

AN IMMUNOELECTROPHORETIC STUDY OF THYROXINE
BINDING PROTEINS IN HUMAN SERUM

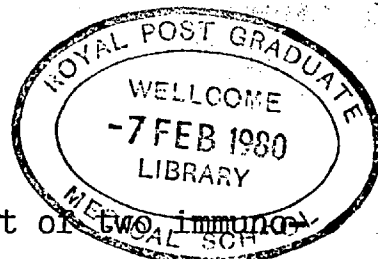
Ian Paul DRYSDALE

Thesis submitted for the Ph.D. Degree
of the University of London.

Registered at the
Royal Postgraduate Medical School

March 1979

ABSTRACT



This study describes the development of two immuno-electrophoretic techniques for the assay of Thyroxine Binding Globulin (TBG). The techniques were designed with the aims of providing (i) a direct and straightforward method for the estimation of TBG concentrations in patients and (ii) a method which could simultaneously estimate TBG concentration and the distribution of thyroxine amongst the various serum proteins capable of binding thyroxine.

The methods devised for this study included several novel features. Multispecific antisera were used so that the entire analysis could be performed simultaneously. Thyroxine incorporating a radioactive isotope ($^{125}\text{I T}_4$) was used to trace both protein position and hormone binding. The electrophoretic medium was modified to incorporate a physiological buffer so that closer approximation to the in vivo milieu could be attained. The modified methods were designed to be straightforward in performance, and simple in measurement of the final result. They were thoroughly evaluated and found to be sensitive and highly reproducible.

Thyroxine Binding Globulin concentrations were measured in patients of either sex with hyper- and hypothyroidism, in women with high circulating levels of oestrogen (during pregnancy or administration of oral contraceptive), in foetal serum, in amniotic fluid, in a family with congenital deficiency of TBG, and in patients with liver disease and following surgery. Where rapid changes in TBG concentration were anticipated, sequential observations were made (during oestrogen administration and surgery).

The relationship between total thyroxine concentration

and percentage binding of thyroxine by TBG was investigated and the quantitative recovery and distribution of thyroxine was examined using physiological and non-physiological buffers.

The techniques which have been developed are considered to be widely applicable in the fields of Biochemistry, Physiology and Pharmacology.

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ACKNOWLEDGEMENTS

My thanks and appreciation are due to many people who have helped and encouraged this project, and to the Authorities at Northwick Park Hospital Clinical Research Centre for the use of their facilities. Principal acknowledgement is due:

to the following for their valuable advice and discussion

Professor B. Lewis
Dr. R.L. Himsworth
Dr. D.B. