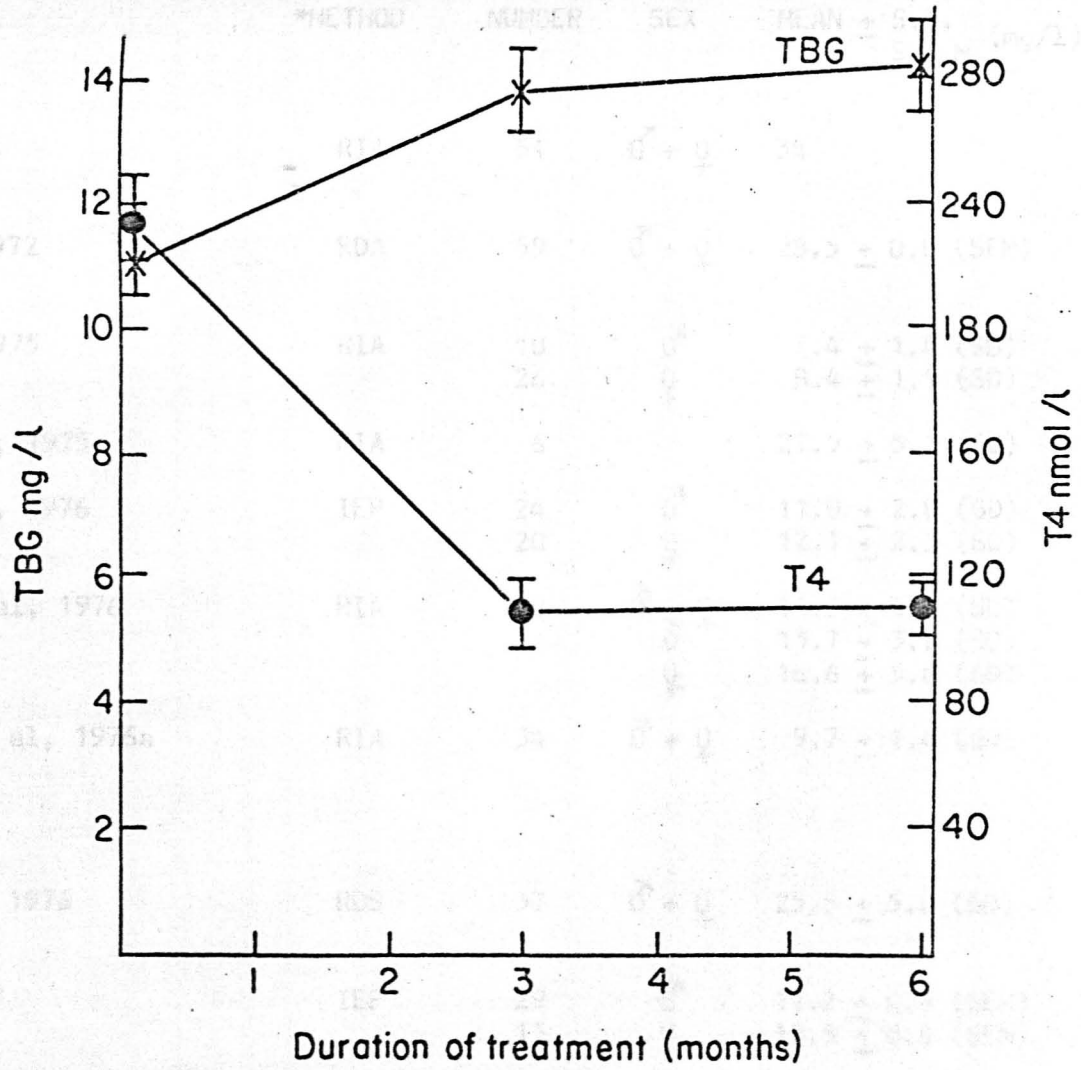


Figure 3.4

T_4 AND TBG CONCENTRATIONS IN 30 THYROTOXIC PATIENTS DURING MEDICAL TREATMENT OF THYROTOXICOSIS WITH CARBIMAZOLE OR PROPYLTHIOURACIL

(see text for significance values).

Table 3.4

AUTHOR AND YEAR	*METHOD	NUMBER	SEX	MEAN ± S.D. S.E.M. (mg/l)	FINDINGS
Levy et al, 1971	RIA	51	♂ + ♀	34	Thyrotoxicosis TBG↓ Myxoedema TBG →
Chopra et al, 1972	RDA	59	♂ + ♀	28.5 ± 0.8 (SEM)	Thyrotoxicosis TBG↓ Myxoedema TBG→
Wagner et al, 1975	RIA	10 26	♂ ♀	7.4 ± 1.4 (SD) 8.4 ± 1.1 (SD)	Thyrotoxicosis TBG↓
Cavalieri et al, 1975	RIA	6		21.5 ± 5.1 (SD)	
X Bradwell et al, 1976	IEP	24 20	♂ ♀	11.0 ± 2.8 (SD) 12.1 ± 2.3 (SD)	
Gershengorn et al, 1976	RIA	98	♂ + ♀ ♂ ♀	14.8 ± 4.6 (SD) 13.7 ± 3.7 (SD) 16.6 ± 5.6 (SD)	Female > Male (21%) log-normal distribution in males and females
X Hesch R. -D et al, 1976a	RIA	34	♂ + ♀	9.7 ± 1.4 (SD)	Thyrotoxicosis (mild) TBG↑ Thyrotoxicosis (severe) TBG→ Myxoedema TBG↑ normal distribution
X Rudorff et al, 1976	RDS	37	♂ + ♀	25.5 ± 5.0 (SD)	Thyrotoxicosis TBG↓ Myxoedema TBG ↑
Burr et al, 1977	IEP	29 13	♂ ♀	11.2 ± 0.5 (SEM) 12.5 ± 0.8 (SEM)	Thyrotoxicosis TBG↓ Myxoedema TBG↑ Female/male difference not significant

DIRECT ASSAYS OF TBG CONCENTRATION

Table 3.4 (Cont.)

AUTHOR AND YEAR	*METHOD	NUMBER	SEX	MEAN ± S.D. S.E.M. (mg/l)	FINDINGS
Kågedal and Källberg, 1977	RID	127 95	♂ ♀	13.8 ± 2.0 (SD) 15.4 ± 2.4 (SD)	Female > Male (12%) Female distribution skewed towards high values Male distribution normal
X Bastomsky et al, 1977	RDA	155	♂ + ♀	31.6 ± 5.4 (SD)	Thyrotoxicosis TBG ↓ Myxoedema TBG ↑ Normal distribution
Parslow et al, 1977	RIA	95	♂	36.5 ± 7.8 (SD)	
X Mulaisho and Utiger, 1977	RDA	38 35	♂ ♀	32.0 ± 6.0 (SD)	Thyrotoxicosis TBG → Myxoedema TBG ↑ Female/Male difference not significant
Horn et al, 1977	RIA		♂ + ♀	23.0 ± 4.0 (SD)	Thyrotoxicosis TBG → Myxoedema TBG → Female/Male difference not significant
De Nayer and Luypaert, 1978	RID	10 18	♂ ♀	18.3 ± 4.5 (SD) 20.2 ± 3.3 (SD)	Female/Male difference not significant
Kallner et al, 1978	RIA	20	♂ + ♀	16.2 ± 3.2 (SD)	Thyrotoxicosis TBG →
Bisset et al, 1978	RIA	29 114	♂ ♀	20.4 ± 4.3 (SD) 21.3 ± 3.7 (SD)	Thyrotoxicosis TBG → Female/Male difference not significant

* RIA = Radioimmunoassay RDA = Radiodisplacement assay RID = Radial immunodiffusion IEP = Immunoelectrophoresis

X Method standardised against another assay

36.5 mg/l (Parslow, Oddie and Fisher, 1977). Gershengorn et al (1976a) measured the maximum T_4-binding capacity and TBG concentration (RIA) in 64 normal individuals and calculated the T_4:TBG binding ratio. By assuming a molecular weight for TBG of 57,000 Daltons, and a 1:1 molar ratio of T_4 to TBG, they predicted a T_4:TBG binding ratio of 13.6 μg T_4/mg TBG, and actually observed a ratio of 13.8 $\pm$ 0.5 μg T_4/mg TBG. They took this to be good evidence that their assay was providing a measurement of the actual TBG concentration.

The assay used in the present studies was standardised against that of Gershengorn et al (1976a). The mean TBG concentration obtained by IEP measurement was 18% less than the normal mean found by Gershengorn. The difference may have arisen because of losses during lyophilisation and reconstitution of the standard serum or may be due to methodological or population differences.

The IEP assay for TBG described in this thesis did not involve the isolation of pure TBG. However, the purification of TBG is not accomplished easily, as evidenced by the nine protein contaminants persisting after the first four purification steps described in Chapter 2.2.B.i. Several preparations of "pure" TBG, already in use for radio-immunoassays, have been examined by 2 DIEP and shown to contain other proteins as contaminants (Bradwell, Burnett and Ramsden, 1978). Such contamination could account for some of the higher TBG concentrations reported. Until an international standard for TBG is agreed upon, there would appear to be no cogent reason for favouring any particular value of TBG.

The finding of a 9% increase in TBG concentration in females compared to males agrees with a previous report (Kågedal and Källberg, 1977) although

Gershengorn et al (1976a) found 20% higher values in females, and others have failed to identify a sex difference (Horn et al, 1977; Mulaisho and Utiger , 1977; De Nayer and Luypaert, 1978; Bisset et al, 1978).

The explanation for the sex difference in TBG concentration almost certainly lies in the effect of oestrogens tending to increase TBG concentrations in the female (Dowling et al, 1965a, b; Robbins and Nelson, 1958), and of androgens tending to depress concentrations in the male (Federman et al, 1958). The effects of oestrogens have been confirmed in the present study in oral contraceptive users and pregnant women.

No effects of the physiological hormonal changes of the menstrual cycle were manifest on the TBG concentration of the one woman in whom this was studied. Her TBG concentrations were rather low, which would tend to diminish the precision of the TBG measurements, and it is also possible that some alteration of TBG concentration might have been revealed if this had been expressed relative to total protein rather than serum volume (Wagner, 1978). Horn et al (1977) also failed to show an association between menstruation and TBG concentration but Drysdale et al (1975) reported that a peak of TBG concentration occurred at about the time of ovulation. However, an oestrogen-induced peak of TBG concentration might be expected to occur later in the menstrual cycle, since approximately 5 - 6 days are required for TBG concentration to reach a peak after β-oestradiol administration in the rhesus monkey (Glinoer et al, 1977a), and oestrogen concentrations are also considerably raised during the luteal phase of the menstrual cycle (Hall et al, 1974).

Oral contraceptive therapy with oestrogen/progestogen mixtures has been reported in the past to cause TBG elevation (Musa et al, 1967; Laurell et al, 1968), but only Drysdale et al (1975) have observed sequential TBG

alterations during therapy. They found that cessation of an oral contraceptive was associated with a slow decline of TBG, reaching minimum levels after 14 - 16 days.

In the present study, oestrogens were not withdrawn completely, but merely stopped for 7 days. TBG concentration reached a minimum approximately 10 - 12 days after cessation of the oral contraceptive, and the mean half-life of TBG calculated during this time was 14.6 days. Since the half life of TBG in normals is 4 - 6 days (Cavalieri et al, 1975; Refetoff et al, 1976), and oestrogen treatment (in rhesus monkeys) affects mainly TBG synthesis rather than degradation (Glinoer et al, 1977 a, b), it would seem likely that there is a lag effect of oestrogen on TBG synthesis persisting for several days after oestrogen withdrawal. There may also be only a gradual increase in TBG synthesis after reintroduction of oestrogens, since TBG falls for 3 - 5 days after reintroduction of the contraceptive pill. This would be in contrast to the finding of Glinoer et al (1977a), who observed a rise in serum TBG concentration within 24 hours of 17β-oestradiol treatment in monkeys. The difference may be due to the low dose of oestrogen in the contraceptive pill.

An association between age and TBG concentration has been reported in the past (Hesch et al, 1976a, b, 1977; Horn et al, 1977). These authors found high TBG concentrations until puberty, with lower levels in adult life and a rise after age 60. In the present study, high TBG concentrations were found in the neonate, which persisted until the age of five and then fell between the ages of five and ten years. During early adult life, there was a tendency for a slight decrease of TBG in males, and an increase in females. After the age of 60, there was a tendency for TBG to rise, and this was more marked in males. The high TBG in neonates presumably resulted from intra-uterine exposure to high concentrations of oestrogen, but this could

not account for the persistence of raised TBG until the age of 5. The alterations in TBG concentration occurring in mature men and women can be explained on the basis of altered oestrogen and androgen concentrations after sexual maturity, and the rise in TBG in senescent men may be due to the decreased testosterone production which is known to occur in elderly males (Vermeulen et al, 1972).

High TBG concentrations in myxoedema have frequently been observed (Hesch et al, 1976a; Rudorff et al, 1976; Burr et al, 1977; Bastomsky et al, 1977; Mulaisho and Utiger, 1977), but in making comparisons between myxoedematous and euthyroid patients it is important to make allowance for the preponderance of females in the myxoedematous population (Means, J. H., 1948). Since we have shown increased TBG in females, the myxoedematous population may well have a higher TBG concentration than the combined male and female euthyroid controls on the basis of sex difference alone. For this reason, we have also compared myxoedematous patients with euthyroid females, and shown that significant differences persist.

Failure to allow for sex differences between the dysthyroid and control populations may also account for the failure of some authors to demonstrate decreased TBG concentration in thyrotoxicosis (Hesch et al, 1976a; Mulaisho and Utiger, 1977). The high proportion of women in the thyrotoxic population (DeGroot and Stanbury, 1975) justifies the comparison of this group also with euthyroid females.

The results now presented clearly demonstrate a 24% increase in TBG concentration during the first three months of medical treatment for thyrotoxicosis. The persistence of TBG elevation after six months precludes the possibility that the TBG increase was due to the induction of transient hypothyroidism, since by six months all patients were unequivocally euthyroid.

It is interesting to note that the most marked elevation of TBG concentration occurred in thyrotoxic women who were taking the contraceptive pill. Thyrotoxicosis is known to elevate serum concentrations of sex hormone-binding globulin (SHBG) (Crépy et al, 1967), and this may cause lowering of free oestrogen levels and reduced oestrogen effect. Lowering of SHBG concentration during treatment of thyrotoxicosis could lead to a rise in free oestrogen concentration with stimulation of hepatic TBG synthesis (Glinoer et al, 1976). A similar mechanism of increased SHBG causing reduced negative and positive oestrogen feedback has been suggested to account for the amenorrhoea of thyrotoxicosis (Anderson, 1974) and for the increased LH levels found in thyrotoxicosis (Chopra et al, 1972; Akande and Anderson, 1975). However, these observations are incompatible with the observation of an increase in free oestradiol-17β in thyrotoxic men (Chopra et al, 1972; Chopra and Tulchinsky, 1974), unless one postulates a sex-difference in the effect of thyrotoxicosis on free oestrogen levels. This may be possible in view of the increased peripheral conversion of testosterone and androstenedione to oestrone and oestradiol reported in thyrotoxic men and women (Southren et al, 1974).

Other factors may account for the apparent antagonism of oestrogen-induced TBG synthesis in thyrotoxicosis. TBG degradation may be increased in thyrotoxicosis (Refetoff et al, 1976) so that any increased synthesis does not lead to increased serum concentration, or thyroid hormones may exert a direct inhibitory effect on hepatic TBG synthesis, which counteracts the oestrogen stimulation (Gershengorn et al, 1976b).

Further investigation, possibly using an in vitro system, would be necessary to resolve these questions.

Chapter 4 THE EFFECTS OF SURGERY, NON-THYROIDAL ILLNESS AND STARVATION ON THYROID HORMONES AND BINDING PROTEINS

1) Introduction - aims and designs of study

2) Patient groups
   a) "major" surgery
   b) "minor" surgery
   c) myocardial infarction
   d) starvation

3) Measurements

4) Results
   a) "major" surgery
   b) "minor" surgery
   c) myocardial infarction
   d) starvation

5) Discussion

Chapter 4 THE EFFECTS OF SURGERY, NON-THYROIDAL ILLNESS AND STARVATION ON THYROID HORMONES AND BINDING PROTEINS

1) INTRODUCTION - AIMS AND DESIGN OF STUDIES

The object of this work was to study the response of thyroid hormones, free thyroid hormones and thyronine-binding proteins to the relatively reproducible stress of a surgical operation. It was hoped that some insight would be gained into the alterations in thyroid hormone metabolism induced by surgical stress, and also that further information regarding the interactions of thyroid hormones, free hormones and binding proteins would be gained.

Having observed some dramatic changes in thyroid hormones and binding proteins after surgical operation in the first part of the study, the observations were extended to a group of patients suffering from acute myocardial infarction, to determine whether other types of illness could elicit similar changes. In addition, a group of patients undergoing therapeutic starvation was also studied, because the profound effect of diet on thyroid function (see Chapter 1.5.b.iii.) indicated that this was an essential control, especially for the surgical patients.

2) PATIENT GROUPS

Patients were all selected from the medical and surgical wards of the Queen Elizabeth Hospital, Birmingham. Informed consent was obtained in every case. All were clinically and biochemically euthyroid.

a) Major surgery

This group consisted of sixteen patients, who underwent elective

surgery of the type shown in Table 4.1.a. All patients in this group underwent general anaesthesia, which was usually induced with a short-acting barbiturate such as thiopentone together with a myo-neural blocking agent such as suxamethonium, and was maintained with inhaled nitrous oxide, oxygen and halothane, together with pancuronium or D-tubocurarine.

Patients were studied by means of blood samples (20 ml), taken at 9.00 a.m. daily for one or two days pre-operatively and at six hours post-operatively and then daily for 6 - 10 days.

b) Minor surgery

These patients (Table 4.1.b.) underwent more minor procedures than group a, and did not undergo opening of a body cavity. One patient (19) received local anaesthesia.

c) Myocardial infarction

Thirteen patients were studied who had undergone recent acute myocardial infarction (MI). Criteria for inclusion in this group were as follows:-

1) History of severe chest pain of less than 24 hours duration, with no antecedent history of angina pectoris or other chest pains.
2) ECG evidence of acute MI.
3) Elevation of serum aspartate aminotransferase.

These patients were studied by 20 ml blood samples, taken as soon after admission to hospital as possible, and daily at 9.00 a.m. thereafter for ten days.

d) Starvation

Studies were performed in eight obese women volunteers, aged 21 - 53

Table 4.1.a

PATIENT	AGE	SEX	DIAGNOSIS	OPERATION	COMMENT
1	76	M	Carcinoma of colon	R. hemicolectomy & cholecystectomy	Gram negative septicaemia died day 16
2	74	F	Carcinoma of head of pancreas	Cholecystogastro-stomy. Anterior gastro-jejunost-omy	Heart failure days 0 - 4
3	81	F	Choledocholithiasis	Cholecystectomy choledocho-duo-denostomy	
4	36	F	Polycystic kidneys	R. nephrectomy	Renal transplant recipient, taking Prednisone, 25 mg daily
5	55	F	Abscess of gall bladder	Cholecystotomy	
6	62	F	Duodenal ulcer	Vagotomy and pyloroplasty	
7	55	F	Gastric ulcer	Partial gastrectomy	
8	71	F	Cholecystitis	Cholecystectomy appendicectomy	Wound infection day 6
9	45	F	Pyloric stenosis	Pyloroplasty jejunal biopsy	
10	57	F	Gastric ulcer	Billroth I partial gastrectomy	
11	49	M	Duodenal ulcer	Vagotomy & pyloro-plasty, appendic-ectomy	
12	31	F	Cholelithiasis	Cholecystectomy	
13	23	F	Idiopathic thrombo-cytopaenia purpura	Splenectomy	Prednisolone 12.5 mg daily PUO days 1 - 7
14	68	M	Fibrosarcoma of diaphragm	Excision of fibrosarcoma	
15	35	F	Ulcerative colitis	Pan-proctocolect-omy & eversion ileostomy	
16	31	M	Duodenal ulcer	Vagotomy & pyloro-plasty	

PATIENTS SUBMITTED TO "MAJOR" SURGERY

Table 4.1.b

PATIENT	AGE	SEX	DIAGNOSIS	OPERATION	COMMENT
17	75	M	Malignant melanoma	Excision of melanoma + skin graft to R. ankle	
18	14	M	Pubertal mammary hypertrophy	Bilateral mastectomy	
19	78	M	Femoral hernia	Herniorrhaphy	Local anaesthetic
20	45	M	Thyroid adenoma	Excision of adenoma	
21	16	M	Pubertal mammary hypertrophy	Bilateral mastectomy	

PATIENTS SUBMITTED TO "MINOR" SURGERY

years, whose body weights ranged from 34 - 140% above the ideal. All were clinically euthyroid, but one had an elevated basal TSH of 11 mU/l and low normal T_4:TBG ratio (Burr et al, 1979) suggesting a degree of thyroid failure. They were hospitalised and completely fasted for 4 days, during which they drank only a minimum of 2 litres tap water daily. Blood samples were taken at 9.00 a.m. daily.

3) MEASUREMENTS AND STATISTICAL ANALYSES

Group a) - Major surgery

T_3, T_4 (Black et al, 1975), TBPA and albumin were measured in all patients. For logistic reasons such as insufficient sample or non-availability of assay, some tests could not be performed on all samples:-

TBG was measured in 13 patients (except patients 7 - 9 inclusive)

TSH was measured in 11 patients (except patients 7 - 9, 12 and 13)

% Free T_3, % free T_4 were measured in 11 patients (except patients 12 - 16 inclusive)

rT_3 (Dr. H. Meinhold) was measured in patients 3, 12, 14, 16 (group a) and 21 (group b)

Group b) - Minor surgery

Serum T_3, T_4 and TBPA were measured in this group.

Group c) - Myocardial infarction

Albumin was not measured in these patients.

% Free T_3 and % free T_4 were measured in 9 patients and rT_3 in only 2.

Group d) - Starvation

T_3, T_4, rT_3 (Griffiths et al, 1976), TSH, TBPA, TBG and albumin were measured in all samples. Free hormone concentration was not measured

directly, but was computed from total hormone and binding protein concentrations by the method of Princé and Ramsden (1978). Serum acetoacetate (Miss S. A. Nutter, Biochemistry Department, General Hospital, Birmingham) was measured by the method of Price, Lloyd and Alberti (1977), with the exception that spectrophotometric measurements were made at 25° C with a Cecil Ultraviolet Spectrophotometer CE. 292 (Cecil Instruments Ltd., Milton Industrial Estate, Cambridge, U.K.). Absorbance readings at 340 nm were taken at 0 mins and 1 min after addition of 3-hydroxybutyrate dehydrogenase, and the rate of change of absorbance calculated. The assay had an intra-assay precision of 4% at a plasma acetoacetate concentration of 0.2 mmol/l, and 3% at a plasma acetoacetate of 0.4 mmol/l. All samples from a single individual in sections a, b, c, and d above were measured in the same assay.

All statistical analyses in this section were by means of paired t-test, comparing baseline and pre-operative or pre-fasting values with samples obtained on subsequent days. In the case of individuals suffering from myocardial infarction, no true baseline reading could be obtained, and initial samples were taken as soon after hospital admission as possible, and not later than 24 hours from the onset of symptoms.

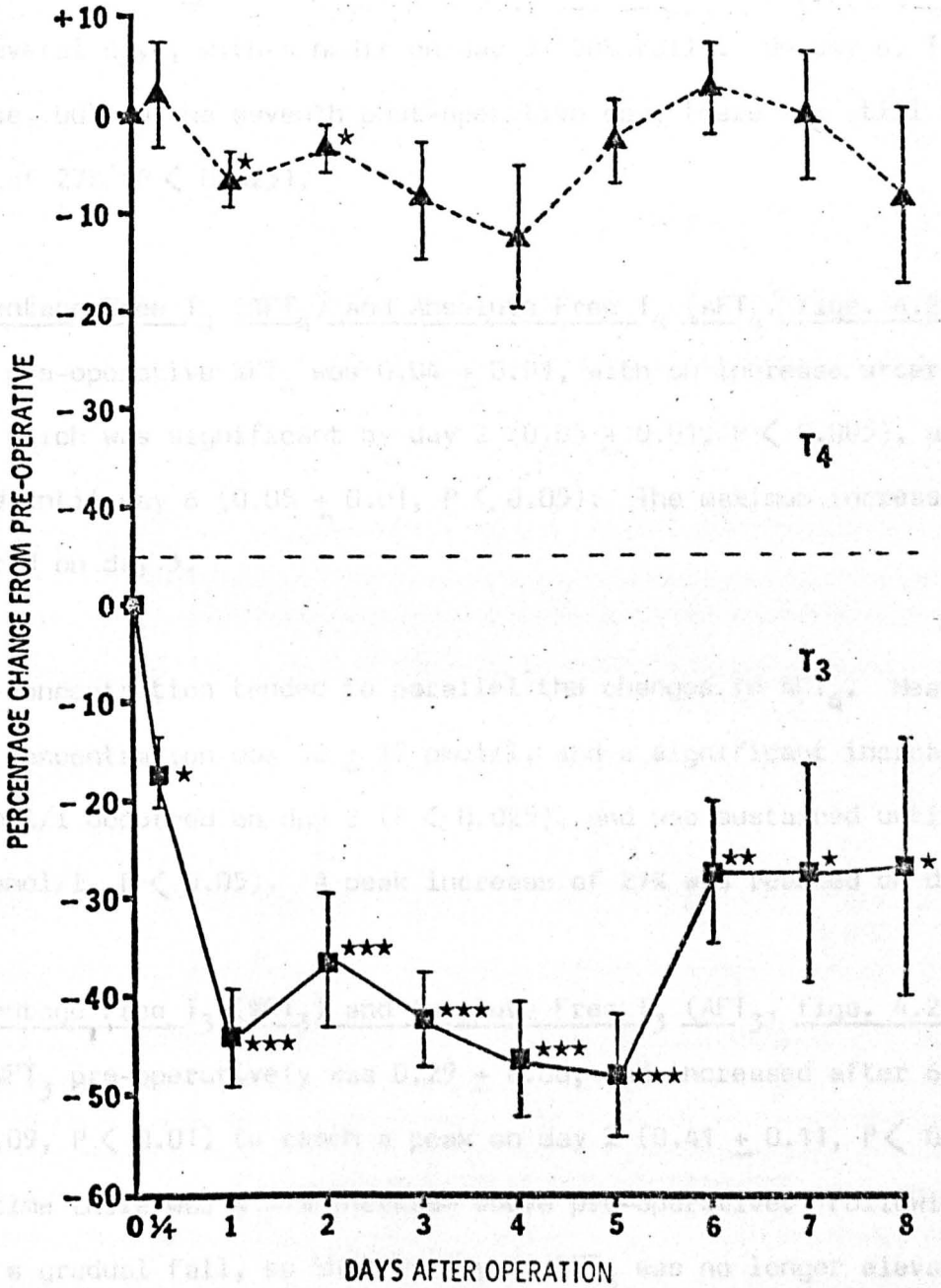
4) RESULTS (mean figures ± one standard deviation are given, and full results are tabulated in appendices B, C, D and E).

a) Major surgery (Tabulated results: appendix B)

Serum T_4 (fig. 4.1)

Mean pre-operative T_4 was 131 ± 32 nmol/l. At 24 and 48 hours after operation, mean values of 123 ± 38 and 125 ± 29 nmol/l were obtained ($P < 0.05$) with subsequent return to pre-operative concentration.

Figure 4.1



SIGNIFICANT DIFFERENCE FROM PRE-OPERATIVE ★ $P < 0.05$
★★ $P < 0.005$
★★★ $P < 0.0005$

PERCENTAGE CHANGE IN T_4 AND T_3 CONCENTRATIONS AFTER SURGICAL OPERATION

Serum T_3 (Fig. 4.1)

Serum T_3 fell rapidly and profoundly. Mean pre-operative concentration was 2.0 $\pm$ 0.5 nmol/l, 6 hours post-operative was 1.5 $\pm$ 0.3 nmol/l ($P < 0.01$), and 24 hours was 1.1 $\pm$ 0.5 nmol/l ($P < 0.0005$). The concentration remained low for several days, with a nadir on day 5 (50% fall). On day 6, T_3 began to increase, but on the seventh post-operative day, there was still a mean reduction of 27% ($P < 0.025$).

Percentage Free T_4 (%FT_4) and Absolute Free T_4 (AFT_4, figs. 4.2 and 4.3)

Mean pre-operative %FT_4 was 0.04 $\pm$ 0.01, with an increase after operation which was significant by day 2 (0.05 $\pm$ 0.01, $P < 0.005$), and was maintained until day 6 (0.05 $\pm$ 0.01, $P < 0.05$). The maximum increase of 33% occurred on day 3.

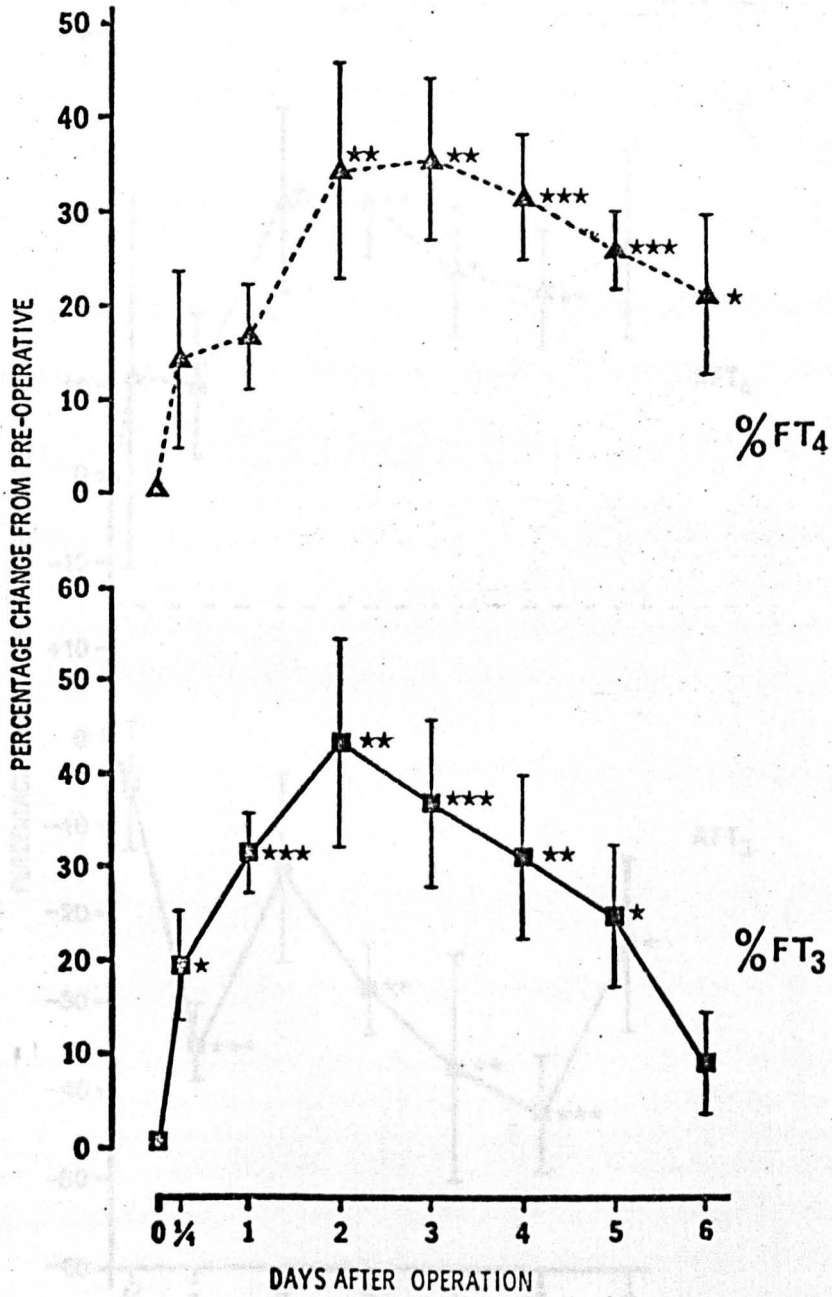
AFT_4 concentration tended to parallel the changes in %FT_4. Mean pre-operative concentration was 52 $\pm$ 15 pmol/l, and a significant increase to 66 $\pm$ 27 pmol/l occurred on day 2 ($P < 0.025$), and was sustained until day 6 (63 $\pm$ 17 pmol/l, $P < 0.05$). A peak increase of 27% was reached on day 3.

Percentage Free T_3 (%FT_3) and Absolute Free T_3 (AFT_3, figs. 4.2 and 4.3)

The %FT_3 pre-operatively was 0.29 $\pm$ 0.06, and increased after 6 hours (0.36 $\pm$ 0.09, $P < 0.01$) to reach a peak on day 2 (0.41 $\pm$ 0.11, $P < 0.0025$), at which time there was a 41% increase above pre-operative. Following this there was a gradual fall, so that by day 6, %FT_3 was no longer elevated.

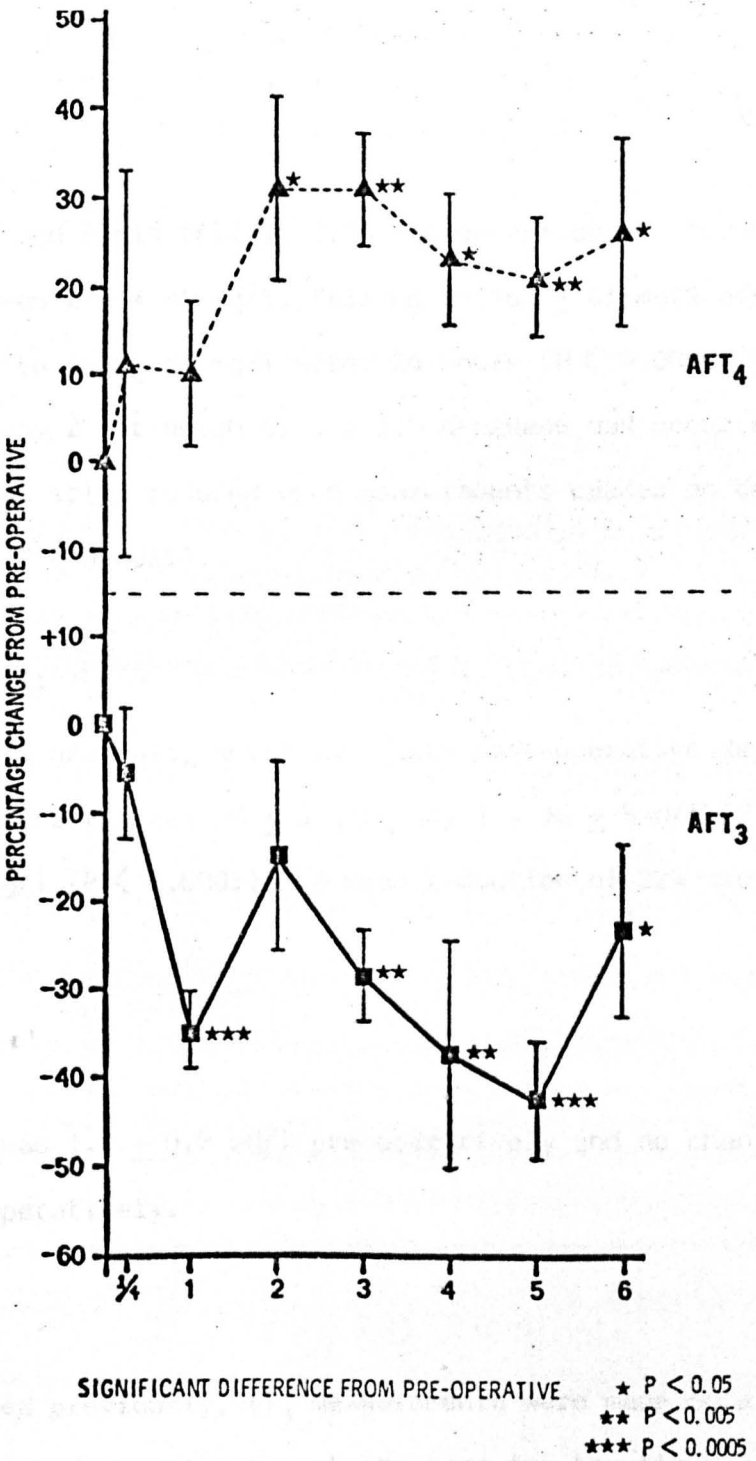
AFT_3 decreased in spite of the increased %FT_3. Pre-operative concentration was 5.5 $\pm$ 1.0 pmol/l, falling to 3.6 $\pm$ 1.3 pmol/l after 24 hours ($P < 0.0025$). After 2 days, there was partial recovery of AFT_3 concentration, but the concentration remained less than pre-operative even after 6 days (4.1 $\pm$ 1.5 pmol/l, $P < 0.05$).

Figure 4.2



PERCENTAGE CHANGE IN % FREE T_4 AND T_3 AFTER SURGICAL OPERATION

Figure 4.3



PERCENTAGE CHANGE IN ABSOLUTE FREE T_4 AND T_3 (AFT_4 AND AFT_3) CONCENTRATIONS AFTER SURGICAL OPERATION

TBG (Fig. 4.4)

There was a slight reduction of TBG post-operatively, from 13.7 ± 2.6 mg/l to 12.9 ± 2.6 mg/l on day 1 ($P < 0.05$) and to 12.4 ± 2.2 mg/l on day 3 ($P < 0.01$), representing a 9% reduction compared to pre-operative concentration.

TBPA (Fig. 4.4)

A profound and rapid fall in TBPA concentration was found. Pre-operative TBPA was 210 ± 84 mg/l, falling to 165 ± 63 mg/l after 6 hours ($P < 0.025$) and to 164 ± 61 mg/l after 24 hours ($P < 0.0005$). The nadir was reached on day 2, at which time a 52% decrease had occurred. The concentration was still reduced when measurements ceased on day 8 (111 ± 58 mg/l, $P < 0.0025$).

Albumin (Fig. 4.4)

Albumin fell gradually until the sixth post-operative day. Pre-operative concentration was 38 ± 8 g/l, day 1 - 36 ± 8 g/l ($P < 0.01$), day 6 - 30 ± 8 g/l ($P < 0.0005$). A mean reduction of 22% was found on day 6.

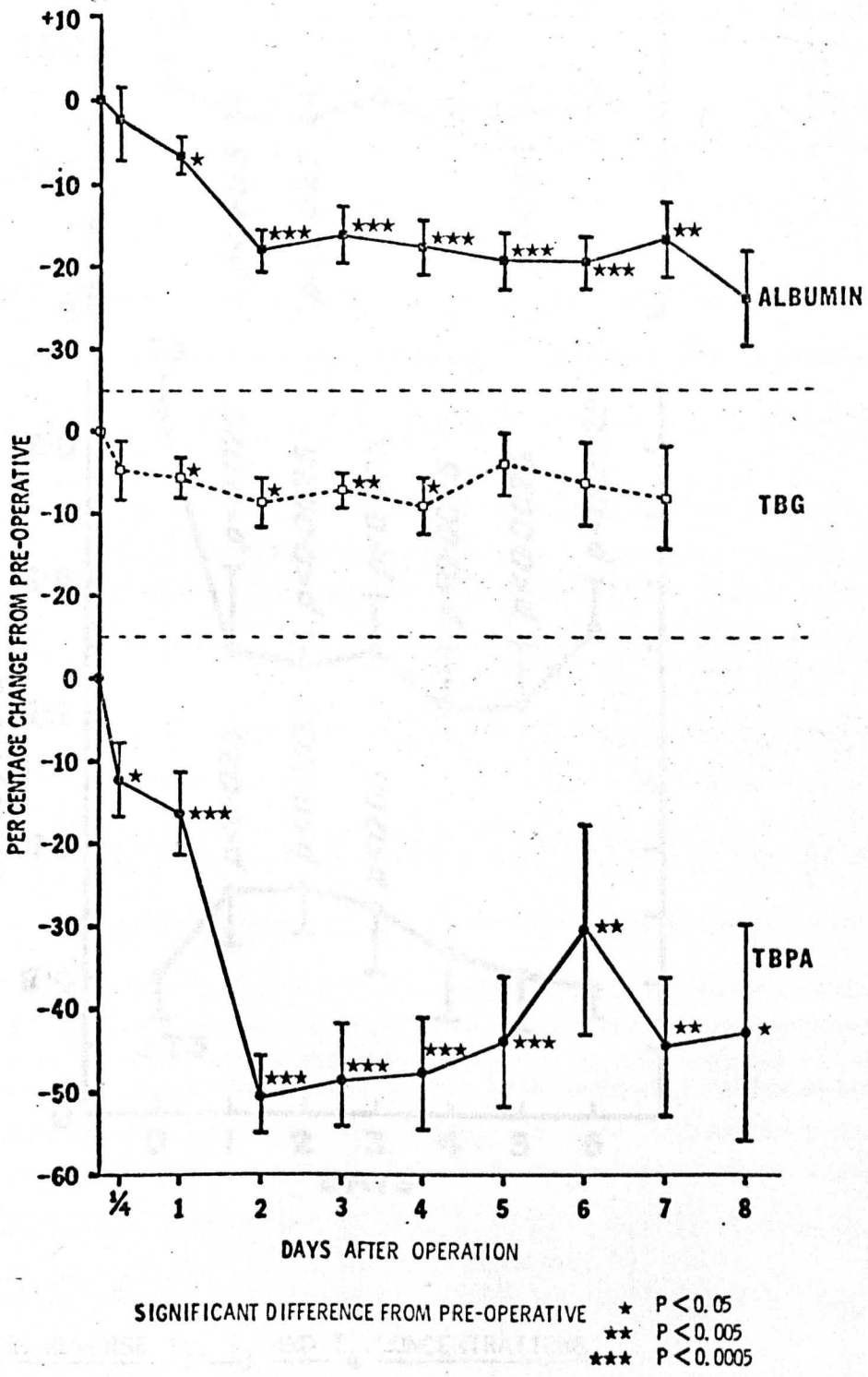
TSH

Serum TSH was 1.7 ± 0.9 mU/l pre-operatively and no change was observed post-operatively.

rT_3 (Fig. 4.5)

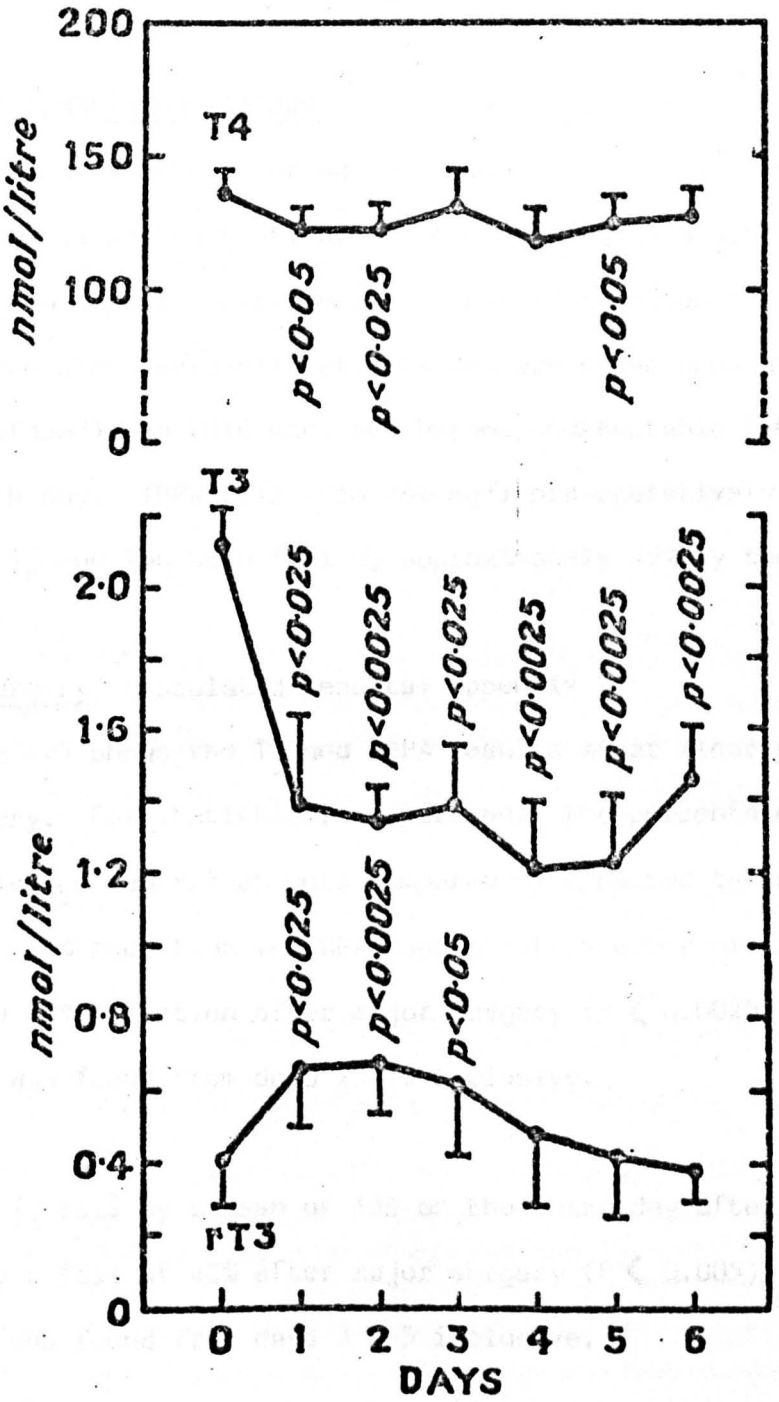
As mentioned previously, rT_3 measurements were made on a sub-group of 5 patients. There was a significant increase for the first 3 days after operation, from 0.40 ± 0.13 nmol/l pre-operatively to a maximum of 0.68 ± 0.13 nmol/l on day 2 ($P < 0.0025$). Figure 4.5 shows rT_3 results for five patients, with T_3 results from the same individuals.

Figure 4.4



PERCENTAGE CHANGE IN ALBUMIN, TBG AND TBPA CONCENTRATIONS AFTER SURGICAL OPERATION

Figure 4.5



SERUM REVERSE T_3, T_3 AND T_4 CONCENTRATIONS

AFTER SURGICAL OPERATION

A summary of the main findings after major surgery is given in Table 4.2.

Effect of overwhelming illness

One patient in the major surgery group (patient 1) developed intra-abdominal sepsis post-operatively, and died of gram-negative septicaemia on the sixteenth post-operative day. Thyroid function tests in this patient differed from other surgical patients and are shown separately in Fig. 4.6. T_3 fell profoundly in this man, and became undetectable ($<$ 0.3 nmol/l) on the fifth day. TBPA fell from 264 mg/l pre-operatively to 19 mg/l on day 8, and T_4 and TBG both fell by approximately 50% by the eighth day.

b) Minor surgery (Tabulated results: appendix C)

Figure 4.7 shows the T_3 and TBPA results after minor surgery and after major surgery. For statistical comparisons, the percentage changes from pre-operative concentration were compared by unpaired t-test. After 2 days, there was a 17% reduction in TBPA concentration after minor surgery, compared to 50% reduction after major surgery ($P < 0.0025$). A statistical difference was found from days 2 - 5 inclusive.

Serum T_3 fell by a mean of 15% on the third day after minor surgery, compared to a fall of 42% after major surgery ($P < 0.005$), and a statistical difference was found from days 3 - 5 inclusive.

c) Myocardial infarction (Tabulated results: appendix D)

Serum T_4 (Fig. 4.8)

On the day of admission, T_4 was 140 $\pm$ 40 nmol/l, and a slight reduction occurred on the following day, with a nadir reached on day 4, (119 $\pm$ 28 nmol/l, $P < 0.05$). T_4 rose after this, to reach pre-illness concentration by day 8 (134 $\pm$ 32 nmol/l, NS).

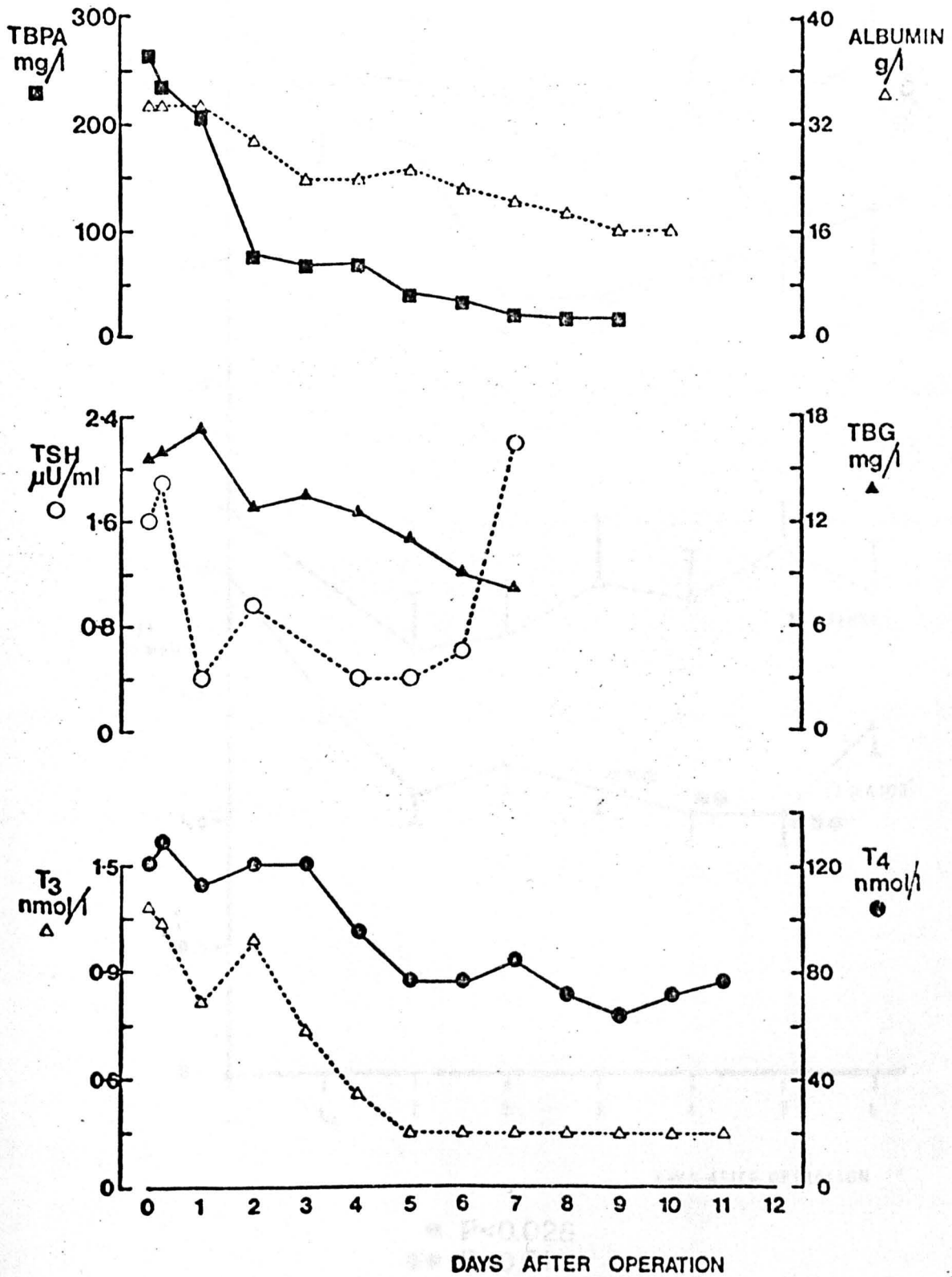
Table 4.2

DAY 0 = DAY OF OPERATION

		MAXIMUM CHANGE
T_4	↓ DAYS 1, 2	↓ 6% (DAY 1)
$\%FT_4$	↑ DAYS 2 - 6	↑ 36% (DAY 3)
AFT_4	↑ DAYS 2 - 6	↑ 27% (DAY 2, 3)
T_3	↓ 6 hrs, DAYS 1 - 8	↓ 49% (DAY 5)
$\%FT_3$	↑ 6 hrs, DAYS 1 - 5	↑ 41% (DAY 2)
AFT_3	↓ DAY 1, 3 - 6	↓ 42% (DAY 5)
rT_3	↑ DAYS 1 - 3	↑ 70% (DAY 2)
TSH	NO SIGNIFICANT CHANGE	
TBPA	↓ 6 hrs, DAYS 1 - 8	↓ 52% (DAYS 2 - 4)
TBG	↓ DAYS 1 - 4	↓ 9% (DAY 4)
ALBUMIN	↓ DAYS 1 - 7	↓ 20% (DAYS 2, 5, 6)

SUMMARY OF FINDINGS AFTER MAJOR SURGERY

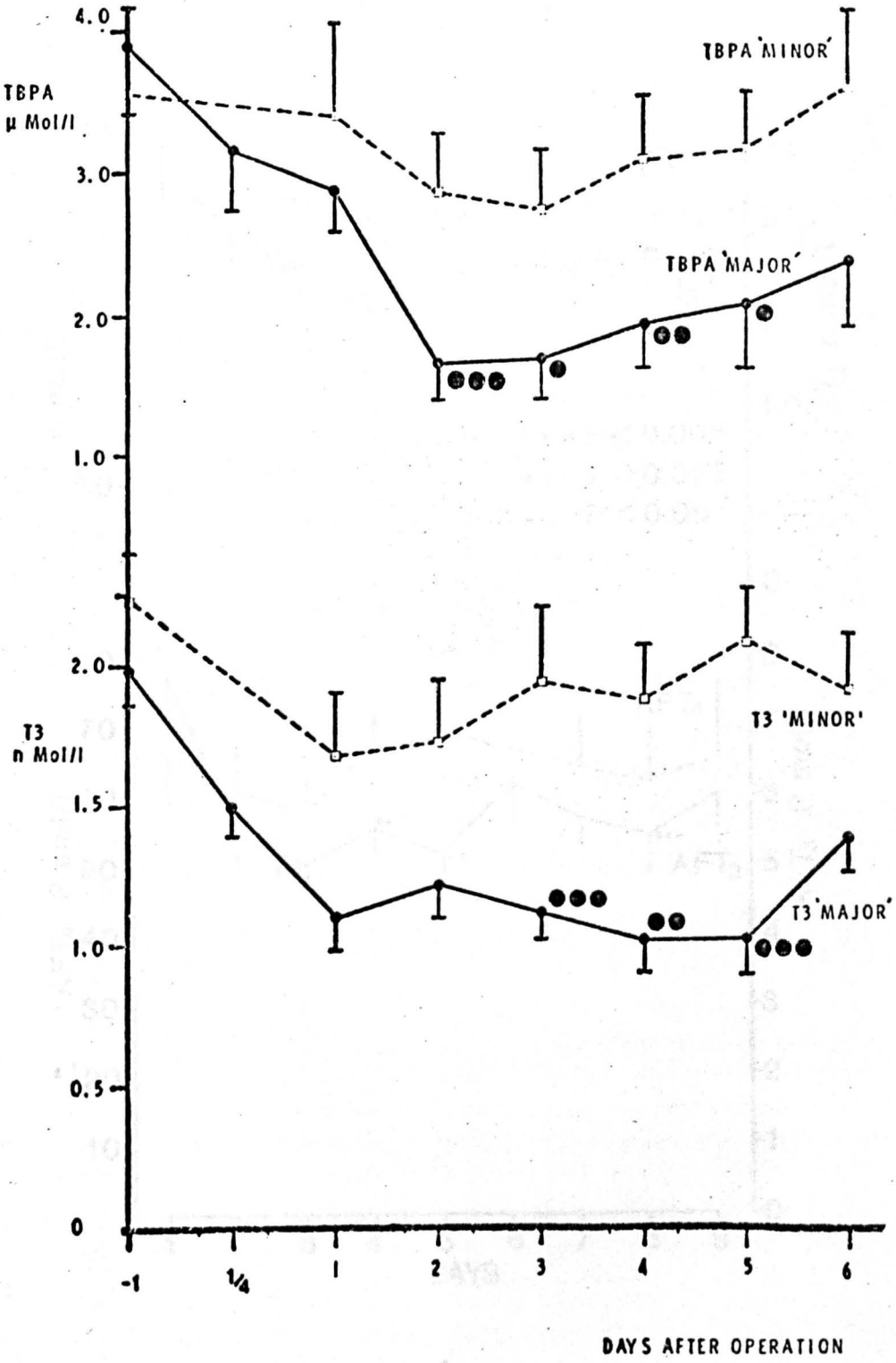
Figure 4.6



THYROID FUNCTION IN SEVERE ILLNESS

PATIENT NUMBER 1 DIED SEPTICAEMIA DAY 16

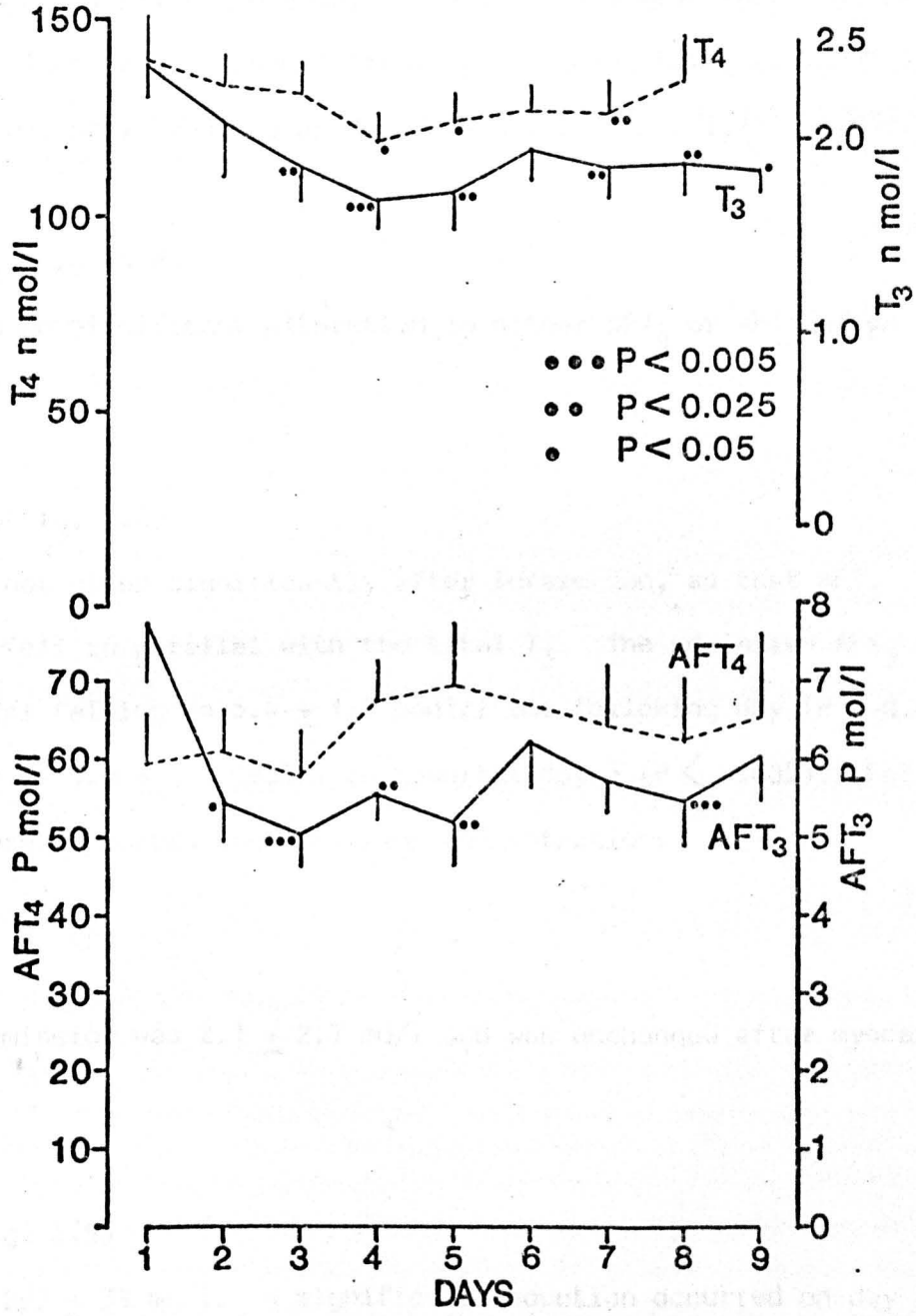
Figure 4.7



● P<0.025
●● P<0.01
●●● P<0.005

T_3 AND TBPA CONCENTRATIONS AFTER MAJOR AND MINOR SURGERY

Figure 4.8



T_4, T_3, AFT_4 AND AFT_3 AFTER MYOCARDIAL INFARCTION

Serum T_3 (Fig. 4.8)

T_3 fell after infarction from the admission level of 2.4 ± 0.6 nmol/l to 1.9 ± 0.6 nmol/l after two days ($P < 0.025$). The nadir was reached on day 4 (1.7 ± 0.6 nmol/l, $P < 0.0025$) at which time a mean reduction of 29% had occurred. There was a gradual recovery following this, although T_3 concentration was still reduced on day 9 (1.8 ± 0.3 nmol/l, $P < 0.05$).

% FT_4 and AFT_4 (Fig. 4.8)

There was no significant alteration in either %FT_4 or AFT_4 after infarction.

%FT_3 and AFT_3 (Fig. 4.8)

%FT_3 did not alter significantly after infarction, so that AFT_3 concentration fell in parallel with the total T_3. The admission AFT_3 was 7.8 ± 2.2 pmol/l falling to 5.4 ± 1.2 pmol/l the following day ($P < 0.05$) and to a nadir of 5.0 ± 1.1 pmol/l on hospital day 3 ($P < 0.005$). Following this AFT_3 returned towards the admission concentration.

Serum TSH

TSH on admission was 2.1 ± 2.7 mu/l and was unchanged after myocardial infarction.

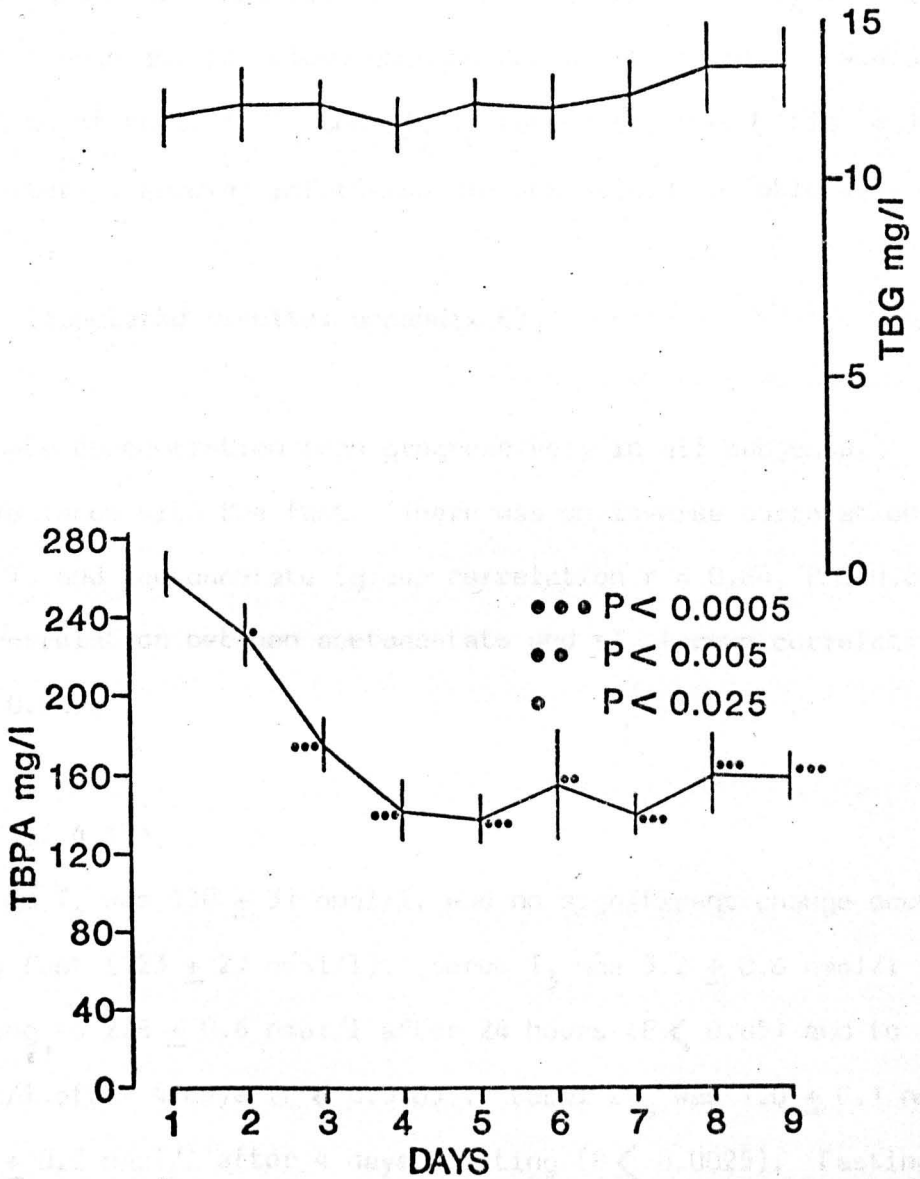
Serum TBPA (Fig. 4.9)

TBPA was 262 ± 39 mg/l. A significant reduction occurred on day 2, and low concentrations persisted until observations ceased on day 9. The lowest TBPA concentration was found on day 5 (136 ± 45 mg/l, $P < 0.0005$).

Serum TBG (Fig. 4.9)

Serum TBG on admission was 11.7 ± 2.4 mg/l and no significant change occurred after myocardial infarction.

Figure 4.9



TBG AND TBPA AFTER MYOCARDIAL INFARCTION

rT_3 (Fig. 4.10)

This was only measured in two patients. In both there was a rise in rT_3 concentration for up to four days after admission, while T_3 fell on day 2, and then gradually increased. Marked fluctuations of T_4 in one patient may have been due to volume changes during treatment of cardiac failure. Because of this, T_3/T_4 and rT_3/T_4 ratios are shown (Fig. 4.10). The findings after myocardial infarction are summarised in Table 4.3.

d) Starvation (Tabulated results: appendix E)

Acetoacetate

Acetoacetate concentration rose progressively in all subjects, confirming compliance with the fast. There was an inverse correlation between serum T_3 and acetoacetate (group correlation $r = 0.84$, $P < 0.0005$) and a direct correlation between acetoacetate and rT_3 (group correlation $r = 0.77$, $P < 0.005$).

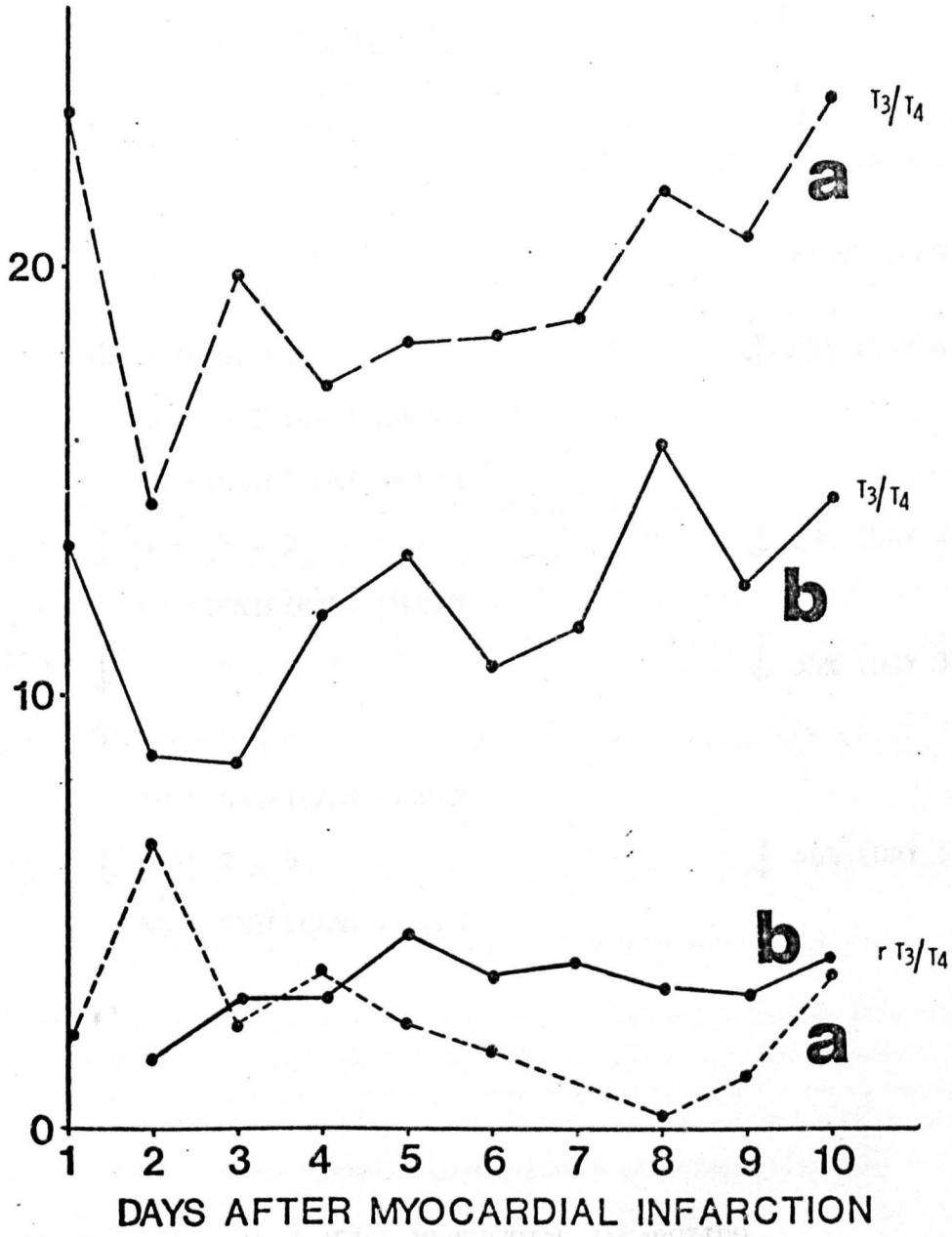
T_4, T_3, rT_3 (Fig. 4.11)

Basal serum T_4 was 130 $\pm$ 31 nmol/l, and no significant change occurred during a 4-day fast (123 $\pm$ 27 nmol/l). Serum T_3 was 3.2 $\pm$ 0.6 nmol/l basally, falling to 2.8 $\pm$ 0.6 nmol/l after 24 hours ($P < 0.05$) and to 1.9 $\pm$ 0.4 nmol/l after 4 days ($P < 0.0005$). Basal rT_3 was 1.0 $\pm$ 0.1 nmol/l, rising to 1.5 $\pm$ 0.3 nmol/l after 4 days' fasting ($P < 0.0025$). Fasting resulted in a 41% reduction in T_3 and a 54% increase in rT_3.

TBG, TBPA and albumin (Fig. 4.12)

TBPA was reduced after 24 hours (basal = 392 $\pm$ 77 mg/l, 24 hours = 387 $\pm$ 65 mg/l, $P < 0.05$) and fell by a mean of 28% to 281 $\pm$ 38 mg/l after 4 days ($P < 0.0005$). TBG fell by a mean of 9% after 4 days (basal = 15.3 $\pm$ 2.7 mg/l, 4 days = 13.9 $\pm$ 2.1 mg/l, $P < 0.005$). Serum albumin was not diminished by fasting.

Figure 4.10



T_3/T_4 AND rT_3/T_4 RATIOS AFTER MYOCARDIAL INFARCTION IN TWO PATIENTS

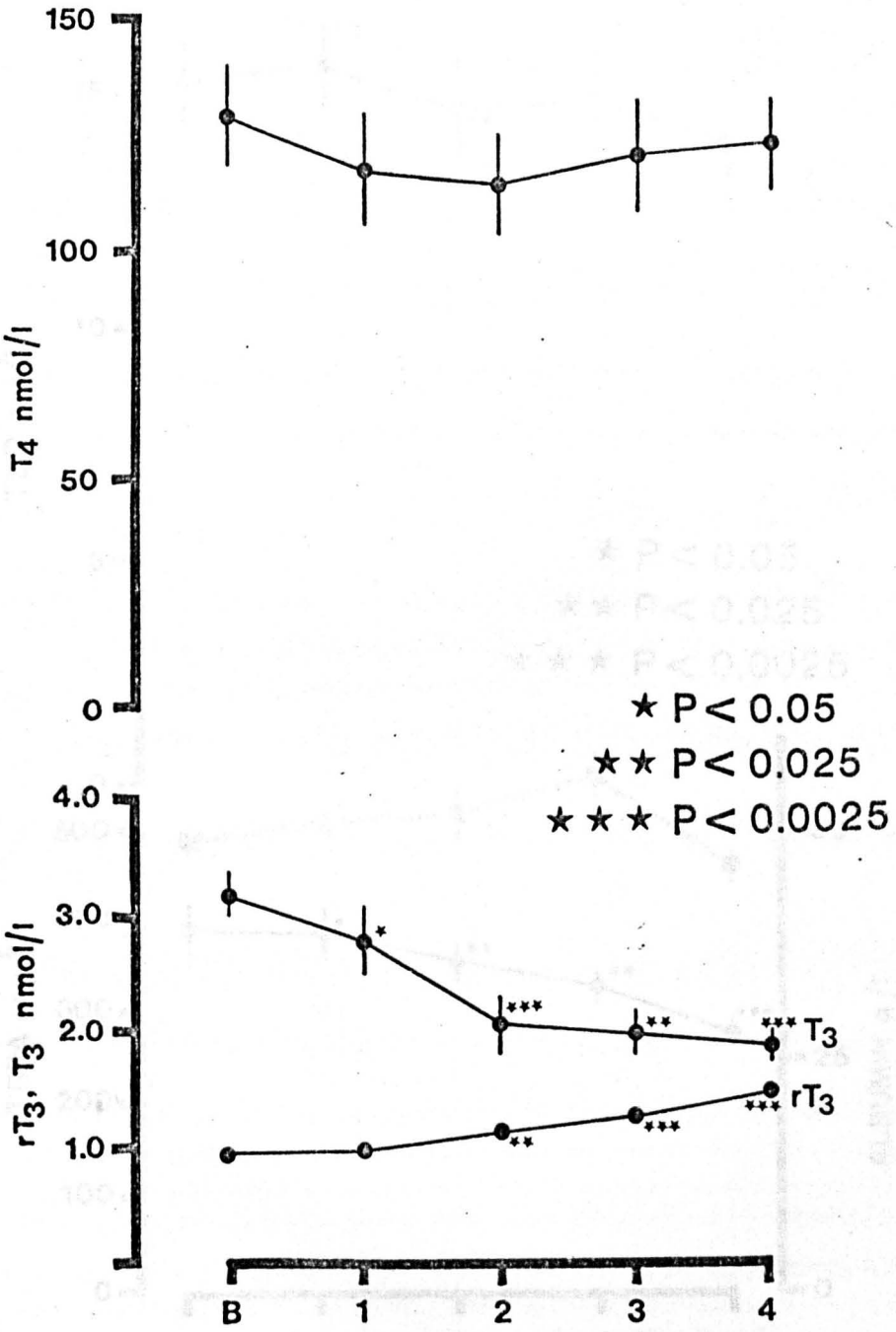
Table 4.3

DAY 1 = ONSET OF INFARCTION

		MAXIMUM CHANGE
T_4	↓ DAYS 4, 5, 7	↓ 15% (DAY 4)
$\%FT_4$	NO SIGNIFICANT CHANGE	
AFT_4	NO SIGNIFICANT CHANGE	
T_3	↓ DAYS 3 - 9	↓ 29% (DAY 4)
$\%FT_3$	NO SIGNIFICANT CHANGE	
AFT_3	↓ DAYS 2 - 5, 8	↓ 35% (DAY 3)
rT_3	↑ (?DAYS 2 - 5)	
TSH	NO SIGNIFICANT CHANGE	
TBPA	↓ DAYS 2 - 9	↓ 48% (DAY 5)
TBG	NO SIGNIFICANT CHANGE	

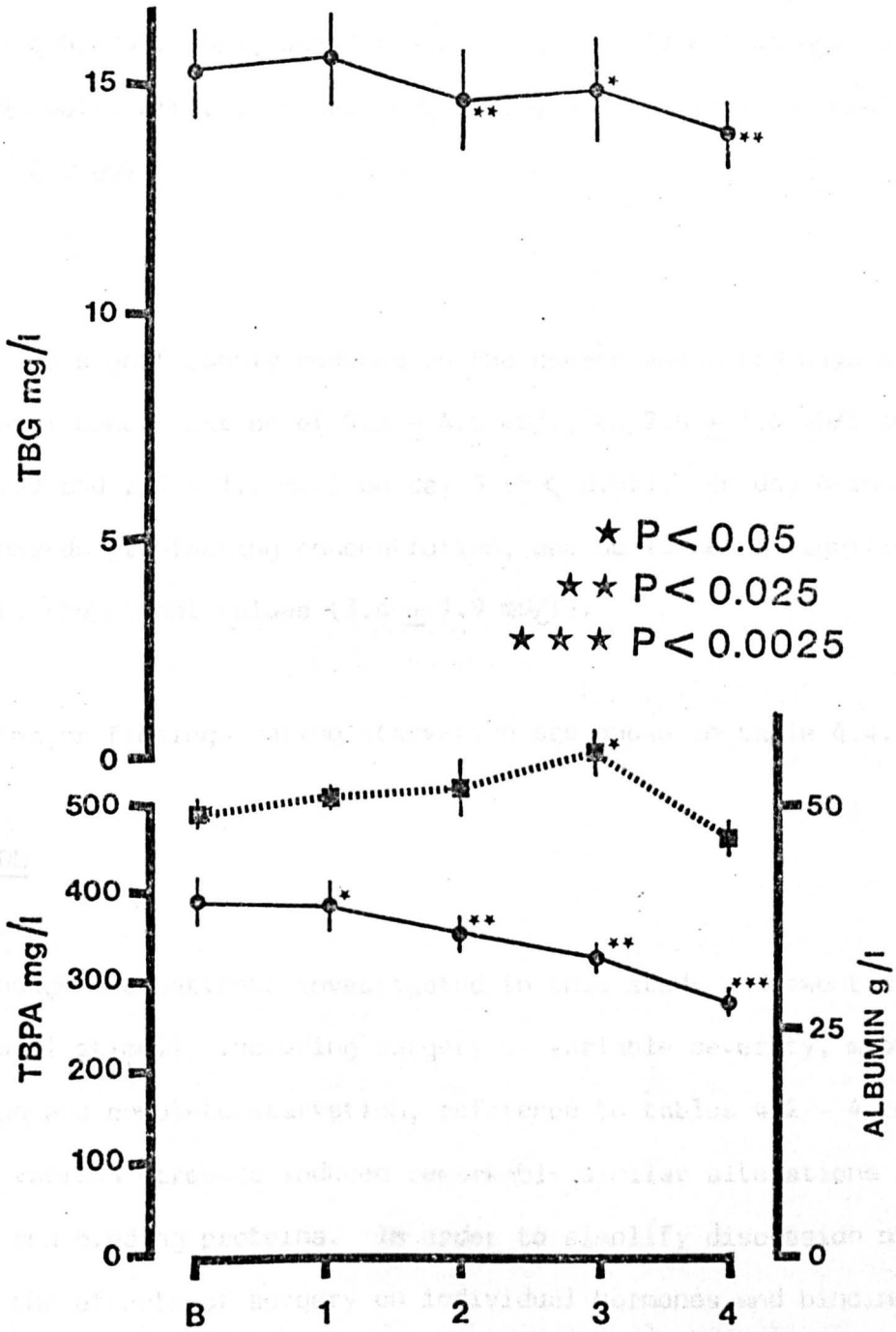
SUMMARY OF FINDINGS AFTER MYOCARDIAL INFARCTION

Figure 4.11



T_3, T_4 AND rT_3 CONCENTRATION DURING STARVATION

Figure 4.12



TBG, TBPA AND ALBUMIN CONCENTRATION DURING STARVATION

AFT_3 and AFT_4 (Fig. 4.13)

Calculated free T_4 was unchanged for the first 3 days of fasting and rose slightly on the fourth day (basal = 35 ± 16 pmol/l, day 4 = 39 ± 16 pmol/l, $P < 0.025$). AFT_3 was 7.0 ± 2.2 pmol/l before fasting, falling to 5.6 ± 1.9 pmol/l after 24 hours ($P < 0.025$) and to 4.8 ± 1.7 pmol/l after 4 days ($P < 0.0025$).

TSH

TSH was significantly reduced on the second and third days of fasting, from a basal concentration of 5.3 ± 4.0 mU/l, to 2.6 ± 1.5 mU/l on day 2 ($P < 0.025$) and 2.7 ± 1.5 mU/l on day 3 ($P < 0.05$). On day 4 there was a return towards pre-fasting concentration, and no longer a significant difference from basal values (3.4 ± 1.9 mU/l).

The major findings during starvation are shown in table 4.4.

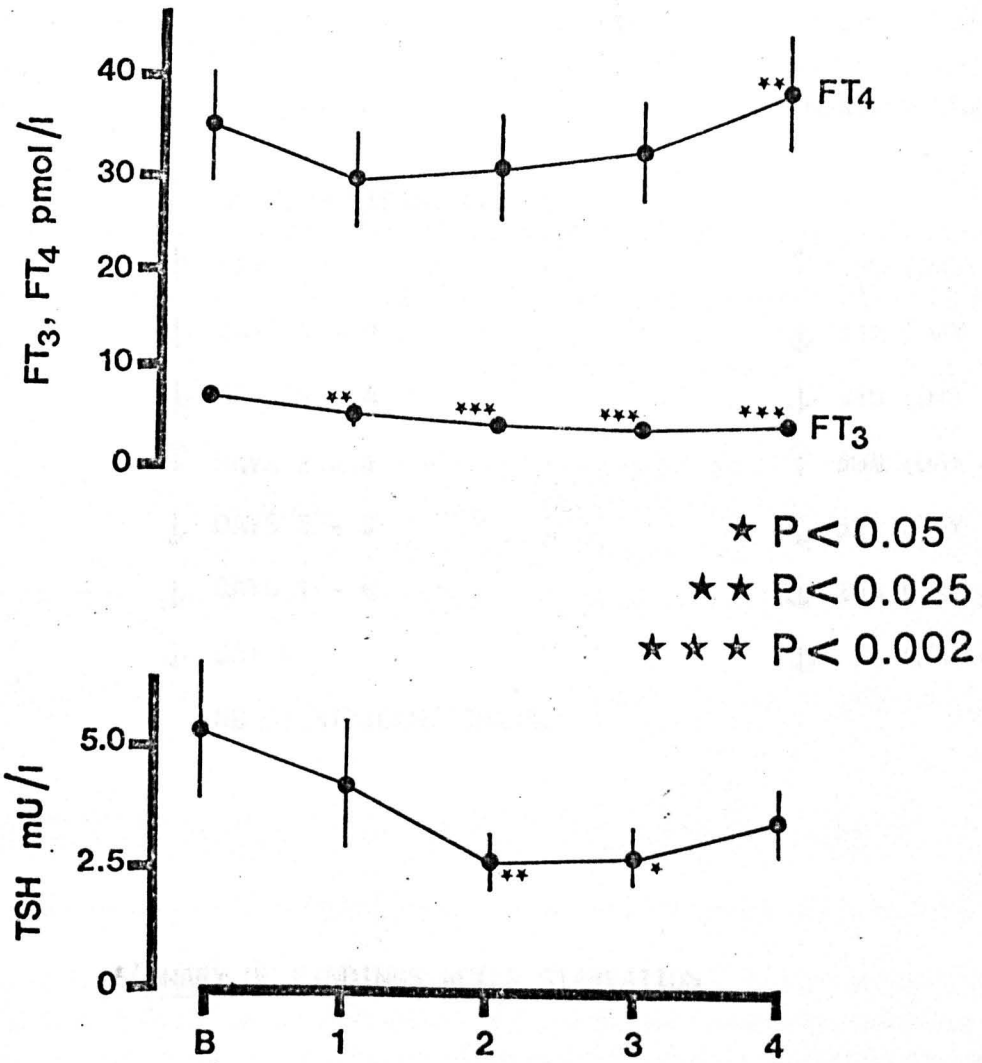
DISCUSSION

Although the patients investigated in this study underwent a variety of stressful stimuli, including surgery of variable severity, myocardial infarction and complete starvation, reference to tables 4.2 - 4 reveals that the various stresses induced remarkably similar alterations in thyroid hormones and binding proteins. In order to simplify discussion of these changes, the effects of surgery on individual hormones and binding proteins will be mentioned in turn, together with any specific alterations noted after myocardial infarction or starvation.

TBPA

Reduction in TBPA after surgery has been noted in the past (Minchin-Clarke et al, 1971; Aronsen et al, 1972; Kirby et al, 1973; Wandall, 1974;

Figure 4.13



FT_3, FT_4 AND TSH CONCENTRATION DURING STARVATION

Table 4.4.

		MAXIMUM CHANGE
T_4	NO SIGNIFICANT CHANGE	
AFT_4	↑ DAY 4	↑ 15% (DAY 4)
T_3	↓ DAYS 1 - 4	↓ 41% (DAY 4)
AFT_3	↓ DAYS 1 - 4	↓ 31% (DAY 4)
rT_3	↑ DAYS 2 - 4	↑ 50% (DAY 4)
TSH	↓ DAYS 2 - 3	↓ 51% (DAY 2)
TBPA	↓ DAYS 1 - 4	↓ 28% (DAY 4)
TBG	↓ DAY 4	↓ 9% (DAY 4)
ALBUMIN	NO SIGNIFICANT CHANGE	

SUMMARY OF FINDINGS AFTER STARVATION

Ramsden et al, 1979). Decrease in TBPA has been noted after anaesthesia and prior to skin incision (Wandall, 1974), and in the present study, a 12% fall was noted by the time of the first post-operative sample after 6 hours. The TBPA reduction is by no means unique to surgery, and has been noted in chronic illness, after myocardial infarction and after fasting (see Chapter 1.5.b.). Calorie withdrawal alone seems to account for only part of the TBPA change after surgery, since a complete fast for 4 days caused only a 28% reduction in TBPA, while "major" surgery caused a 52% reduction, minor surgery 23% and myocardial infarction 48%. It is not known whether tissue injury or inflammation contribute to the TBPA change, and the role of catecholamines has not been investigated. The adrenocortical response to stress is unlikely to be a causative factor since synthetic steroids cause an increase in TBPA concentration (Oppenheimer and Werner, 1966; Burr et al, 1976).

Studies using radio-iodinated purified TBPA have shown that the low concentrations after surgery and illness are probably due to reduced synthesis (Socolow et al, 1965; Prince, 1978).

TBG

Binding capacity measurements of TBG have been reported to show no consistent alteration after operation (Surks and Oppenheimer, 1964; Kirby et al, 1973), while a number of studies have claimed that the number of T_4-TBG binding sites (measured as resin uptake) is reduced after surgery (Brandt et al, 1976; Adami et al, 1978; Prescott et al, 1979). The above tests are either insensitive, indirect, or both, and cannot be considered to be strong evidence against the present finding of a slight (9%) but definite post-operative reduction of TBG. No change of TBG concentration was found after myocardial infarction, but starvation caused a similar decrease to that found after surgery. In surgical patients, intravenous

fluid therapy and post-operative fluid retention may have contributed to the reduced TBG concentration. There have been no kinetic studies of TBG metabolism after surgery, although increased catabolism with normal TBG synthesis was found in one severely ill patient (Refetoff et al, 1976). Reduced TBG concentration has been described previously in severe illness (Harvey, 1971), and a 50% reduction was noted eight days after surgery in a septicaemic patient in this study.

Albumin

A 22% reduction in serum albumin was found after surgery, and similar changes have been reported previously after surgery (Birke et al, 1959; Mouridsen, 1967), and in infection (Grossman, Yalow and Weston, 1960). The catabolic rate of albumin has been found to increase after operation (Birke et al, 1959), although Mouridsen (1967) found no change in catabolic rate, and attributed the fall in concentration to a shift into the extra-vascular space, especially the wound area.

It seems probable that the reduction in albumin concentration is mediated, at least in part, via the action of adrenal corticosteroids (Grossman et al, 1960).

T_4

A slight post-operative reduction of T_4 was found, and has been observed in some other studies (Adami et al, 1978; Chan et al, 1978). It has been suggested that the fall in T_4 is due to reduced thyroidal secretion, (Chan et al, 1978) but another explanation would be that the T_4 reduction is secondary to the reduction of TBG concentration which occurred at the same time.

It appears that T_4 may undergo different responses after surgery

compared to those found after other stresses. Harland et al (1972a) found that T_4 production rate was increased after surgery, while no increase occurred after myocardial infarction (Harland et al, 1972b). These results have to be interpreted with caution, since certain anaesthetic agents, notably halothane, may have profound effects in displacing T_4 from protein binding sites (Oyama, 1973), and in addition it has been suggested that anaesthetic agents may cause large alterations in T_4 distribution between intravascular and extravascular fluids (Adami et al, 1978; Chan et al, 1978).

In the present study, there was a slight reduction in T_4 concentration after myocardial infarction, although no change in TBG occurred. Starvation caused no significant change in T_4.

$\underline{T_3 + rT_3}$

The marked changes in T_3 and rT_3 concentration found in the present study after surgery, myocardial infarction and starvation confirm the reports of many other groups with respect to changes in acute and chronic illness and malnutrition, after surgery, and after fasting (see Chapter 1.5.b.).

Kinetic studies of T_4, T_3 and rT_3 metabolism in the above situations suggest that the changes result from diminished T_3 production, together with normal or increased rT_3 production, and diminished rT_3 degradation (Chopra, Sacks and Fisher, 1975; Vagenakis et al, 1977; Eisenstein et al, 1978; Suda et al, 1978).

The results are compatible with the suggestion that there are separate deiodinases for thyroxine , acting at the 5' position to produce T_3 from T_4 and 3, 3' T_2 from rT_3, and at the 5 position to produce rT_3 from T_4 and 3, 3' T_2 from T_3. Relative inhibition of the 5'-deiodinase

would lead to the changes described above.

With regard to the reduction of T_3 and increase of rT_3 after surgery, it has been suggested that adrenocortical steroids might be responsible, since pharmacological doses of corticosteroids induced similar changes (Duick et al, 1974; Burr et al, 1976). However, this suggestion became untenable when Brandt et al (1976) showed that the changes of thyroid hormones aftersurgery occurred with epidural anaesthesia, which blocked the cortisol response to surgery. The above authors speculated as to the role of psychic stress or of increased catecholamines in causing altered thyroid hormone metabolism.

Another factor of possible aetiological significance, which was given little consideration in earlier studies, is that of altered dietary intake. In the present study, T_3 was decreased by 49% after major surgery, by 25% after minor surgery, by 29% after myocardial infarction and by 41% after fasting. The calorie intake of surgical and myocardial infarction patients was not monitored, but it seems likely that major surgical patients would have been fasted completely for up to sixteen hours, whereas myocardial infarction and minor surgery patients would have had some reduction of calorie intake. The findings of the present study therefore raise the possibility that the low T_3 concentrations after surgery and myocardial infarction may be due entirely to reduced calorie intake (Davidson and Chopra, 1979). However, further analysis of the timing of T_3 changes in relation to surgery and fasting, shows that T_3 was reduced by 25% within 6 hours of major surgery, while starvation resulted in only a 12.5% fall after 24 hours. The timing of T_3 changes after surgery and infarction would therefore suggest that there are additional influences apart from simple calorie withdrawal.

Free T_3 and free T_4

A marked increase in percentage free T_4 and in free T_4 concentration was found post-operatively, and confirmed previous reports (Surks and Oppenheimer, 1964; Kirby et al, 1973; Chan et al, 1978; Prescott et al, 1979). This was also consistent with the findings of Ramsden et al (1979), who found an increase in urinary T_4 after surgery which may have resulted from an increase in free hormone. The increased free T_4 after surgery has been attributed to the low TBPA concentration (Surks and Oppenheimer, 1964), although other workers have maintained that additional factors must be invoked to account for such a marked increase (Kirby et al, 1973). Increased free T_4 in some illnesses has been attributed to the presence of inhibitors of T_4 binding (Lutz et al, 1969), and it may be pertinent to seek for evidence of such inhibitors after surgery. In order to do this, the measurements of total T_3, T_4, TBPA, TBG and albumin in the surgical patients have been used to derive predicted values of free T_3 and free T_4 concentration, using a computer-based model of thyroid hormone - TBP interaction (Prince and Ramsden, 1977). These results were then compared with actual free hormone measurements obtained in the same sera by the method of Finucane and Griffiths (1976).

The results are given in Table 4.5. This shows that although there is a systematic difference between measured and predicted concentration, especially with respect to free T_4, when the respective percentage changes are compared, there is remarkably good agreement. This suggests that the binding protein changes are sufficiently large to account directly for changes in free T_4 and free T_3, without invoking the presence of binding inhibitors. After myocardial infarction, there were no significant changes in measured free T_4, and only slight changes were predicted by the computer model (Table 4.5). Direct measurements of free hormone were not made after starvation, but a significant increase of free T_4 was calculated on day 4.

Table 4.5

SURGERY TIME	AFT_4 MEASURED pmol/l	AFT_4 PREDICTED pmol/l	AFT_4 MEASURED % CHANGE	AFT_4 PREDICTED % CHANGE	AFT_3 M* pmol/l	AFT_3 P pmol/l	AFT_3 M %	AFT_3 P %
0	52.4	44.6	-	-	5.5	5.4	-	
6 hrs	67.4	55.6	+29	+25	5.4	4.8	- 2	-11
Day 1	57.0	46.1	+ 9	+ 3	3.6	3.3	-35	-39
" 2	65.8	55.5	+26	+24	4.5	4.2	-18	-22
" 3	66.3	55.9	+27	+25	4.0	3.8	-27	-30
" 4	63.6	52.1	+21	+17	3.6	3.6	-35	-33
" 5	62.9	55.9	+20	+25	3.2	3.5	-42	-35
" 6	63.3	59.2	+21	+33	4.1	4.9	-25	- 9
MYOCARDIAL INFARCTION								
Day 1	59.5	54.9	-	-	7.8	7.9	-	-
" 2	60.8	51.6	+ 2	- 6	5.4	6.7	-31	-15
" 3	57.7	54.2	- 3	-13	5.0	6.3	-36	-20
" 4	67.3	50.4	+13	- 8	5.6	5.8	-28	-27
" 5	69.2	52.0	+16	- 5	5.2	5.8	-33	-27
" 6	-	53.0	-	- 3	6.2	6.6	-21	-13
" 7	64.2	52.1	+ 8	- 5	5.7	6.2	-27	-22
" 8	62.5	53.0	+ 5	- 3	5.5	6.0	-29	-24

* M : Measured P : Predicted

COMPARISON BETWEEN MEASURED AND PREDICTED FREE T_4 AND FREE T_3 CONCENTRATION AFTER SURGERY AND MYOCARDIAL INFARCTION

Measured and predicted free T_3 concentration were reduced after surgery, infarction and starvation, and this occurred in spite of an increase in percentage free T_3.

TSH

No change in TSH concentration occurred after surgery in the present study, and this is in agreement with several previous reports (Kirby et al, 1973; Chan et al, 1978; Prescott et al, 1979). An early (12 - 24 hour) post-operative reduction of TSH was however noticed by Charters et al (1969) and by Kehlet et al (1979). A significant reduction of TSH on the second and third days of fasting was noted in the present study, and there is considerable conflict in the literature regarding the effect of starvation on TSH. No change in basal TSH was found by several groups (Portnay et al, 1974; Carlson et al, 1977; Merimee and Fineberg, 1977), while reduced basal TSH was noted in other studies (Rothenbuchner et al, 1973; Croxson et al, 1977; Palmblad et al, 1977; Azizi, 1978). Conflicting TSH results have also been reported after TRH injection during fasting. The response has been found to be normal (Portnay et al, 1974; Gardner et al, 1979) or reduced (Vinik et al, 1975; Carlson et al, 1977; Azizi, 1978). In spite of the conflicting results reported above, it is evident that both the TSH basal concentration and response to TRH injection in fasting are either normal or reduced, but are nevertheless inappropriate for the marked reduction in free T_3 concentration which occurs. The same is also true of the normal or low TSH concentration after operation, in spite of very low free T_3 concentration. In the case of post-surgical patients however, there is a clear increase of free T_4, while in starvation, an increase of free T_4 was not noted until day 4, when TSH concentration was returning to normal. It is possible that an increase of free T_4 concentration after surgery may compensate for a low free T_3 concentration and this raises the question of the relative importance of free T_4 and free T_3 in pituitary

feedback inhibition of TSH. Recent work by Larsen and co-workers (Silva and Larsen, 1978; Larsen et al, 1979) has demonstrated that a significant proportion of pituitary intra-nuclear T_3 is derived from T_4 deiodination within the pituitary cell. This serves to emphasise the possible importance of free T_4 in governing TSH levels.

In conclusion, the results of the present study would suggest that after surgery TSH is unchanged in spite of low free T_3 concentration, and that this may be due to feedback inhibition by increased free T_4. In contrast, in starvation, TSH levels are reduced at a time when free T_3 concentration is low, and no increase in free T_4 concentration has occurred. It is suggested that in starvation there are additional factors which mediate TSH suppression at either hypothalamic or pituitary level.

Chapter 5 HEREDITARY ABNORMALITIES OF TBG CONCENTRATION

1) Introduction

2) Patients and families studied

3) Results
   a) Case reports 1 - 9
   b) Immunological and electrophoretic properties of TBG in familial TBG abnormality
   c) Serum TBPA concentration
   d) Inheritance of TBG abnormality
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      i) TBG elevation
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4) Discussion
   a) Electrophoretic and immunological characteristics of TBG
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Chapter 5 HEREDITARY ABNORMALITIES OF TBG CONCENTRATION

INTRODUCTION

The first report of inherited variation of TBG was that of Beierwaltes and Robbins (1959), who described a family with increased TBG. Sporadic cases of decreased and absent TBG were also reported, (Tanaka and Starr, 1959a, b) but the first family with diminished TBG was described by Nicoloff, Dowling and Patton (1964). It is now established that the mode of inheritance of increased, decreased and absent TBG is of the sex-linked, codominant type (see Chapter 1).

Most individuals with abnormal TBG concentration have been clinically euthyroid, but an association of diminished TBG and thyrotoxicosis was found in five individuals by Horwitz and Refetoff (1977) and in another two cases by Strunge (1974) and Gerstner and Caplan (1976). In one well-documented case of thyrotoxicosis, hereditary TBG elevation was inferred from study of the patient's family (Hodgson and Wahner, 1972), but in four other patients with increased TBG who were treated for thyrotoxicosis, the evidence for thyrotoxicosis was unconvincing (Florsheim et al, 1962; Ingbar, Waterhouse and Cushman, 1964; Fialkow, Giblett and Musa, 1970; Shane, Seal and Jones, 1971).

In the years 1975 - 1979, during which TBG assays were performed for the present study, a number of individuals with hereditary TBG abnormalities were identified. From the clinical histories of these patients, it was apparent that considerable problems could be associated with the diagnosis of TBG abnormality, especially when this co-existed with thyroid disease. For this reason, the case histories of several individuals have been examined in an attempt to identify the causes of diagnostic difficulty.

The typical biochemical features of TBG abnormality have been tabulated, and immunoelectrophoretic studies have been carried out to confirm earlier reports that hereditary TBG abnormality results from abnormal concentrations of an immunologically and electrophoretically normal protein (Refetoff et al, 1972, 1976).

2) PATIENTS AND FAMILIES STUDIED

A total of 32 index cases with abnormal TBG concentration were studied. These comprised individuals seen by the author, as well as other cases referred from hospitals in the Birmingham area. Serum samples for individual and family studies were also received from other hospitals in the United Kingdom, with a particularly large contribution from Dr. W. van't Hoff, Consultant Physician, North Staffordshire Royal Infirmary, Stoke-on-Trent. Full clinical details were obtained of those individuals referred from other centres.

Family studies were carried out which confirmed the hereditary nature of TBG abnormality in 19 families (A - S). In 13 other individuals hereditary TBG abnormality was inferred from the absence of any other factors known to alter TBG concentration. A total of 15 males with absent TBG and 15 females heterozygotic for absent TBG were identified; 14 males had excess TBG and 44 females were heterozygotic for increased TBG. 48 family members were found to have normal TBG, and comprised the control population for statistical comparisons.

Although the majority of individuals with abnormalities of TBG concentration were eventually shown to be euthyroid, 4 individuals were found to have co-existent TBG excess and thyrotoxicosis, and 3 individuals had TBG excess and myxoedema.

3) RESULTS

a) Clinical material

The clinical details and family studies of 9 probands are included (Families A-I), together with 2 further individuals in whom family studies were not performed. In general, the cases have been selected to demonstrate the mode of inheritance of TBG abnormalities, together with the diagnostic and management problems associated with these patients.

Family trees of a further ten probands (J-S) are given in appendix F.

Case reports

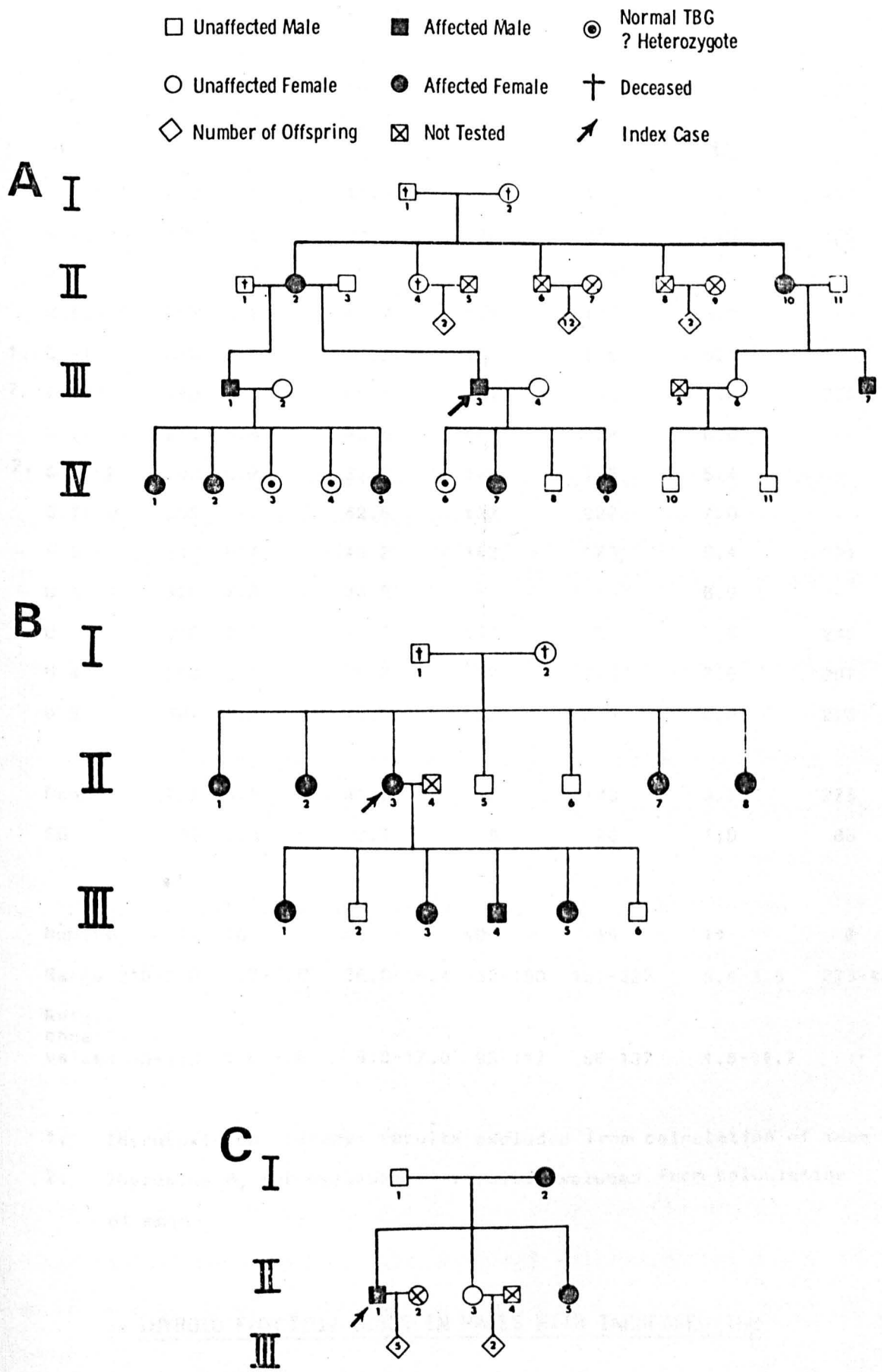
Euthyroid individuals with increased TBG

1) Family A III 3 (Fig. 5.1, Table 5.1). This 33-year-old male presented with an 8-month history of lassitude, increased sweating, heat intolerance, loose bowel actions and weight loss of 3 kg. in spite of good appetite. On examination his pulse was 80 beats/minute and regular, there was no finger tremor and the thyroid was not palpable.

Investigations: T_4 300 nmol/l, T_3 uptake 130%, FTI 231, T_3 6.8 nmol/l. 24-hour thyroidal ^{131}I-uptake 49.9% basal, and after L-T_3 40 µg thrice daily for one week, 16.8%. TRH test (200 µg iv at time 0) TSH 0 min = 7 mU/l, 30 min = 16 mU/l, 60 min = 15 mU/l.

The patient was thought to be thyrotoxic on the basis of clinical history and raised FTI and T_3. However, the absence of a palpable thyroid and the presence of raised T_3 uptake led to a suspicion of raised T_4-binding protein. Dynamic tests excluded thyrotoxicosis, and serum TBG was found to be elevated at 43.6 mg/l, with normal T_4:TBG ratio (6.9). The patient was treated for anxiety with propranolol for 3 months. This was

Figure 5.1



HEREDITARY TBG INCREASE, FAMILIES A - C

Table 5.1

	Case	T4 (nmol/l)	T3 (nmol/l)	TBG (mg/l)	T3U (%)	FTI	T4:TBG	TBPA (mg/l)
	A III 1	260	3.4	41.9	150	173	6.2	430
	A III 3	300	6.8	43.6	138	217	6.9	225
	A III 7	267	4.6	40.2	160	167	6.6	-
	B III 4	257	5.8	40.7	141	182	6.3	-
1.	C II 1	240	4.5	47.0	154	156	5.1	-
2.	F III 2	440	-	49.8	161	273	8.8	225
	G II 1	250	5.8	42.0	144	174	6.0	-
2.	G II 2	203	6.2	37.8	140	145	5.4	-
	Q II 1	300	-	42.6	132	227	7.0	-
	S I 1	242	6.4	45.2	142	170	5.4	321
	U 1	320	7.8	36.0	-	-	8.9	-
	U 3	210	7.2	36.3	139	151	5.8	246
	U 4	300	5.6	39.7	139	216	7.6	207
	U 5	301	3.2	45.4	133	226	6.6	223
	Mean	273	5.7	41.2	142	190	6.7	275
	SD	33	2.3	3.1	8	28	1.0	86
	Number	11	10	11	10	10	11	6
	Range	210-320	3.2-7.8	36.0-45.4	132-160	151-227	5.4-8.9	223-430
	Reference values	60-130	1.6-3.6	6.0-17.0	93-117	56-137	4.8-11.7	-

1. Thyrotoxic on therapy: results excluded from calculation of mean
2. Thyroxine P_X for myxoedema: results excluded from calculation of mean

THYROID FUNCTION TESTS IN MALES WITH INCREASED TBG

discontinued, and eighteen months later the patient remains well on no medication.

Family studies (Fig. 5.1) confirmed the presence of raised TBG in the patient's mother, step-brother and maternal aunt. Two daughters had elevated TBG, but one daughter had thyroid function tests within normal limits (Fig. 5.1, Table 5.2, Case A IV 6).

Elevated FTI and serum T_3 together with suggestive history very nearly caused mis-diagnosis of hyperthyroidism in this patient. However, abnormal elevation of T_3 uptake suggested the need for further tests which established the correct diagnosis. This illustrates how misleading FTI can be when TBG is markedly elevated.

2) Case U1 (Table 5.1). This man presented at the age of 46 in June, 1978. He had atrial fibrillation and congestive cardiac failure of unknown cause. Serum T_4 was estimated as a routine and was found to be 265, 255 and 320 nmol/l on three occasions, T_3 was 7.8 nmol/l. The patient was thought to be thyrotoxic and was treated with digoxin and referred for ablative therapy with ^{131}I. 24-hour ^{131}I thyroidal uptake was found to be normal (28%), and serum TBG was subsequently found to be elevated at 36 mg/l, while T_4:TBG ratio (8.9) was normal. The patient has remained clinically euthyroid during follow-up for one year with no antithyroid medication.

3) Family B II 3 (Fig. 5.1, Table 5.2). This female patient aged 42 years, presented with symptoms of lethargy, cold intolerance and hair loss, and was noted to have a small firm goitre. Thyroxine was 211 nmol/l, T_3 uptake 141% and FTI 148. Four hour thyroidal ^{131}I-uptake was 21.9% (normal range 6 - 36%), and basal TSH was 3.8 mU/l, rising to 10.6 mU/l 30 minutes after TRH.

Table 5.2

	Case	T4 (nmol/l)	T3 (nmol/l)	TBG (mg/l)	T3U (%)	FTI	T4:TBG	TBPA (mg/l)
	A II 2	204	4.8	20.6	129	158	9.9	215
	A II 10	168	2.0	22.2	131	128	7.6	-
	A IV 1	149	4.6	19.8	126	118	7.5	245
	A IV 2	184	4.8	28.1	140	131	6.5	210
1.	A IV 3	123	-	12.5	124	99	9.8	230
1.	A IV 4	150	-	13.2	121	124	11.4	185
	A IV 5	260	-	29.5	145	179	8.8	200
1.	A IV 6	111	-	12.8	119	93	8.7	190
	A IV 7	185	-	26.0	122	152	7.1	150
	A IV 9	147	3.8	18.6	124	119	7.9	225
	B II 1	202	3.1	34.4	131	154	5.9	298
	B II 2	226	4.7	46.9	141	160	4.8	276
	B II 3	211	4.7	32.9	141	150	6.4	-
	B II 7	250	4.4	45.0	139	180	5.6	250
	B II 8	204	3.7	38.8	145	141	153	253
	B III 1	199	3.4	32.3	131	152	6.2	316
2.	B III 3	269	4.3	47.8	145	186	5.6	237
2.	B III 5	283	3.8	51.7	141	201	5.5	310
	C I 2	206	5.0	49.3	127	162	4.2	-
2.	C II 5	343	5.1	57.6	129	266	6.0	-
	D II 3	114	3.0	16.8	108	106	6.8	-
3.	D II 4	236	4.7	30.4	135	175	7.8	-
	D III 1	142	3.0	25.8	118	120	5.5	-
3.	E I 2	280	4.9	32.7	135	207	8.6	-
	E II 3	153	2.6	21.0	111	138	7.3	-
	F II 1	300	3.9	45.5	152	197	6.6	245
	F III 1	276	9.0	37.6	153	180	7.3	-

THYROID FUNCTION TESTS IN FEMALES WITH INCREASED TBG

Table 5.2 (Cont.)

	Case	T4 (nmol/l)	T3	TBG (mg/l)	T3U (%)	FTI	T4:TBG	TBPA (mg/l)
	G I 2	226	41	35.0	134	169	6.5	-
	H II 2	204	4.2	35.3	126	162	5.8	-
	H II 3	183	3.8	22.8	122	150	8.0	382
4.	H II 4	80	2.8	19.1	121	66	4.2	346
	H III 2	144	3.7	29.5	118	122	4.9	240
	J II 7	210	5.3	30.4	130	162	6.9	200
	J III 4	174	4.6	18.9	127	137	9.2	-
	J IV 1	190	4.4	21.1	142	134	9.0	-
	Q III 2	140	2.9	18.1	111	126	7.7	-
	S II 1	128	4.0	21.1	116	110	6.0	327
	S II 2	166	3.7	21.4	120	138	7.8	211
	S II 3	172	4.0	23.0	125	138	7.5	321
3.	U 2	376	11.2	29.6	122	308	12.7	-
	U 6	186	4.0	25,2	12.8	145	7.4	-
	U 7	205	3.6	26.0	140	146	7.9	-
	U 8	282	3.4	29.0	126	224	9.7	248
	U 9	230	4.5	44.7	-	-	5.1	353
	Mean	189	4.1	27.9	129	145	7.2	251
	SD	47	1.1	10.0	11	27	1.6	58
	Number	37	32	37	36	36	37	23
	Range	111-300	2.0-9.0	12.5-49.3	108-153	93-224	4.2-11.4	150-382
	Reference Values	60-130	1.6-3.6	6.0-17.0	93-117	56-137	4.8-11.7	-

1. TBG normal, presumed heterozygote
2. Oral contraceptive P_X: results excluded from calculation of mean
3. Thyrotoxic on therapy: results excluded from calculation of mean
4. Borderline hypothyroid: results excluded from calculation of mean

Myxoedema had been suspected clinically, but the elevation of T_4 and T_3 uptake suggested elevation of TBG, and this was confirmed with a serum TBG of 32.9 mg/l and normal T_4:TBG ratio of 6.4. Dynamic studies confirmed that the patient was euthyroid and showed that four sisters and four of the patient's six children also had high TBG (Fig. 5.1).

Thyrotoxic individuals with increased TBG

4) Family C II 1 (Fig. 5.1, Table 5.1). This 41-year-old man presented to another hospital on 26.08.75 with a 7-month history of weight loss (20 kg), sweating, diarrhoea and protrusion of the eyes. He was noted to have a pulse rate of 120, fine finger tremor, proximal myopathy and pretibial myxoedema. He had severe proptosis, with limitation of eye movements in all directions, bilateral papilloedema, conjunctival oedema and exposure conjunctivitis and keratitis. T_4 was $>$ 12 µg/100ml ($>$ 154 nmol/l). He was treated by orbital decompression, and received carbimazole 10 mg three times daily and propranolol 40 mg three times daily for three months. Shortly after the drugs were discontinued, he redeveloped heat intolerance and weight loss. His physicians were puzzled by an apparent discrepancy between raised serum T_4 levels combined with an elevated T_3 uptake. Treatment was not recommenced over the course of several months, and the patient was lost to follow-up.

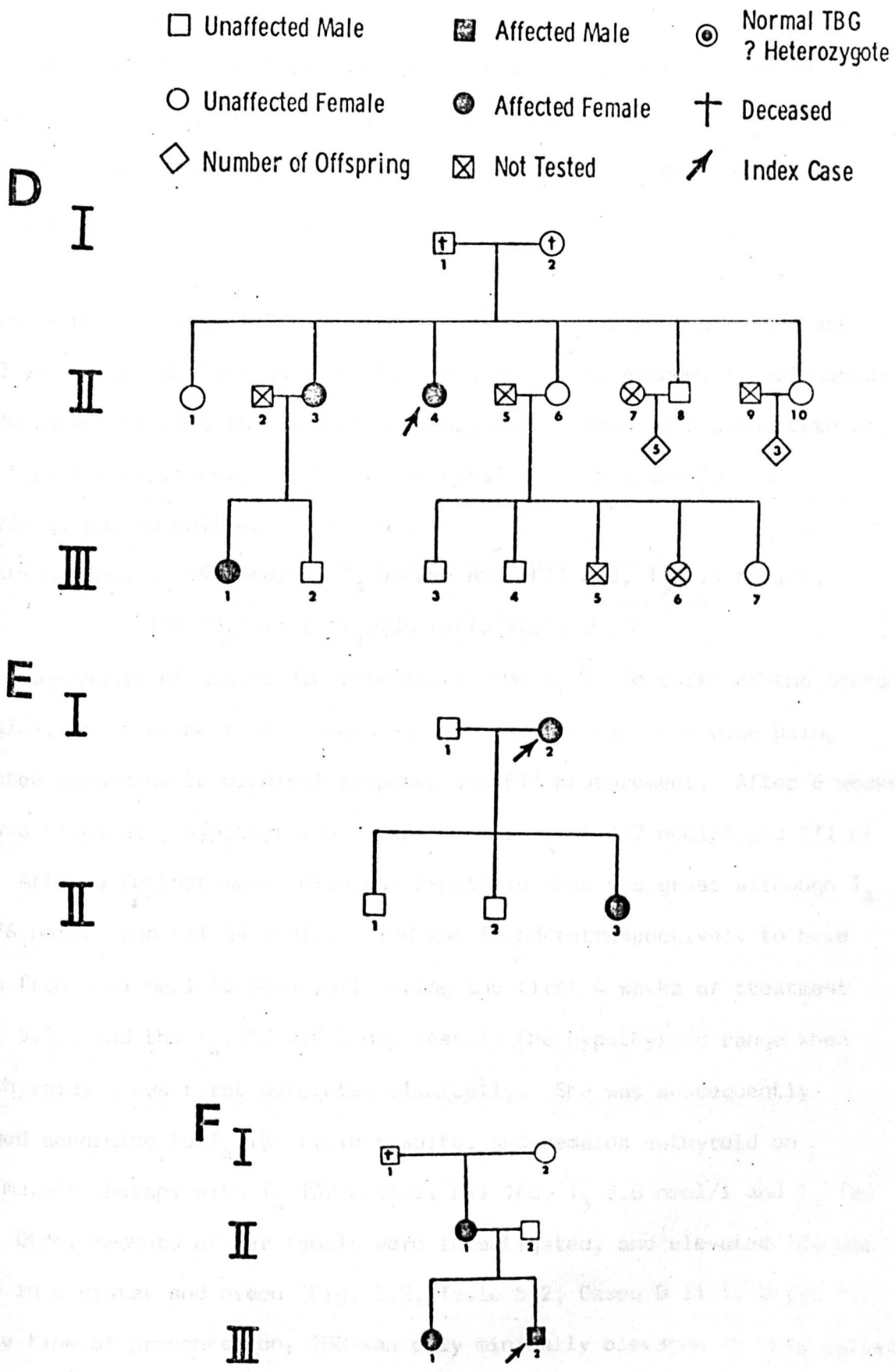
On 16.12.76 he presented at the Queen Elizabeth Hospital, Birmingham, and was grossly thyrotoxic with bilateral proptosis, ophthalmoplegia and severe chemosis.

Investigations: T_4 505 nmol/l, T_3 uptake 118%, FTI 428.

He was treated with ^{131}I 5 mCi, and subsequently with carbimazole. The chemosis resolved and the proptosis diminished.

Serum TBG was found to be elevated at 47 mg/l, and family studies

Figure 5.2



HEREDITARY TBG INCREASE, FAMILIES D - F

confirmed high TBG in his mother and one sister (Fig. 5.1, Table 5.2, Cases CI 2 and C II 5).

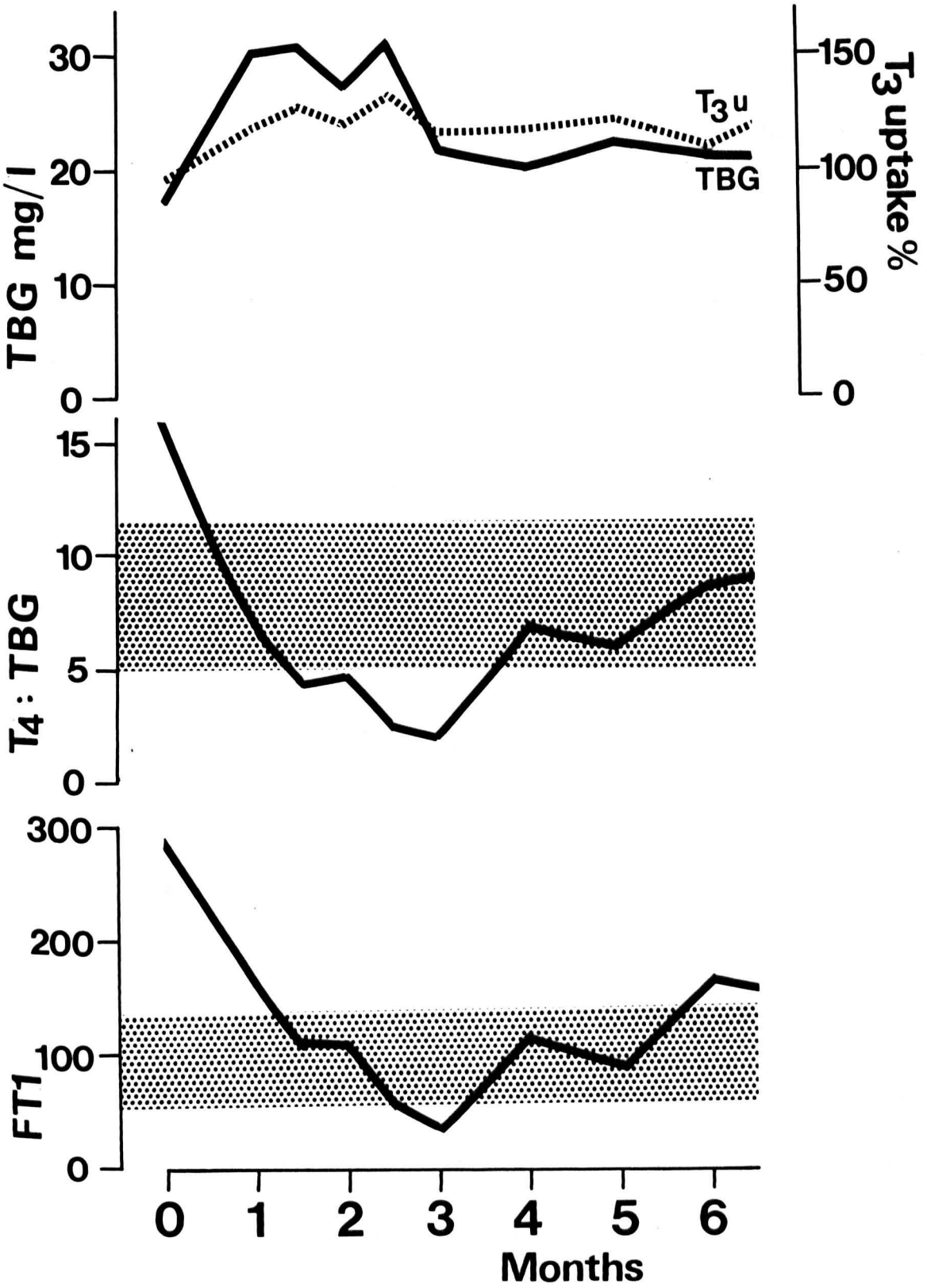
This patient's thyrotoxicosis was left untreated for several months because of confusion resulting from the combination of raised T_4 and raised T_3 uptake results. The delay may have caused deterioration of his eye condition.

5) Family D II 4 (Fig. 5.2, Table 5.2). This lady presented at the age of 50 years with protuberance of the eyes, heat intolerance, tremulousness and 20 kg weight loss in spite of good appetite. She had a pulse rate of 120, fine finger tremor, bilateral exophthalmos with chemosis and scleritis, but no goitre.

Investigations: T_4 390 nmol/l, T_3 uptake 88%, FTI 443, T_3 3.3 nmol/l, TBG 17.1 mg/l, T_4:TBG ratio 22.8.

A diagnosis of Graves' thyrotoxicosis was made, in spite of the normal T_3 value, and treatment was commenced with carbimazole, the dose being adjusted according to clinical response and FTI measurement. After 6 weeks, she was clinically hypothyroid, in spite of a T_4 of 137 nmol/l and FTI of 108. After a further month clinical hypothyroidism was gross although T_4 was 76 nmol/l and FTI 58 nmol/l. TBG was found retrospectively to have risen from 17.1 mg/l to 30.4 mg/l during the first 4 weeks of treatment (Fig. 5.3), and the T_4:TBG ratio had been in the hypothyroid range when hypothyroidism was first suspected clinically. She was subsequently managed according to T_4:TBG ratio results, and remains euthyroid on carbimazole therapy with T_4 189 nmol/l, FTI 160, T_3 3.0 nmol/l and T_4:TBG 9.0. Other members of her family were investigated, and elevated TBG was found in a sister and niece (Fig. 5.2, Table 5.2, Cases D II 3, D III 1). At the time of presentation, TBG was only minimally elevated in this patient, and further TBG measurements were not performed routinely because large

Figure 5.3



THYROID FUNCTION TESTS IN PATIENT D II 4

T_4:TBG ratio becomes subnormal after six weeks', while FTI remains normal until three months'. Also shown is the marked increase in TBG during control of thyrotoxicosis.

alterations of TBG concentration were not anticipated. Measurement of T_4: TBG ratio was found to correlate well with her clinical state and simplified management.

6) Family E I 2, (Fig. 5.2, Table 5.2). This lady presented at the age of 37 with a history of heat intolerance, palpitations, exertional dyspnoea and weight loss of 8 kg. She was found to have a pulse rate of 100, sweaty palms and a fine finger tremor.

Investigations: T_4 336 nmol/l, T_3 uptake 118%, FTI 285, 20-minute thyroidal ^{99m}Tc uptake 5.7% (normal limit 4%), 4 hour thyroidal ^{131}I uptake 57.5% (normal limit 36%).

The clinical diagnosis of thyrotoxicosis was confirmed, and she was treated with carbimazole for 2 years. Problems were encountered during treatment because of a persistent elevation of FTI in the face of clinical hypothyroidism. After two months without drug therapy, T_4 was found to be 280 nmol/l and FTI 207. Relapse was suspected, but TRH injection (200 µg iv) produced a TSH rise from 5.9 mU/l to 11.1 mU/l after 30 minutes. TBG was found to be elevated (32.8 mg/l), with a normal T_4:TBG ratio of 8.5. Family studies confirmed the presence of raised TBG in her daughter (Fig. 5.2, Table 5.2, Case E II 3).

7) Case U 2 (Table 5.2). This lady underwent partial thyroidectomy in 1958 and presented in 1974 at the age of 53 years. She was thought to be thyrotoxic and was treated with carbimazole until 1977. In 1978, she relapsed clinically and investigations revealed a T_4 of 351 nmol/l, T_3 uptake 120%, FTI 293 and serum T_3 20.8 nmol/l. The 20-minute ^{99m}Tc uptake was 15.8% (normal up to 4%), and there was no TSH response to TRH injection, 200 µg iv. After treatment with ^{131}I and carbimazole she became clinically euthyroid, but her physicians were puzzled by persistent elevation of T_4 and

FTI (T_4 = 286 nmol/l, T_3 uptake 138%, FTI 207). The problem was resolved when she was found to have elevated TBG (29.6 mg/l) and normal T_4:TBG ratio (9.7).

Over a 2-year period there were management problems with this thyrotoxic patient because of persistent elevation of T_4 and FTI which did not correspond with her clinical state.

Myxoedematous individuals with increased TBG

8) Family F III 2, (Fig. 5.2, Table 5.1). This boy presented in 1967 at the age of 4 months. His birth weight was 3.8 kg, and he had been jaundiced for the first 10 days of life. After 6 weeks, his mother noticed his large tongue, and that he was a slow feeder.

On examination he had cretinous facies, pallor, supraclavicular fat pads and an umbilical hernia. PBI was 0.5 µg/100 ml (equivalent to a T_4 of approximately 10 nmol/l), bone age was retarded at the tenth centile.

Hypothyroidism was diagnosed, and treatment with L-thyroxine 12.5 µg daily was commenced. The dose was increased to 75 µg daily and three months later PBI measured 15.0 µg/100 ml ($T_4 \sim$ 300 nmol/l). T_4 was reduced to 50 µg daily, and PBI rose to 22.8 µg/100 ml ($T_4 \sim$ 450 nmol/l). T_4 was then reduced to 25 µg daily. At age 2 years he was constipated, at the 3rd centile for height and with a wide fontanelle. Bone age was 6 months, and PBI 9.6 µg/100 ml ($T_4 \sim$ 190 nmol/l). T_4 was increased to 50 µg daily, and at age 4 years, to 100 µg daily, despite the fact that PBI determinations remained above the limit of assay ($>$ 15 µg/100 ml, $T_4 >$ 300 nmol/l). At the age of 6, bone age was less than 3 years, serum T_4 219 nmol/l and T_3 uptake 152%, and at 8 years, bone age was 5 years, T_4 300 nmol/l, T_3 uptake 126%, FTI 238 and TSH 19.5 mU/l. Serum TBG measured at this time

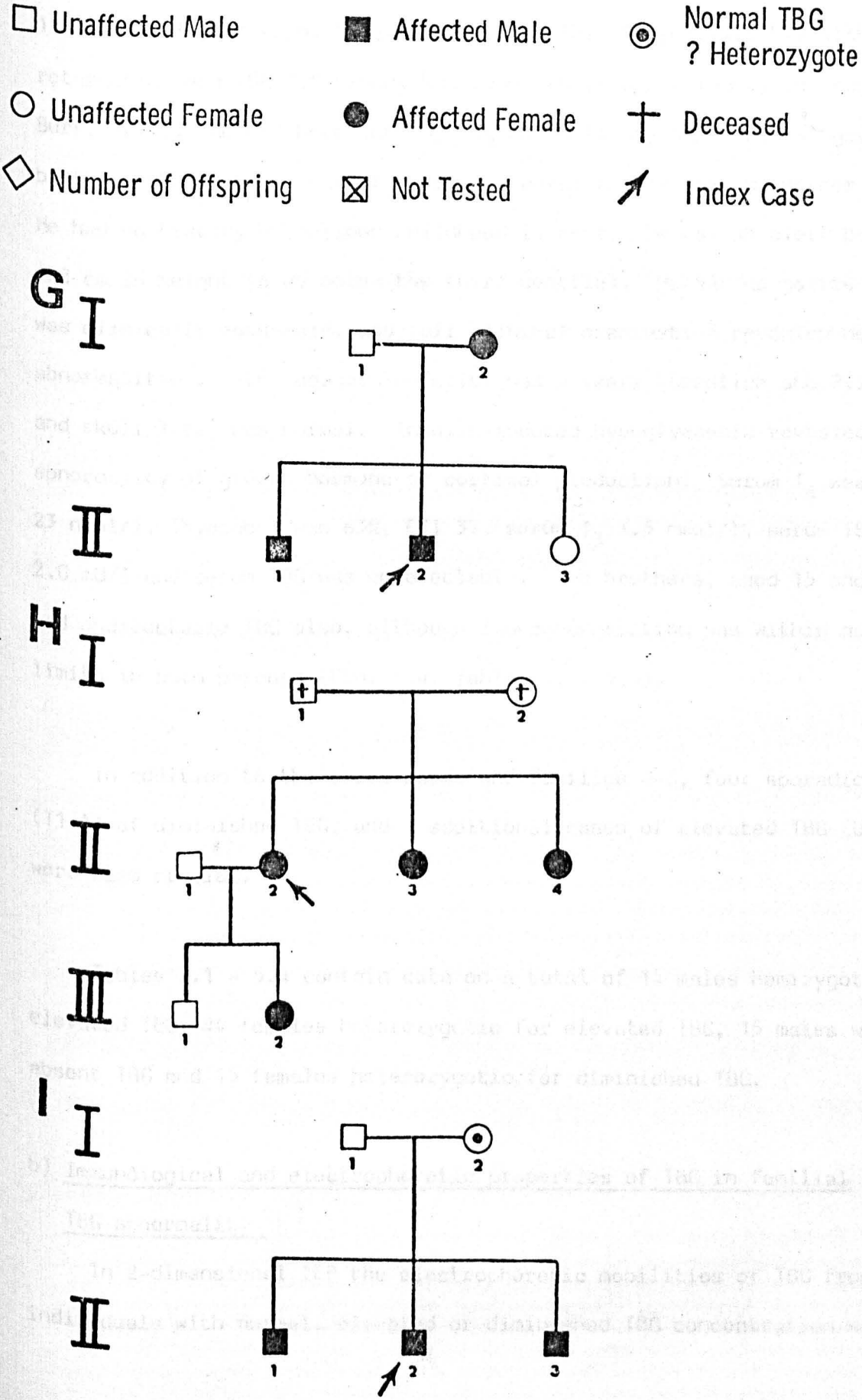
was 49.8 mg/l, and elevated TBG was also found in the patient's mother and sister (Fig. 5.2, Table 5.2, Cases F II 1 and F III 1). The T_4 dose was increased to 150 and then 200 µg daily. On this dose, serum T_4 was 440 nmol/l, FTI 273, T_4:TBG ratio 8.8 (normal) and TSH undetectable.

Three years later the patient is progressing normally. Elevated PBI and T_4 estimations caused by his hereditary TBG elevation resulted in under-treatment of his hypothyroidism for the first eight years of life. Inadequate treatment was confirmed by the advent of TSH assay.

9) Family G II 2, (Fig. 5.4, Table 5.1). This 19-year-old male was commenced on thyroxine treatment for hypothyroidism at the age of thirteen months. Some doubts arose concerning the diagnosis when thyroid function tests revealed a T_4 of 203 nmol/l and a serum T_3 of 6.2 nmol/l while on treatment with 100 µg of L-T_4 daily. The doubts were resolved by the finding of an elevated TBG concentration of 37.8 mg/l and family studies confirmed the hereditary nature of the condition (Fig. 5.4). A raised serum TSH of 16 mU/l suggested that he was not on adequate replacement therapy, in spite of an elevated FTI of 145.

10) Family H II 2, (Fig. 5.4, Table 5.2). Thyroid function tests were performed in this 56-year-old lady to exclude myxoedema as a cause of obesity. T_4 was 204 nmol/l, T_3uptake 126%, FTI 162, TBG 35.3 mg/l and T_4:TBG ratio normal at 5.8. Family studies confirmed hereditary TBG elevation in the patient's two sisters and daughter (Fig. 5.4, Table 5.2 cases H II 3, H II 4 and H III 2). However, in one sister (H II 4) serum T_4 was 80 nmol/l, T_3 uptake 121%, FTI 66, TBG 19.1 mg/l and T_4:TBG ratio 4.2 (low). Serum TSH was elevated at 39 mU/l, although serum T_3 was normal (2.8 nmol/l). In this relative, who was clinically euthyroid, some degree of thyroid insufficiency was suggested by the T_4:TBG ratio and confirmed

Figure 5.4



HEREDITARY TBG INCREASE AND DECREASE FAMILIES G - I

by TSH measurement, although FTI was within normal limits, and serum T_4 was well within normal limits.

11) Family I II 2 (Fig. 5.4, Table 5.3). This 11-year-old boy with growth retardation and TBG deficiency has been reported previously (Barragry and Burr, 1977). He had been born by vaginal delivery at 38 weeks' gestation, birth weight 1932 g. Parental heights were: father 156 cm, mother 144 cm. He had no history of serious childhood illness. He was an alert boy, 122 cm in height (6 cm below the third centile). He had no goitre and was clinically euthyroid, and full clinical examination revealed no other abnormalities. Bone age at the wrist was 5 years (Greulich and Pyle, 1950), and skull X-ray was normal. Insulin-induced hypoglycaemia revealed no abnormality of growth hormone or cortisol production. Serum T_4 was 23 nmol/l, Thyopac 3 was 63%, FTI 37, serum T_3 1.5 nmol/l, serum TSH was 2.0 mU/l and serum TBG was undetectable. Two brothers, aged 15 and 9 years had undetectable TBG also, although TBG concentration was within normal limits in both parents (Fig. 5.4, Tables 5.3, 5.4).

In addition to the above cases and families J-S, four sporadic cases (T1-4) of diminished TBG, and 7 additional cases of elevated TBG (U 3-9) were also studied.

Tables 5.1 - 5.4 contain data on a total of 14 males hemizygotic for elevated TBG, 44 females heterozygotic for elevated TBG, 15 males with absent TBG and 15 females heterozygotic for diminished TBG.

b) Immunological and electrophoretic properties of TBG in familial TBG abnormality

In 2-dimensional IEP the electrophoretic mobilities of TBG from individuals with normal, elevated or diminished TBG concentration were

Table 5.3

Case	T4 (nmol/l)	T3 (nmol/l)	TBG (mg/l)	T3U (%)	FTI	TBPA (mg/l)
I II 1	17	1.8	ND	64	27	243
I II 2	23	1.5	ND	63	36	-
I II 3	23	1.8	ND	63	37	325
K I 1	30	1.0	ND	76	39	374
L II 1	40	1.0	ND	64	63	346
L II 3	43	1.2	ND	69	62	379
M I 1	17	0.8	ND	51	33	-
N II 1	16	1.5	ND	52	31	225
N II 2	25	0.6	ND	47	53	236
O II 1	30	1.9	ND	-	-	164
P I 1	43	1.3	ND	73	59	566
R III 3	24	1.1	ND	59	41	432
T 2	21	0.8	ND	51	41	-
T 3	17	-	ND	42	40	-
T 4	16	-	ND	61	26	-
Mean	26	1.3		60	42	329
SD	10	0.4		10	12	118
Number	15	13		14	14	10
Range	16-43	0.6-1.9		42-76	27-63	164-566
Reference values	60-130	1.6-3.6	6.0-17.0	93-117	56-137	-

THYROID FUNCTION TESTS IN MALES WITH ABSENT TBG

Table 5.4

THYROID FUNCTION TESTS IN FEMALES WITH DECREASED TBG

	Case	T4 (nmol/l)	T3 (nmol/l)	TBG (mg/l)	T3U (%)	FTI	T4:TBG	TBPA (mg/l)
1.	I I 2	71	3.1	9.5	100	71	7.5	401
	K II 3	55	1.6	4.7	57	96	11.7	278
1.	L III 4	68	1.6	6.9	86	79	9.9	312
	M II 2	57	1.1	5.0	66	86	11.4	-
1.	M II 3	74	1.8	8.0	76	97	9.3	-
	M II 4	70	1.3	5.0	80	88	14.0	-
	N I 2	43	1.2	1.0	66	65	43.0	333
1.	O I 2	78	1.8	7.1	81	96	11.0	306
	P II 1	94	-	5.6	-	-	16.8	414
	R II 2	28	1.4	0.5	55	51	56.0	-
1.	R II 4	-	-	6.2	-	-	-	-
	R III 4	59	1.6	4.5	78	76	13.1	317
	R III 6	38	1.5	3.4	75	51	11.2	368
	R III 7	61	1.9	3,3	83	73	18.5	408
	T 1	25	-	2.4	-	-	10.4	-
	Mean	59	1.7	4.9	75	77	17.4	349
	SD	20	0.5	2.5	13	16	14.1	50
	Number	14	12	15	12	12	14	9
	Range	25-94	1.1-3.1	0.5-9.5	55-100	51-97	7.5-56	278-414
	Reference values	60-130	1.6-3.6	6.0-17.0	93-117	56-137	4.8-11.7	-

1. TBG normal, presumed heterozygote.

THYROID FUNCTION TESTS IN FEMALES WITH DECREASED TBG

similar (Fig. 5.5). In addition, near-perfect Gaussian peaks were obtained in both TBG elevation and deficiency, which would suggest that no heterogeneity of electrophoretic mobility was present. A band of immunological identity was found between TBG from normal persons and individuals with TBG elevation, as shown in the composite IEP plate in figure 5.6

c) Serum TBPA concentration

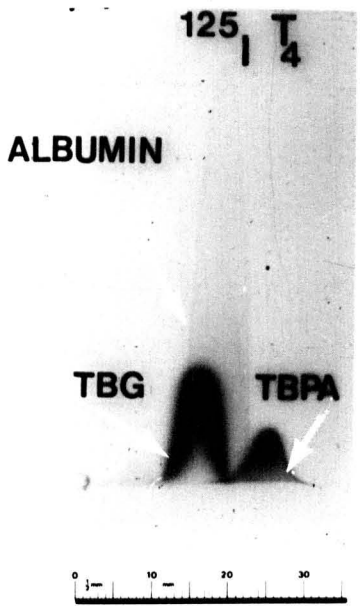
In males and females with hereditary TBG elevation, mean serum TBPA ($\pm$ S.D.) in 31 euthyroid individuals was 257 $\pm$ 63 mg/l. This was significantly less than the concentration found in 24 relatives with normal TBG concentration (327 $\pm$ 82 mg/l, $P < 0.0005$). TBPA concentration in 19 euthyroid males and females with absent or diminished TBG was 338 $\pm$ 91 mg/l, which was not significantly different from the concentration in relatives with normal TBG concentration.

d) Inheritance of TBG abnormalities

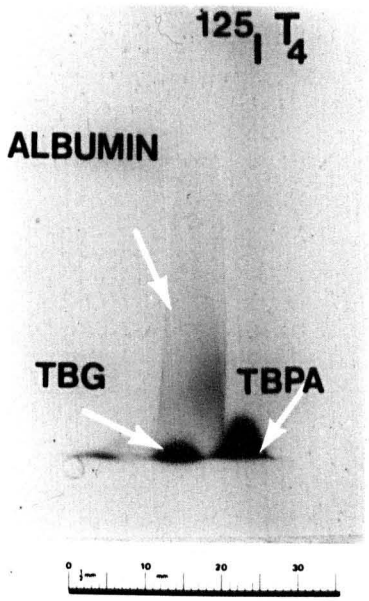
Table 5.5 summarises the inheritance of TBG abnormalities in 19 families.

No cases of father-to-son transmission occurred out of 9 possibilities (6 for high TBG, 3 for absent TBG). Fathers with high TBG produced daughters with normal TBG concentration on 3 occasions (cases A IV 3, A IV 4 and A IV 6 - table 5.2), while on 9 occasions the daughters had elevated TBG. On two occasions, fathers with absent TBG had daughters with normal TBG (cases L III 4 and M II 3, table 5.4). Out of a total of 42 male and female offspring of mothers with high or low TBG, 28 inherited the condition while 14 were normal.

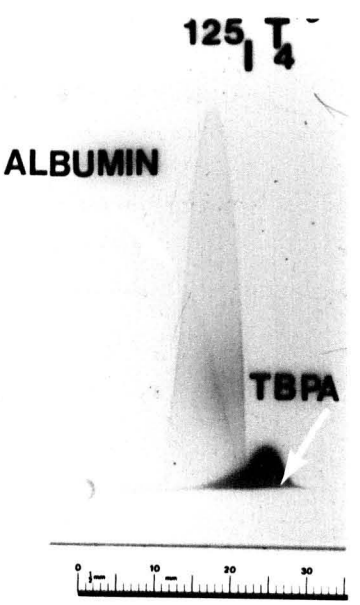
Figure 5.5



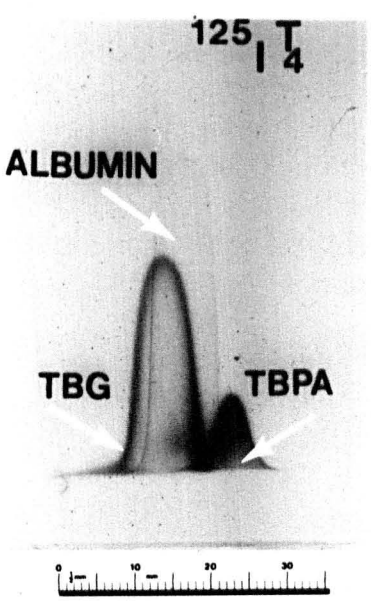
Normal TBG



Low



Absent

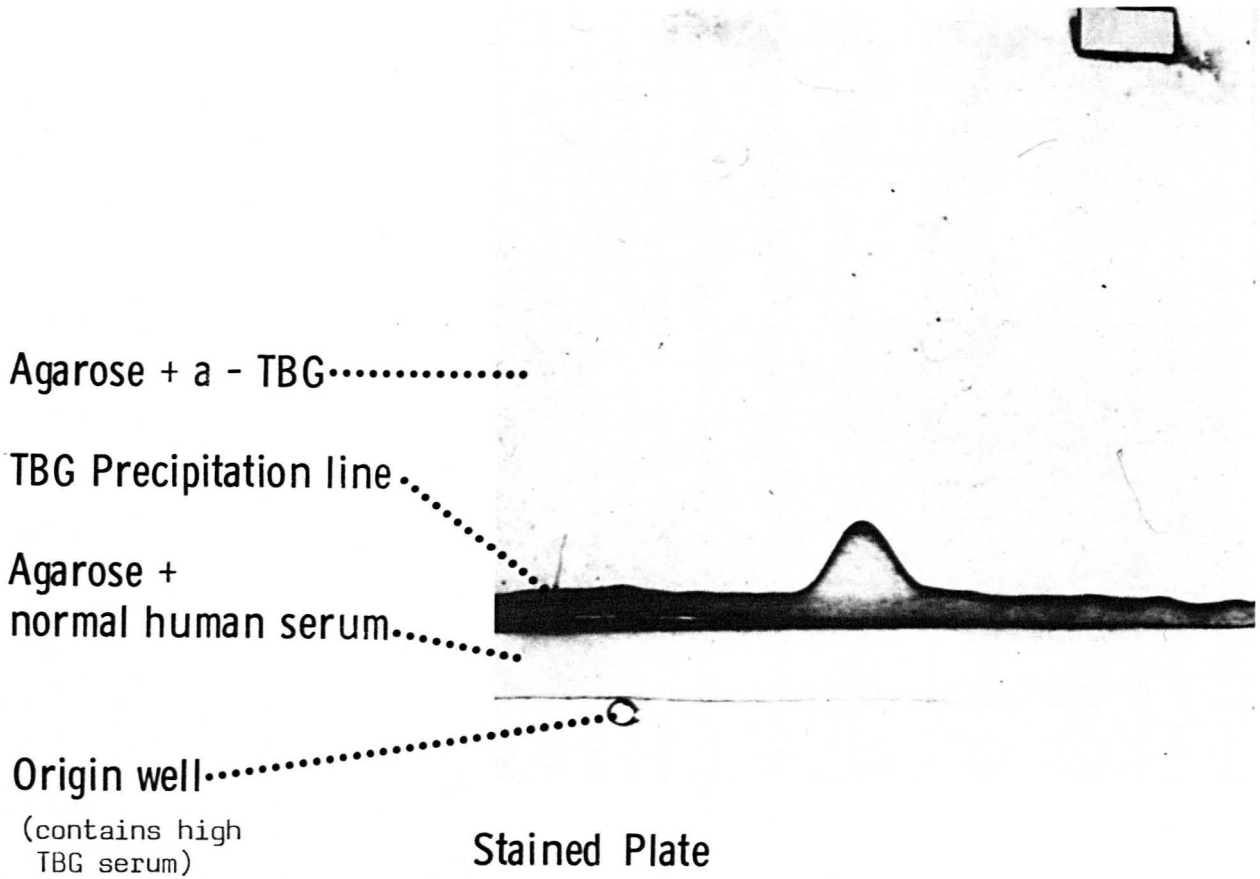


High

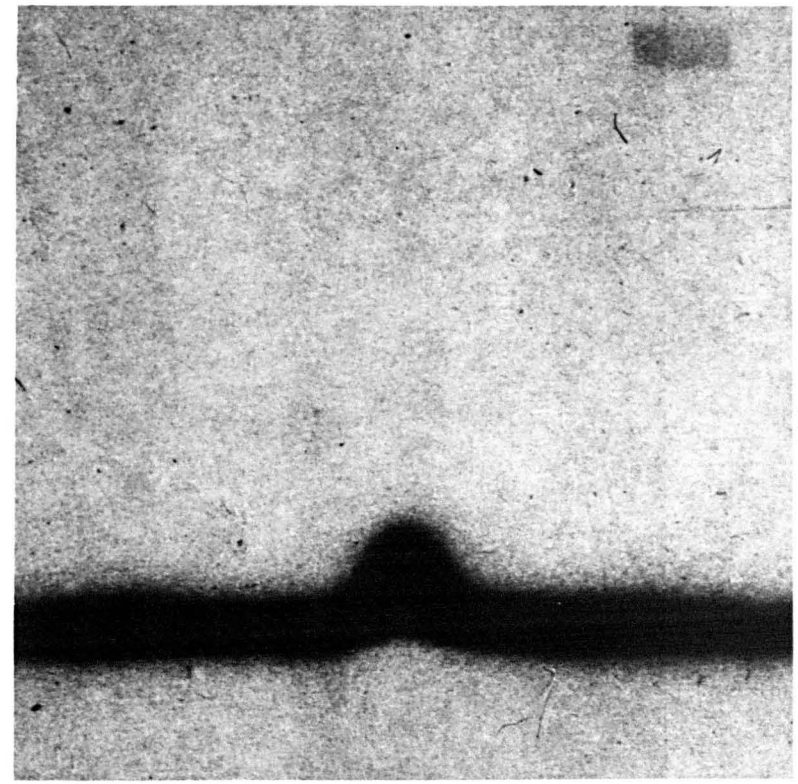
2 DIEP OF SERUM FROM PATIENTS WITH FAMILIAL TBG ABNORMALITY

^{125}I-T$_4$ LABELLING + AUTORADIOGRAPHY

Figure 5.6



Stained Plate

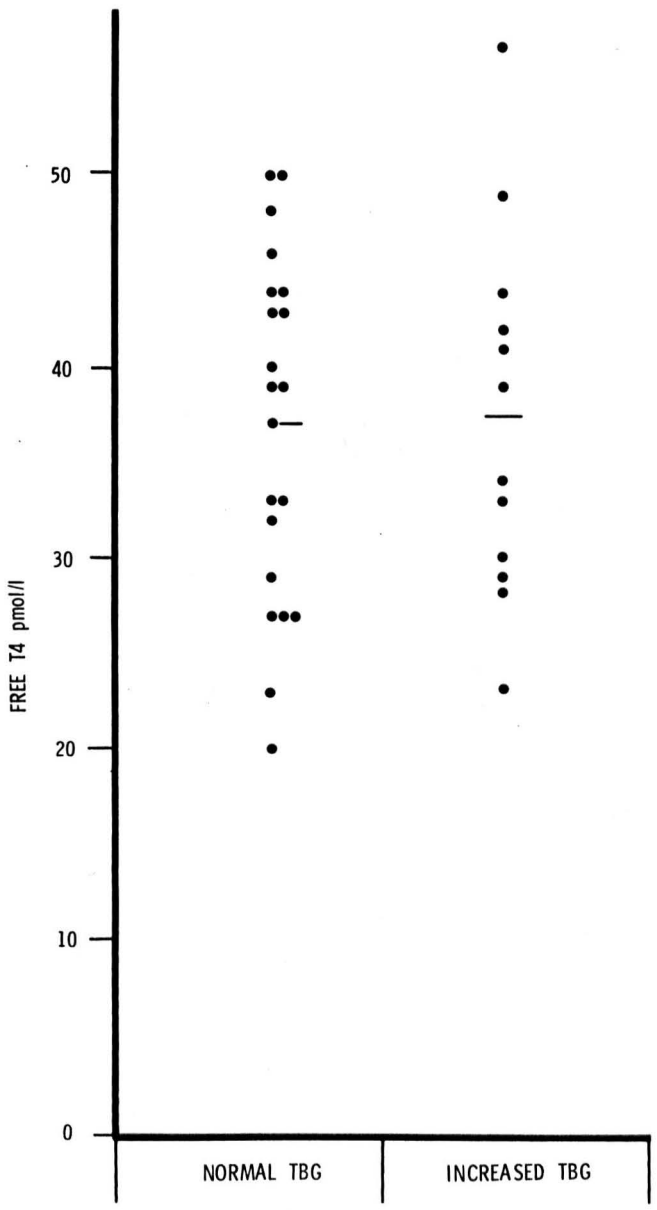


Autoradiograph

COMPOSITE IEP PLATE OF NORMAL + ABNORMAL TBG SERUM

2DIEP of serum from an individual with hereditary TBG elevation (with added $^{131}I\text{-}T_4$) electrophoresed through a gel containing normal human serum into anti-TBG.

Figure 5.7



AFT_4 CONCENTRATION IN HEREDITARY TBG INCREASE AND NORMALS

e) Thyroid function tests in TBG abnormality

i) TBG elevation

TBG concentration in the presumed hemizygous males with TBG increase was remarkably constant (Table 5.1), with a mean (± S.D.) of 41.8 ± 2.7 mg/l. Serum T_4 was uniformly elevated (269 ± 31 nmol/l), and serum T_3 usually raised (5.4 ± 1.4 nmol/l). T_3 uptake was elevated (142 ± 8%), and FTI was above the normal range in all cases (mean = 190 ± 28, $P < 0.0005$ compared to normal relatives). T_4:TBG ratio (6.4 ± 0.6) was significantly lower than the ratio in normal relatives ($P < 0.0005$), but in no case did the ratio fall outside the normal range.

In females who were presumed to be heterozygous for increased TBG, there was a wide scatter of TBG values, ranging from normal to the high values found in hemizygous males (mean ± S.D. = 29.1 ± 10.8 mg/l, Table 5.2). The highest TBG concentrations noted in this study occurred in 2 women who were presumed to be heterozygous for TBG excess and who were taking the contraceptive pill (cases B III 5, C II 5 - Table 5.2). In heterozygous women, thyroid function tests were affected to an intermediate and highly variable degree, but FTI was significantly greater than in normal relatives (149 ± 30, $P < 0.0005$) and exceeded the normal range in 25/39 individuals. T_4:TBG was significantly less than in normals (7.2 ± 1.6, $P < 0.0005$), but only fell below the normal range in 1 out of 40 individuals. Serum free T_4 concentration was measured in 12 individuals (10 male, 2 female) with high TBG, and in 21 normal controls. Mean control concentration was 37 ± 9 pmol/l, with a range of 27 - 47 pmol/l, and the concentration did not differ in TBG elevation (mean = 37 ± 10 pmol/l, range 23 - 57 pmol/l, Fig. 5.7).

ii) TBG decrease

Mean values of thyroid function tests for hemizygous males with absent TBG are shown in Table 5.3. FTI (42 ± 12) was significantly

lower than in normals ($P < 0.0005$), and fell below the normal range in 11/14 individuals. T_4:TBG ratio could not be applied.

Heterozygous females (Table 5.4) had TBG concentrations ranging from 0.5 mg/l up to the normal range. FTI (77 ± 16) was significantly reduced ($P < 0.0025$) and fell below the normal range in 2/12 individuals, while T_4:TBG ratio was significantly elevated (17.4 ± 14.1, $P < 0.0005$) and exceeded the normal range in 8/14 individuals. Free T_4 was not measured in TBG decrease.

4) DISCUSSION

Electrophoretic and immunological characteristics of TBG

The results of electrophoretic and immunological studies reported here support the suggestion that the inherited abnormalities of TBG are due to varying concentrations of an immunologically normal protein. Serum thyroid hormone concentrations would further suggest that the protein is functionally normal. These results are in agreement with the studies of Hamada, Takemura and Sterling (1971), Hansen and Siersbaek-Nielsen (1972) and Refetoff et al (1972). Refetoff et al (1976) have further shown that the probable defect in TBG abnormality lies in alterations in TBG synthetic rate.

Serum TBPA

A significant decrease in TBPA concentration was found in individuals with TBG elevation, when compared with normal family members, but no significant alterations were found in TBG decrease or absence. The co-existence of absent TBPA and increased TBG (both measured as binding capacity) has been reported on one occasion in the past (Thomson et al, 1972), although in view of the poor sensitivity of TBPA capacitance assays,

(Premachandra and Wu, 1979), total absence of TBPA was not convincingly proved. There are several interesting examples of reciprocity between TBG and TBPA levels. In active acromegaly TBG capacity has been found to be diminished, while TBPA capacity was increased (Inada and Sterling, 1967c). Adrenal corticosteroids (Oppenheimer and Werner, 1966) and anabolic steroids (Braverman and Ingbar, 1967) apparently produce the same effects, but no satisfactory explanation for these findings has been advanced. The present finding of decreased TBPA in hereditary TBG increase could be taken to be evidence in favour of a genetic influence on TBPA concentration. This has already been proposed for a link observed between decreased TBPA and α-1-antitrypsin deficiency (Premachandra and Wu, 1979). However, another explanation lies in the fact that TBPA concentration varies with age (Braverman, Dawber and Ingbar, 1966), and our family populations were not age-matched.

The failure to find TBPA increase in the absence of TBG argues against any simple reciprocal relationship between TBPA and TBG accommodation.

c) Inheritance of TBG abnormality

The first case reports of TBG abnormality were thought to show an autosomal dominant inheritance (Beierwaltes and Robbins, 1959; Nicoloff et al, 1964), but were also compatible with an X-linked dominant inheritance which was suggested in later studies (Nikolai and Seal, 1966; Marshall, Levy and Steinberg, 1966). Refetoff et al (1972) reviewed 20 families with abnormal TBG concentration and described 4 new kindreds. From a total of 19 families with diminished or absent TBG and 6 with excess TBG, they derived strong support for X-linked inheritance of TBG decrease and excess.

The nineteen families described here in general satisfy the criteria for X-linkage enunciated by Refetoff et al (1972), namely a) full expression

of the defect is manifest in hemizygous subjects (possessing a single X chromosome), b) heterozygous subjects have intermediate expression of the defect and have two X-chromosomes, c) all female offspring of affected males are heterozygous for the trait, d) all male offspring of affected males are normal, e) affected males are offspring of heterozygous females, and f) heterozygous females are offspring of affected males or females.

With regard to criterion a) above, it should be mentioned that although no female with absent TBG has been found, a concentration of only 0.5 mg/l occurred in subject R II 2 (Table 5.4), and in females with high TBG, subjects B III 5 and C II 5 (Table 5.2), who were taking oestrogens, had TBG concentrations higher than the hemizygous males. These extremes of TBG concentration, occurring in females, do not necessarily disprove X-linkage, but may be due to an unusually great degree of inactivation of the normal X-chromosome, according to the Lyon hypothesis of random X-chromosome inactivation (Lyon, 1962). The Lyon hypothesis may also explain why some presumably heterozygotic females had TBG concentrations in the normal range (e.g. A IV 3, A IV 4, and A IV 6, Table 5.2). The final proof of the heterozygotic state of these subjects will depend on testing of their eventual offspring.

With the above exceptions, X-linkage was fulfilled and no case of father to son transmission occurred out of nine possible instances (Table 5.5).

The marked elevation of TBG concentration in the two women with TBG elevation who were also taking the contraceptive pill is an interesting observation. Although the additional TBG increase above the levels normally seen in women with familial TBG increase may be due to the response of hepatocytes bearing a "normal" X chromosome, the extreme

Table 5.5

MALES	NO. OF FATHERS	SONS AFFECTED	SONS NORMAL	DAUGHTERS AFFECTED	DAUGHTERS NORMAL
High TBG	5	0	6	9	3
Absent TBG	4	0	3	4	2
FEMALES	NO. OF MOTHERS				
High TBG	11	8	7	10	4
Low TBG	5	7	3	3	0

PROGENY OF AFFECTED PARENTS

elevation of TBG which was found suggests the possibility of an increased sensitivity to the effects of oestrogens. There is some conflict among reports of the effect of oestrogens in TBG excess. Some authors have found an increase in both men and women (Jones and Seal, 1967; Shane et al, 1971) while others found no effect in a woman (Ingbar et al, 1964). The results are of considerable theoretical interest, because if the defect in familial TBG excess lies in a repressor gene, one might expect that oestrogen would no longer control TBG synthesis, whereas an exaggerated response would be more consistent with a gene duplication (Robbins, 1973; Jones and Seal, 1967). These questions could be resolved by studies involving oestrogen administration to hemizygous males with TBG excess, but for ethical reasons, these were not undertaken in the present study.

d) Association of TBG abnormality and thyroid dysfunction

TBG abnormality has been reported in association with growth retardation (Penfold et al, 1971; Leiba et al, 1972; Barragry and Burr, 1977), with mental subnormality (Thomson et al, 1972) and with hypertrophic cardiomyopathy (Kallee et al, 1978). An association with thyroid disease was first reported by Shane et al (1971) who studied a large kindred with TBG increase and goitre. Apart from probable mis-diagnosis (Florsheim et al, 1962; Ingbar et al, 1964, Fialkow et al, 1970; Shane et al, 1971), the only reported link between increased TBG and thyroid dysfunction was a thyrotoxic woman with suspected TBG increase reported by Hodgson and Wahner (1972). The four cases of thyrotoxicosis and three cases of myxoedema documented here would appear to be the only cases in which an association with TBG elevation has been directly confirmed. Out of a total of 58 individuals with TBG elevation, 7 had thyroid dysfunction, but because of bias in case selection, we cannot take this to be definite evidence in favour of a positive association between TBG increase and thyroid dysfunction. No cases of thyroid disease and low or absent TBG were found in this series,

although an association between thyrotoxicosis and TBG deficiency has been reported in the past (Strunge, 1974, Gerstner and Caplan, 1976, Horwitz and Refetoff, 1977).

e) Diagnostic and management problems associated with TBG abnormality

In past years patients have been subjected to radioiodine treatment because of TBG elevation (Ingbar et al, 1964). In the present series, no ablative antithyroid therapy was incorrectly given, although diagnostic problems almost led to this error in one patient. An error of this type could result from placing reliance on T_4 measurement, without measurement of binding protein, or from reliance on the FTI result, which has been shown in the present study to give spurious high values in TBG excess (see Chapter 6).

When thyrotoxicosis co-existed with TBG excess (C II 1, D II 4, E I 2 and U 2), problems were encountered with biochemical assessment, and at least one patient was untreated for several months in spite of severe thyrotoxicosis (C II 1). In another individual (D II 4), difficulties in biochemical assessment arose because of a 78% increase in TBG during control of thyrotoxicosis. This led in turn to errors in management because of misleading elevation of the FTI in a hypothyroid individual. Management was simplified by TBG measurement and use of T_4: TBG ratio in controlling drug dosage.

Considerable morbidity seems to have occurred due to under-treatment of myxoedema in one patient with TBG excess (case F III 2), and a further instance of inadequate thyroxine replacement therapy was found in another individual with co-existent myxoedema and TBG excess (case G II 2). These problems resulted from the fact that FTI was spuriously high even when the patient was hypothyroid and had an elevated TSH.

In TBG deficiency, the usual diagnostic problem consists of distinguishing the condition from myxoedema because of the low T_4 and FTI results which occur in this condition.

There is considerable disagreement in the literature regarding absolute free T_4 values in TBG abnormality, with some authors finding abnormal free T_4 (Bayley et al, 1969; Heinonen, Lamberg and Virtaino, 1970; Henneman, Docter and Dolman, 1971; Thomson et al, 1972; Dussault et al, 1973) and others normal free T_4 (Refetoff and Selenkow, 1968, Jones and Seal, 1967; Siersbaek-Nielsen, Hansen and Hippe, 1969). Free T_4 was found to be normal in TBG elevation in this study, and the fact that TSH responses to TRH are normal in TBG deficiency would suggest that effective free hormone levels are normal in this condition also (Hansen et al, 1975; Konno, 1976).

In the patients with TBG excess, it was found that the T_4:TBG ratio, although significantly lower than in normal persons, still gave results within the euthyroid range, and was diagnostically helpful. In subjects with TBG absence or deficiency, both T_4:TBG ratio and FTI gave misleading results. Knowledge that serum TBG was absent or very low has proved helpful in clinical practice, because of the consistent biochemical findings in this condition (Table 5.3), which allowed some prediction of appropriate T_4 and T_3 levels. In general, where TBG concentration was very low, it was found necessary to rely on other tests such as TSH and dynamic tests of thyroid function to resolve the diagnosis.

f) Prevalence of TBG abnormality

The prevalence of hereditary increased TBG is not known, while that of hereditary decreased TBG has been reported to be 0.06% (Horwitz and Refetoff, 1977), or 0.006% (Dussault, Coulombe and Laberge, 1975).

In a 4-year period using a direct assay of TBG, 88 individuals with probable familial TBG abnormality have been encountered. These patients were highly selected, but nevertheless, it was felt that the hereditary abnormalities of TBG concentration may be more common than previously recognised, and may be more frequently recognised with increasing use of TBG assays.

Chapter Six TBG MEASUREMENT IN THE ASSESSMENT OF THYROID FUNCTION

1) Introduction: aims and design of study

2) Patients and methods

3) Results:

   a) T_3-uptake and TBG concentration

   b) Relationship of T_4 and TBG concentration

   c) T_4:TBG ratio and FTI

   d) Retrospective comparison of T_4:TBG and FTI

4) Discussion

Chapter 6 TBG MEASUREMENT IN THE ASSESSMENT OF THYROID FUNCTION

1) INTRODUCTION: AIMS AND DESIGN OF STUDY

As mentioned in Chapter 5.4, the study of euthyroid individuals with hereditary TBG abnormalities led to the discovery that conventional thyroid function tests were not always reliable when TBG concentration was very high or very low. In particular, the Free Thyroxine Index (FTI = $\frac{T_4}{T_3 \text{ uptake}} \times 100$, (Clarke and Horn, 1965) gave results in the thyrotoxic range in hereditary TBG elevation, and in the myxoedematous range in TBG deficiency. Since free T_4 concentration in hereditary TBG abnormality is generally considered to be normal (see Chapter 5.3.e.i.), the abnormality of FTI implies either a defect of T_4 measurement or T_3 uptake measurement. Problems may be encountered with T_4 RIA, when TBG concentration is high, because of inadequate blockade of TBG-binding sites with 8-anilino-1-naphthalene sulphonic acid or merthiolate (Burr, Evans and Hogan, 1977; Evans, Burr and Hogan, 1977). Allowance was made for this in the present studies by re-measurement of T_4 in diluted samples. Abnormalities of FTI persisted, suggesting that the problem was due to defects of T_3 uptake measurement.

The theoretical basis of the FTI lies in a simplified statement of the law of mass action as applied to thyroid hormones and binding proteins:-

$$[FT_4] = \frac{[\text{bound } T_4]}{k[\text{unbound TBG}]} \quad - (1)$$

In euthyroid serum, total T_4 approximates to bound T_4, and [unbound TBG] may be considered equal to [total TBG - total T_4]. Substituting in equation (1):-

$$[FT_4] = \frac{[\text{total } T_4]}{k\left(\frac{[\text{total TBG}]}{[\text{total } T_4]}\right)-1} \quad - (2)$$

$$= \frac{1}{k}\left(\frac{[\text{total } T_4]}{[\text{total TBG}]} - 1\right) \qquad - (3)$$

Equations (1 - 3) represent a very simplified view when compared with the multi-ligand/multi-protein-binding site system described earlier (Chapter 1.4.a.v.), but because of the predominance of TBG among the T_4-binding proteins, the assumptions may be legitimate and of practical value. It follows from equation (3), that any alteration in serum TBG will be accompanied by appropriate factorial changes in total T_4, so as to keep free T_4 constant. It also follows that the ratio of T_4:TBG should maintain a factorial relationship to free T_4, and may therefore be of diagnostic value.

The work described in this section had several aims:-

1) To evaluate the T_3 uptake test as a measure of [unbound TBG] over a wide range of TBG concentrations.
2) To examine the relationship between total T_4 and total TBG concentration in euthyroid individuals.
3) To compare the ability of FTI and T_4:TBG ratio to discriminate between thyrotoxic, euthyroid and myxoedematous individuals.
4) To compare FTI and T_4:TBG ratio in individuals with abnormal TBG concentration.
5) To test the diagnostic value of FTI and T_4:TBG ratio in a hospital-based trial.

2) PATIENTS AND METHODS

Serum samples were taken from one hundred and sixteen healthy, euthyroid volunteers, and 122 euthyroid hospital in-patients, comprising together 138 men and 100 women. One hundred and one patients with thyrotoxicosis and 36 patients with myxoedema were also studied, together

with 65 persons with high TBG concentration (9 males and 37 females with hereditary TBG elevation, 10 pregnant women and 9 women taking oral contraceptives). Twenty individuals with low or absent TBG were studied (16 male, 4 female), and ten patients on treatment with diphenylhydantoin (DPH). TBG, T_3 uptake and T_4 (Evans et al, 1977) were measured in all sera. FTI ($T_4/T_3U \times 100$) and T_4:TBG ratio were calculated for all patients.

In order to compare the diagnostic value of T_4:TBG ratio and FTI, TBG and T_3uptake were measured in 104 consecutive in-patients with $T_4 > 110$ nmol/l and 40 consecutive patients with $T_4 < 40$ nmol/l. The case notes of all individuals in whom T_4:TBG ratio or FTI gave results outside the 95% confidence limits for normal subjects were examined to establish their thyroid status.

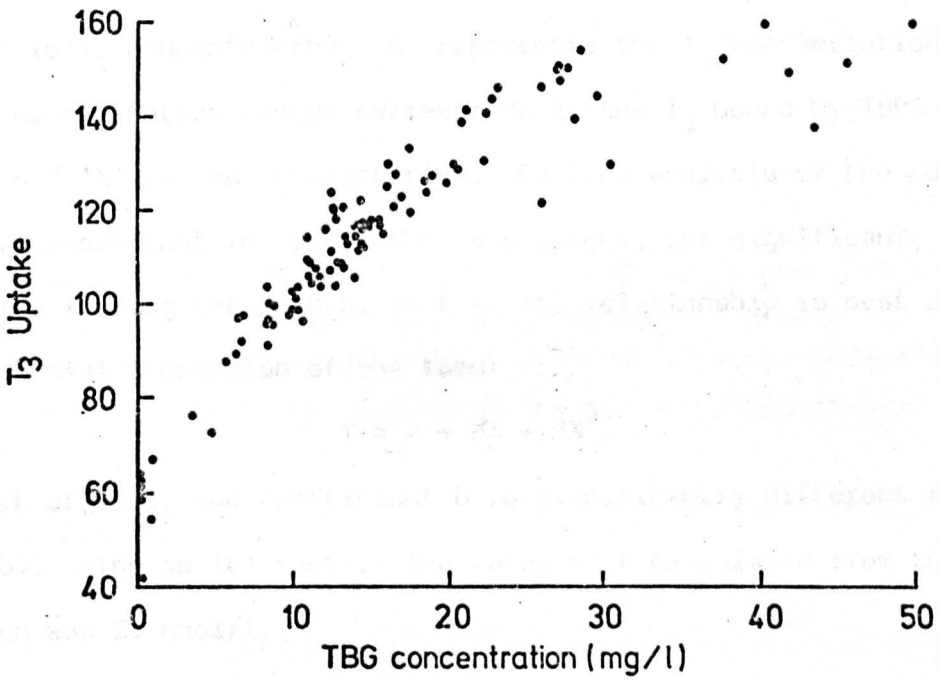
3) RESULTS

a) T_3-uptake and TBG concentration

Figure 6.1 shows the relationship between T_3 uptake and TBG concentration in 82 euthyroid individuals with a wide range of TBG concentrations. The subjects comprised normal people (42), contraceptive pill-treated (9), pregnant (10) and those with hereditary TBG abnormalities (21).

At high concentrations of TBG, the relationship became non-linear, with T_3 uptake values reaching a plateau at about 130%, above which little or no increase occurred despite a doubling of TBG concentration from 25 to 50 mg/l. In TBG absence (Chapter 5 - Table 5.3), mean T_3 uptake ($\pm$S.D.) was 60 $\pm$ 10%, with a range of 42 - 76%.

Figure 6.1



T_3 UPTAKE AND TBG CONCENTRATION

Results are shown for 82 euthyroid individuals with a wide range of TBG concentration.

b) Relationship of T_4 and TBG concentration

In 315 euthyroid individuals, there was a linear correlation between T_4 and TBG (Fig. 6.2, $r = 0.92$, $P < 0.0005$, falling to $r = 0.54$, $P < 0.0005$ when individuals with abnormal TBG concentration were excluded).

The relationship between TBG and T_4 can be expressed as a simple linear equation:

$$Y = A + BX$$

where 'Y' is T_4 concentration, 'A' represents the T_4 concentration at zero TBG concentration (which corresponds to the T_4 bound by TBPA and albumin) and 'X' is TBG concentration. Further analysis of the points in figure 6.2 shows that in fact there is a slight, but significant, curvature to the line linking the points, so that the relationship is best described by a polynomial expression of the form:

$$Y = A + BX + DX^3.$$

In support of this, the coefficient D is significantly different from 0 ($P < 0.005$, using an 'F' test). The value of A calculated from the above expression was 23 nmol/l.

c) T_4:TBG ratio and FTI

The mean T_4:TBG ratio ($\pm$ S.D.) in healthy volunteers (8.2 $\pm$ 1.6) did not differ from the ratio in euthyroid in-patients (8.2 $\pm$ 1.7). The 95% confidence limits for the T_4:TBG ratio derived from the control population of 238 were 4.8 - 11.5. Figure 6.3a shows the T_4 and TBG concentrations in 77 euthyroid individuals with raised or low TBG, plotted with the 95% confidence limits for the T_4:TBG ratio. The ratio cannot be applied to the males with undetectable TBG, but in a few individuals with low TBG, the ratio exceeds the 95% confidence limits. In individuals with raised TBG, the T_4:TBG ratio remains within these limits in all but one case.

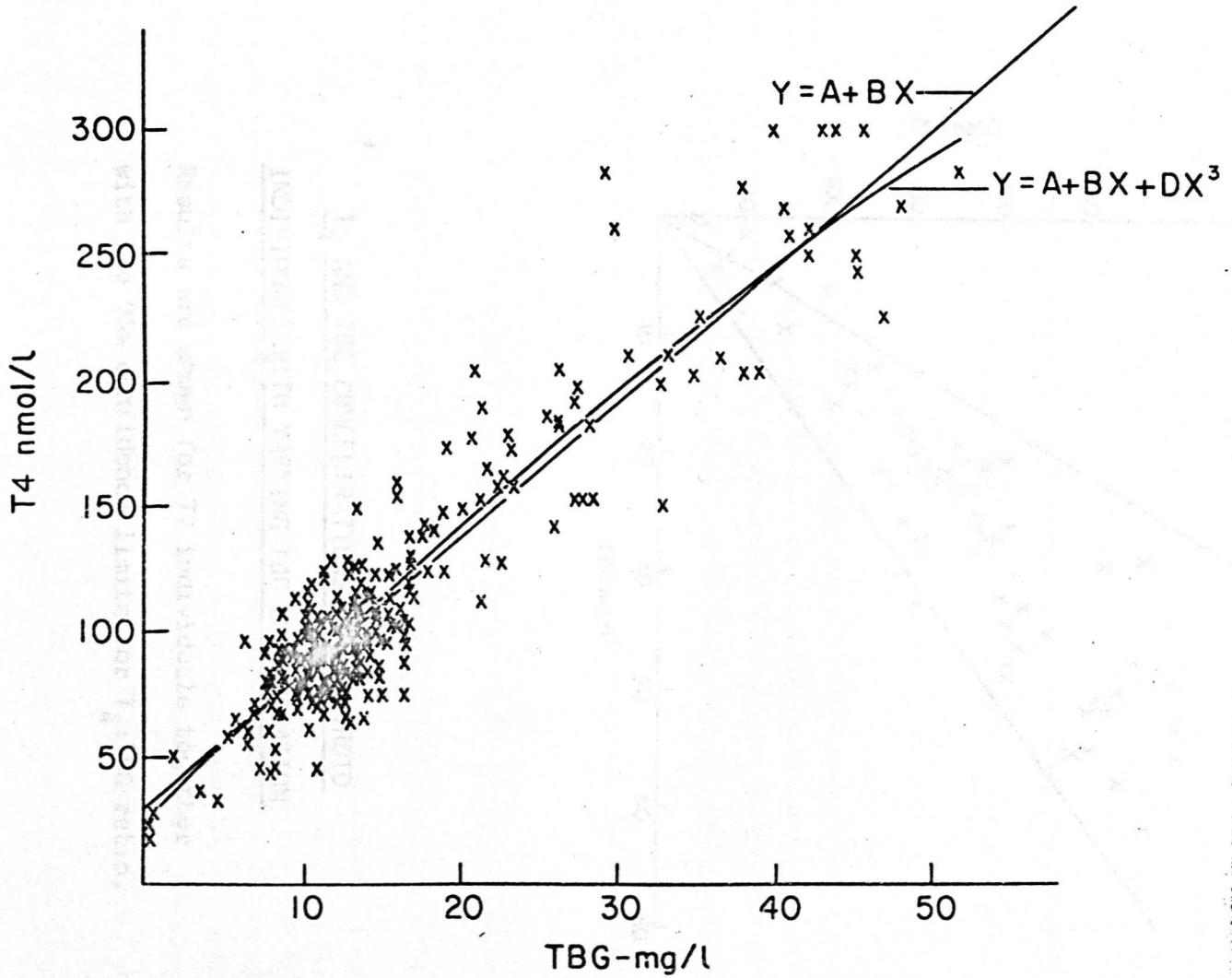
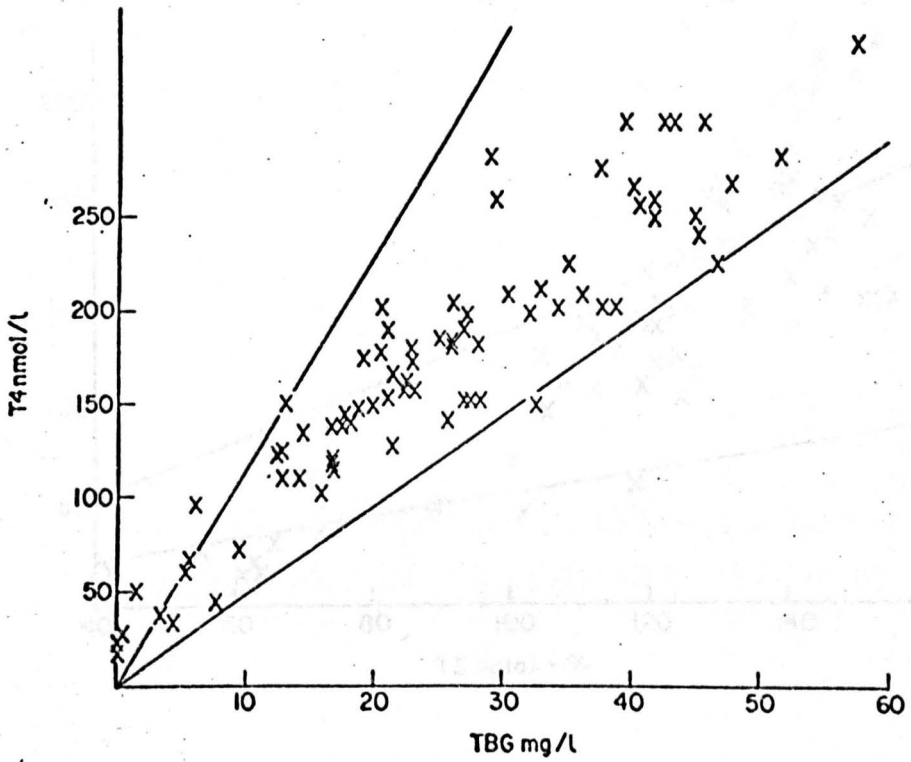


Figure 6.2

THE RELATIONSHIP BETWEEN T_4 AND TBG CONCENTRATION

Results shown are for 315 euthyroid individuals with varying TBG concentrations.

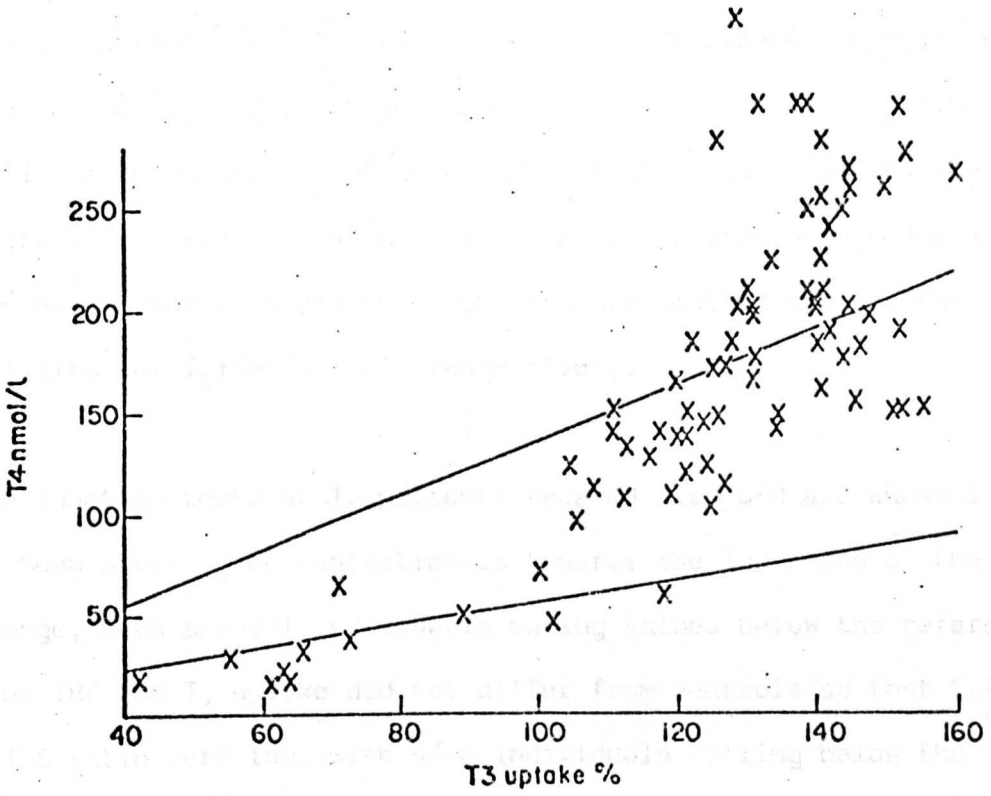
Figure 6.3.a



T_4 AND TBG CONCENTRATIONS IN EUTHYROID INDIVIDUALS WITH VARYING TBG CONCENTRATION

Results are shown for 77 individuals together with the 95% confidence limits for T_4:TBG ratio.

Figure 6.3.b



T_4 AND T_3 UPTAKE IN EUTHYROID INDIVIDUALS WITH VARYING TBG CONCENTRATION

Results are shown for the same individuals as in Figure 6.3.a. together with 95% confidence limits for FTI.

Figure 6.3b shows the T_4 and T_3 uptake results for the same individuals plotted with the 95% limits for the FTI (56 - 137). When TBG is absent or low, the FTI lies below the confidence limits, and when TBG is high, FTI frequently exceeds these limits. The T_4:TBG ratio is clearly superior to FTI in correctly assigning euthyroid status to these patients. T_4:TBG ratio and FTI are comparable in their ability to distinguish patients with abnormal thyroid function from normal, as shown in figures 6.4a and 6.4b, in which thyrotoxic and myxoedematous patients are plotted against the 95% confidence limits for T_4:TBG and FTI respectively.

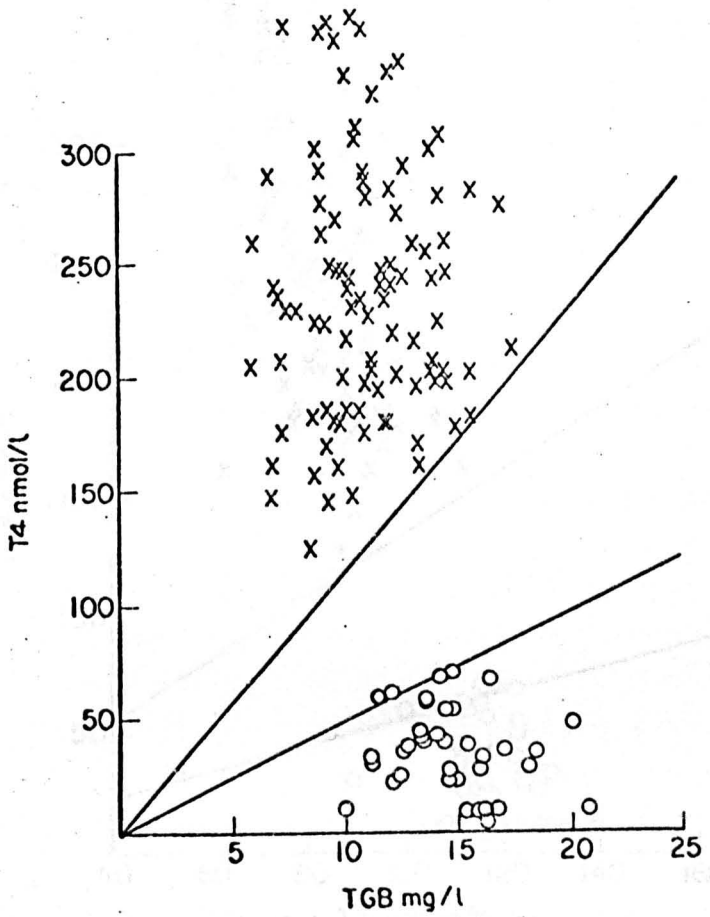
Thyroid function tests of 10 patients treated with DPH are shown in Table 6.1. Mean serum T_4 concentration is towards the lower end of the reference range, with several individuals having values below the reference range. Serum TBG and T_3 uptake did not differ from controls so that both FTI and T_4:TBG ratio were low, with some individuals falling below the reference range.

d) Retrospective comparison of T_4:TBG and FTI

The T_4:TBG ratio and FTI exceeded the 95% limits in 43 of the 144 patients selected because of high or low T_4 concentration, and details are given in Table 6.2. In 23 patients both tests were abnormal and thyroid disease was confirmed. In 20 patients the tests were discordant, but unfortunately a firm diagnosis could only be made in 9 patients.

The FTI gave two false negative results, euthyroid values being obtained in one thyrotoxic and one myxoedematous patient. Six false positives were obtained with FTI, comprising three normal persons in whom myxoedema was predicted, and three normals incorrectly assigned as thyrotoxic. T_4:TBG ratio gave one false negative in a thyrotoxic patient and no false positives.

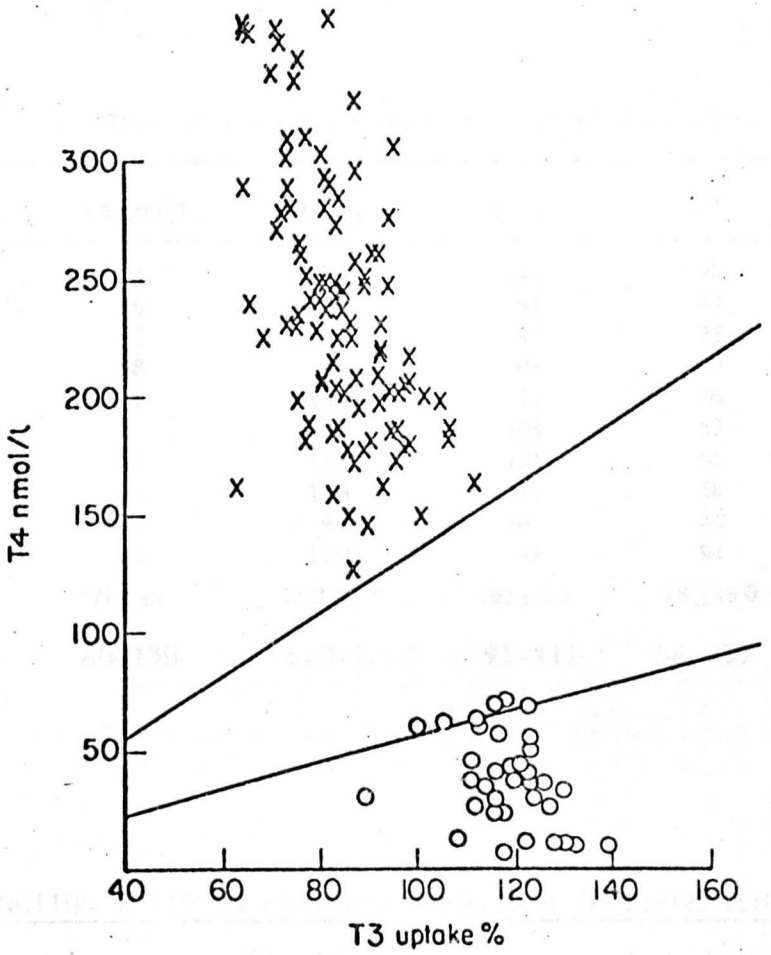
Figure 6.4.a



T_4 AND TBG CONCENTRATIONS IN THYROTOXIC AND MYXOEDEMATOUS PATIENTS

101 thyrotoxic and 36 myxoedematous patients are shown together with 95% confidence limits for T_4:TBG ratio.

Table 6.4.b



T_4 AND T_3 UPTAKE IN THYROTOXIC AND MYXOEDEMATOUS PATIENTS

Results are shown for the same patients as in Figure 6.4.a. together with 95% confidence limits for FTI.

Table 6.1

Thyroid function tests in patients treated with diphenylhydantoin

Patient No.	T4 nmol/l	TBG mg l	T3 U	FTI	T4:TBG
1	101	17·2	112	90	5·9
2	46	11·3	93	49	4·1
3	92	13·3	97	95	6·9
4	68	7·8	98	69	8·7
5	63	12·2	112	56	5·2
6	62	13·4	108	57	4·6
7	68	11·7	104	65	5·8
8	68	12·4	118	58	5·5
9	46	9·5	105	44	4·8
10	92	11·2	98	94	8·2
Mean(±SD)	71±19	12·0±2·5	105±8·0	68±19·0	6·0±1·5
Reference values	60–130	6.0–17.0	93–117	56–137	4.8–11.7

THYROID FUNCTION TESTS IN PATIENTS TREATED WITH DIPHENYLHYDANTOIN

Table 6.2

ABNORMALITY	NUMBER	NUMBER DIAGNOSED	DIAGNOSIS	COMMENT
$\uparrow T_4$:TBG, $\uparrow$FTI	21	21	17 Thyrotoxic 4 T_4 Treatment	
$\uparrow T_4$:TBG, N FTI	9	1	1 Thyrotoxic	1 x False negative FTI
$N T_4$:TBG, $\uparrow$FTI	4	4	3 Euthyroid (2 with TBG) 1 Thyrotoxic	3 x False positive FTI 1 x False negative T_4:TBG
$\downarrow T_4$:TBG, $\downarrow$FTI	2	2	1 Myxoedema 1 T_3 Treatment	
$\downarrow T_4$:TBG, N FTI	1	1	1 Myxoedema	1 x False negative FTI
$N T_4$:TBG, $\downarrow$FTI	6	3	3 Euthyroid	3 x False positive FTI

ABNORMAL T_4:TBG AND FTI RESULTS IN PATIENTS WITH T_4 $\geq$110 NMOL/L OR $\leq$40 NMOL/L

DISCUSSION

The relationship between T_3 uptake and TBG concentration was non-linear, with the result that T_3 uptake tended to underestimate TBG concentration when this was high. The effect was most pronounced in hereditary TBG elevation, but also occurred with T_3 uptake values as low as 130, which are found during oral contraception and pregnancy. An underestimate of TBG binding sites would account for the high FTI values noted in TBG elevation in this study and in previous reports (Goolden, Gartside and Sanderson, 1967; Souma et al, 1968). One explanation of the limitations of the T_3 uptake (and red-cell uptake) tests lies in the fact that they are related to TBG capacity by an expression of the type $y = mx + c$, and not the $y = mx$ relationship which is assumed for calculation of the FTI (Osorio et al, 1961). Another technical defect (for Thyopac 3 measurement) may be that insufficient tracer $^{125}I - T_3$ is supplied to saturate large amounts of TBG (Davies and Allison, 1977).

The relationship between T_4 and TBG in euthyroid persons remained very nearly linear through a wide range of TBG concentrations. The slight tendency towards a curvilinear relationship may have been caused by a slight reduction in TBPA when TBG was elevated (v.s Chapter 5.3.c.). From the graph relating T_4 and TBG concentrations (Fig. 6.2), it was calculated that 23 nmol/l of T_4 would be associated with TBPA, albumin and other binding proteins in the absence of TBG. In practice, a figure of 26 $\pm$ 10 nmol/l was found in fifteen men with absent TBG (v.s. Table 5.3). The linear relationship between T_4 and TBG forms the basis for suggesting the use of a T_4:TBG ratio in thyroid diagnosis. The use of this simple ratio does not take account of the contribution of other binding proteins mentioned above. However, reference to Fig. 6.3.a. shows that the ratio

provides a useful index of thyroid status throughout a wide range of TBG concentrations. A spurious elevation of T_4:TBG ratio only occurs at extremely low TBG concentrations such as those found in TBG deficiency. In these rare cases, an indication of thyroid status might be gained by a plot of T_4 against TBG concentration, as in Fig. 6.2. T_4:TBG ratio appears to be diagnostically superior to FTI, which gives falsely low values in TBG deficiency and falsely high values in TBG excess.

Patients on treatment with DPH are known to have low serum T_4 and FTI values (Heyma et al, 1977). This may be due to displacement of T_4 from TBG binding sites (Oppenheimer and Tavernetti, 1962), or due to a stimulation of T_4 catabolism (Larsen et al, 1970), or a combination of both (Heyma et al, 1977). It was therefore anticipated that T_4:TBG ratio would be low, and results confirmed this.

The retrospective analysis of hospital patients with high and low T_4 concentrations was undertaken in an attempt to assess the frequency of false positive and false negative diagnoses using FTI and T_4:TBG ratio. In a high proportion of cases there was insufficient clinical data to arrive at a definite diagnosis, but such results as were obtained suggested that T_4:TBG ratio may produce fewer false positives and negatives than FTI.

In conclusion, the results obtained in this study and in Chapter 5 suggest that T_4:TBG ratio has considerable advantages over FTI in the diagnosis of thyroid dysfunction. This is particularly true in the common occurrence of TBG elevation due to oestrogen treatment or pregnancy. The ideal test of thyroid function would provide some measure of thyroid hormone activity at tissue level; failing this, an assay of free hormone in serum would be valuable. At present, such assays are not routinely available, or are insufficiently tested (Boss, Diahanbakheh and Kingstone, 1978;

Ekins, 1979), and the T_4:TBG ratio appears to provide the best alternative for the routine assessment of thyroid function.

CHAPTER 7 DISCUSSION

The purification of human TBPA and partial purification of human TBG by chromatographic techniques have been described in this thesis. 2DIEP was used to assess the success of protein purification, and in the case of TBG, 2DIEP was used to isolate a TBG/anti-TBG complex.

Purified TBPA and the TBG/anti-TBG complex were used to raise monospecific antisera in rabbits and sheep respectively, and these antisera were used in monorocket IEP assays for TBPA and TBG. The assays were found to be rapid, inexpensive, simple and precise.

The IEP assay for TBG represented a considerable advance over existing radioimmunoelectrophoretic techniques in allowing direct visualisation and measurement of the protein peak, hence dispensing with the need for handling radio-isotopes and performing auto-radiography. The assay also had some advantages over RIA measurement of TBG since it did not require chromatographic isolation and possible denaturation of TBG. Use of IEP was found to be suitable for assay of proteins present in serum in concentrations above 50 μg/l, and was therefore suitable for in vivo studies of TBG and TBPA. The relatively low sensitivity of IEP compared to RIA does however impose limitations on the use of IEP for in vitro studies.

The IEP assay was used to measure TBG in healthy persons, and the variation of TBG concentration with age and gender was noted. Elevation of TBG concentration in myxoedema, and reduced TBG in thyrotoxicosis with recovery on treatment were clearly shown.

In Chapter 4, the alterations of TBG, TBPA and albumin in surgery and non-thyroidal illness were studied, together with the inter-relationships of changes of free thyroid hormones and binding proteins. It was found that the alterations of binding protein and hormone concentration were sufficiently great to account for post-operative changes in free hormone concentration without invoking the presence of binding inhibitors.

Comparison of the binding protein and hormone changes after surgery, illness and starvation showed that although there was considerable similarity in the changes after all three, there were differences in timing of changes which suggested that additional factors other than calorie restriction were involved.

Use of the TBG assay allowed identification of a large number of individuals with hereditary TBG abnormalities, including seven individuals with combined TBG abnormality and thyroid disease. IEP techniques were used to confirm that in hereditary TBG excess and deficiency there are abnormal concentrations of electrophoretically and immunologically normal TBG.

The FTI in TBG excess and deficiency was frequently observed to give misleading results. It was shown that this probably resulted from insensitivity of the T_3-uptake test to changes in TBG concentration. It was shown that in theory a T_4:TBG ratio should provide an index of thyroid function. The T_4:TBG ratio was compared retrospectively with FTI, and was shown to differentiate at least as well as FTI between thyrotoxic, myxoedematous and euthyroid individuals. In addition, T_4:TBG ratio was able to correctly assign individuals with abnormal TBG concentration to a euthyroid status. In a hospital-based prospective trial, T_4:TBG ratio

gave fewer false positive and false negative diagnoses than FTI.

In conclusion, the work contained in this thesis does suggest that thyroxine-binding proteins may have a physiological role in certain situations in governing free hormone concentration. TBPA, TBG and albumin may all contribute in this respect. However, studies of hereditary increase and deficiency of TBG suggest that this protein can only be of secondary physiological significance.

The most important clinical significance of TBG lies in its influence on diagnostic tests of thyroid function, especially measurement of T_4 and T_3 concentration. This work shows that the direct measurement of TBG concentration would beneficially replace indirect measurements such as the T_3uptake test in the routine assessment of thyroid function.

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APPENDIX A

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ACUTE AND CHRONIC ILLNESS: Bellabarba et al, 1968; Harland et al, 1972; Johansson et al, 1972; Carter et al, 1974; Chopra et al, 1974; Bermudez et al, 1974; Chopra et al, 1975; McLarty et al, 1975; Ramsden et al, 1978; Ratcliffe et al, 1978; Helenius and Liewendahl, 1979.

FEBRILE ILLNESS: Gregerman and Solomon, 1967; Shambaugh and Beisel, 1967; Lutz et al, 1972; Burger et al, 1976; Talwar et al, 1977; Maharajan et al, 1978.

APPENDIX B

	DAY							
	0	¼	1	2	3	4	5	6
T_4 nmol/l	131± 32	139±26	123±38	125±29	128±36	121±37	126±34	133±38
P <		NS	0.05	NS	NS	NS	NS	NS
T_3 nmol/l	1.98±0.48	1.49±0.30	1.09±0.45	1.21±0.45	1.10±0.35	1.06±0.51	1.01±0.46	1.37±0.44
P <		0.01	0.0005	0.0005	0.0005	0.0005	0.0005	0.0025
rT_3* nmol/l	0.40±0.32	-	0.67±0.45	0.68±0.31	0.62±0.48	0.48±0.48	0.42±0.42	0.38±0.20
P <			0.025	0.0025	0.05	NS	NS	NS
TBG mg/l	13.7±2.6	13.1±2.7	12.9±2.6	12.4±2.3	12.7±2.2	12.4±2.5	12.4±2.6	12.4±2.5
P <		NS	0.05	0.01	0.005	0.01	NS	NS
TBPA mg/l	210±84	165±63	164±61	103±56	101±59	101±48	107±60	133±70
P <		0.025	0.0005	0.0005	0.0005	0.0005	0.0005	0.0025
ALBUMIN g/l	38±8	34±7	36±8	31±7	31±7	32±10	31±9	30±8
P <		NS	0.01	0.0005	0.0005	0.0005	0.0005	0.0005

* Measured in 5 patients only

APPENDIX B (Cont.)

	DAY 0	¼	1	2	3	4	5	6
TSH mU/l	1.7±0.9	1.7±0.9	1.6±1.3	1.9±1.5	1.6±1.0	1.5±0.8	1.4±0.9	1.7±1.1
P <		NS	NS	NS	NS	NS	NS	NS
$\%FT_4$	0.042±0.008	0.048±0.012	0.048±0.009	0.053±0.012	0.056±0.009	0.054±0.008	0.052±0.009	0.049±0.008
P <		NS	NS	0.005	0.0025	0.0005	0.0005	0.05
AFT_4 pmol/l	52±15	67±22	57±19	66±27	66±21	64±19	63±19	63±17
P <		NS	NS	0.025	0.0025	0.025	0.005	0.05
$\%FT_3$	0.29±0.06	0.36±0.09	0.38±0.09	0.41±0.11	0.40±0.07	0.37±0.08	0.35±0.08	0.31±0.04
P <		0.01	0.0005	0.0025	0.0005	0.005	0.01	NS
AFT_3 pmol/l	5.5±1.9	5.4±1.5	3.6±1.3	4.5±1.6	4.0±1.1	3.6±1.8	3.2±1.8	3.2±1.5
P <			0.0025	NS	0.0025	0.01	0.0005	0.025

APPENDIX C

	DAY 0	1	2	3	4	5	6
T_4 nmol/l	102±18	101±21	93±18	105±21	104±28	107±22	100±15
P<		NS	NS	NS	NS	NS	NS
T_3 nmol/l	2.3±0.4	1.7±0.5	1.7±0.5	1.9±0.6	1.9±0.5	2.1±0.5	1.9±0.4
P<		0.0125	0.0025	NS	0.05	NS	0.05
TBPA mg/l	191±67	182±69	154±44	147±46	166±48	169±42	173±78
P<		NS	NS	0.05	NS	NS	NS

THYROID FUNCTION AND MINOR SURGERY

APPENDIX D

	DAY 1	2	3	4	5	6	7	8	9
T_4 nmol/l	140± 40	133± 31	131± 28	119± 28	124± 24	127± 17	126± 29	134± 32	130± 24
P <		NS	NS	0.05	0.05	NS	0.25	NS	NS
T_3 nmol/l	2.4± 0.6	2.1± 0.9	1.9± 0.6	1.7± 0.6	1.7± 0.7	2.0± 0.5	1.9± 0.6	1.9± 0.5	1.8± 0.3
P <		NS	0.025	0.0025	0.01	NS	0.01	0.025	0.05
TBG mg/l	11.7± 2.4	12.0± 2.9	12.0± 2.1	11.5± 2.5	12.0± 2.2	12.0± 2.2	12.2± 2.8	12.8± 3.1	12.8± 2.8
P <		NS	NS	NS	NS	NS	NS	NS	NS
TBPA mg/l	262± 39	230± 52	174± 46	141± 53	136± 45	153± 76	138± 36	158± 56	157± 34
P <		0.0025	0.0005	0.0005	0.0005	0.005	0.0005	0.0005	0.0005
$\%FT_4$	0.045± 0.017	0.053± 0.018	0.053± 0.015	0.059± 0.019	0.056± 0.014	-	0.052± 0.014	0.048± 0.011	0.051± 0.020
P <		NS	NS	NS	NS	-	NS	NS	NS

THYROID FUNCTION AFTER MYOCARDIAL INFARCTION

APPENDIX D (Cont.)

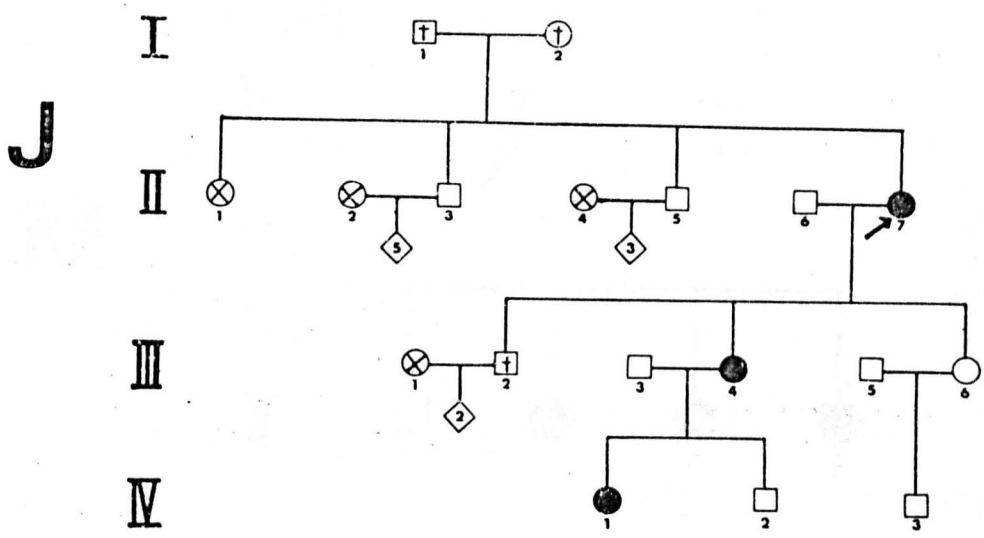
	DAY 1	2	3	4	5	6	7	8	9
AFT_4 pmol/l	60± 16	61± 15	58± 15	67± 15	69± 25	-	64± 21	63± 17	65± 26
P <		NS	NS	NS	NS	-	NS	NS	NS
$\%FT_3$	0.34± 0.12	0.33± 0.03	0.34± 0.05	0.40± 0.12	0.33± 0.06	-	0.34± 0.04	0.33± 0.07	0.32± 0.06
P <		NS	NS	NS	NS	-	NS	NS	NS
AFT_3 pmol/l	7.8± 2.2	5.4± 1.2	5.0± 1.1	5.6± 1.0	5.2± 1.7	-	5.7± 1.2	5.5± 1.3	6.0± 1.3
P <		0.05	0.005	0.025	0.025	-	NS	0.0005	NS
TSH mU/l	2.1± 2.7	1.1± 0.9	1.0± 0.6	1.4± 1.5	1.2± 1.2	1.0± 0.4	1.5± 1.5	1.5± 0.8	1.5± 1.3
P <		NS	NS	NS	NS	NS	NS	NS	NS

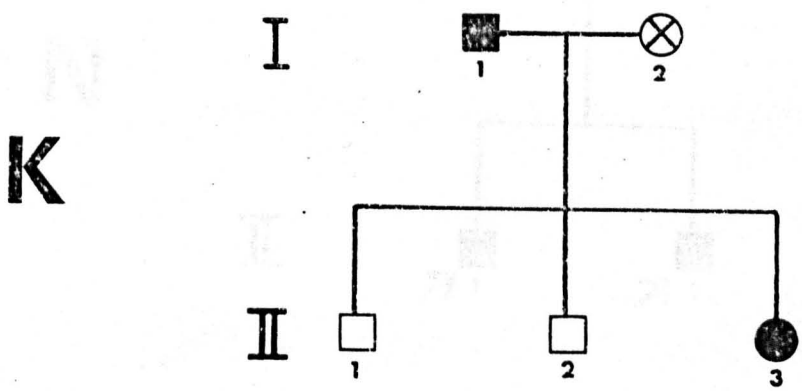
APPENDIX E

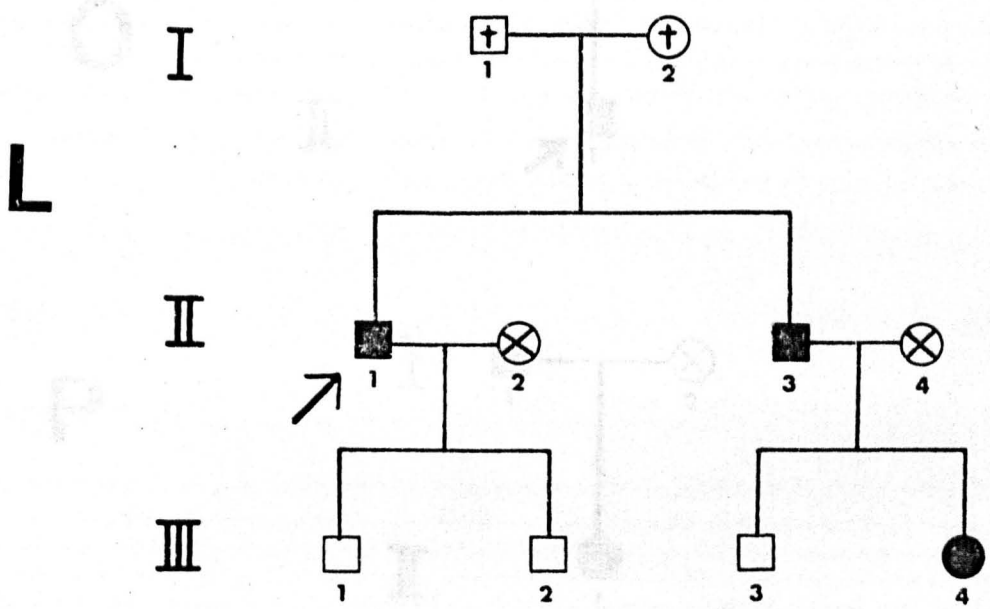
	DAY 0	1	2	3	4
T_4 nmol/l	130±31	118±29	115±27	121±29	123±27
P <		NS	NS	NS	NS
T_3 nmol/l	3.2±0.6	2.8±0.6	2.1±0.5	2.0±0.6	1.9±0.4
P <		0.05	0.0025	0.005	0.0005
rT_3 nmol/l	1.0±0.1	1.0±0.1	1.1±0.1	1.2±0.1	1.5±0.3
P <		NS	0.025	0.0025	0.0025
TBG mg/l	15.3±2.7	15.6±2.5	14.7±2.7	14.9±2.6	13.9±2.1
P <		NS	0.025	0.05	0.005
TBPA mg/l	392±77	387±65	357±47	329±38	281±38
P <		0.05	0.025	0.01	0.0025
ALBUMIN g/l	49.0±4.7	50.7±3.5	51.7±7.5	55.6±6.3	45.9±6.3
P <		NS	NS	0.05	NS
TSH mU/l	5.3±4.0	4.2±3.2	2.6±1.5	2.7±1.5	3.4±1.9
P <		NS	0.025	0.05	NS
AFT_4 pmol/l	35.0±15.6	29.5±12.1	30.8±13.3	32.7±12.8	38.7±15.8
P <		NS	NS	NS	0.025
AFT_3 pmol/l	7.0±2.2	5.6±1.9	4.6±1.6	4.3±1.3	4.8±1.7
P <		0.025	0.0025	0.0025	0.0025

THYROID FUNCTION DURING COMPLETE STARVATION

APPENDIX F (For key see Fig.5.1)

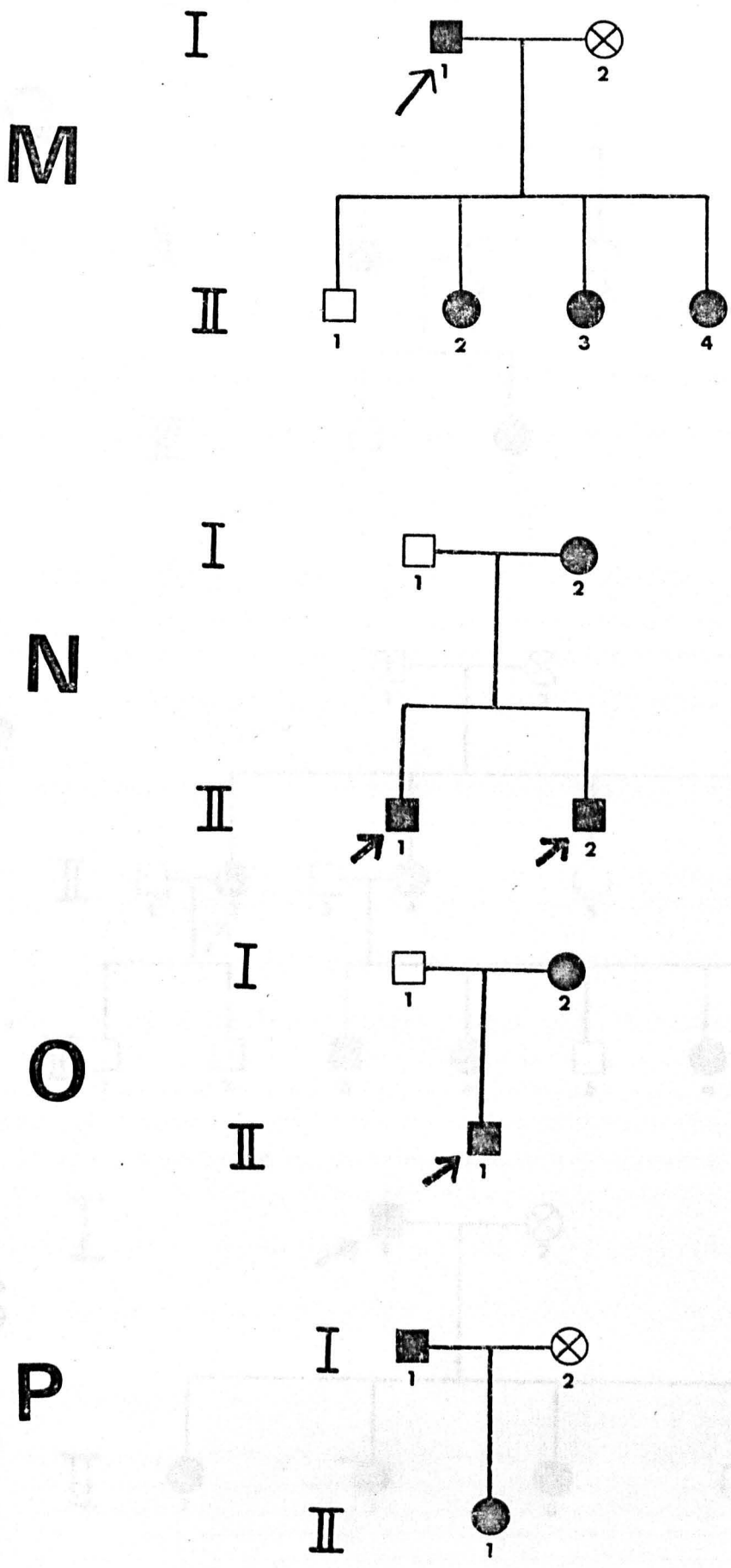






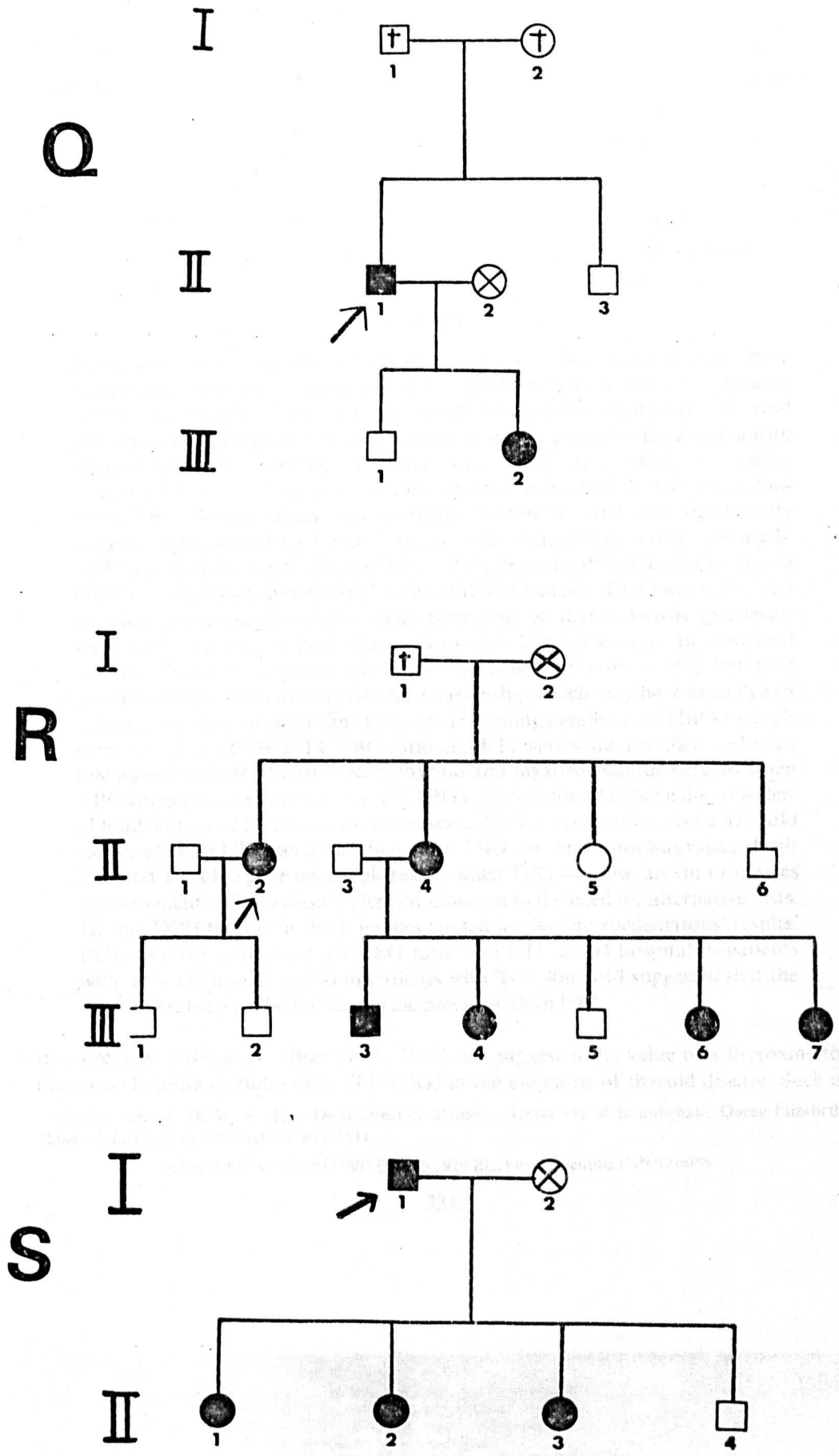
HEREDITARY TBG INCREASE (FAMILY J) AND DECREASE (FAMILIES K AND L)

APPENDIX F (Cont.)



HEREDITARY TBG DECREASE (FAMILIES M, N, O AND P)

APPENDIX F (Cont.)



HEREDITARY TBG INCREASE (FAMILIES Q AND S) AND DECREASE (FAMILY R)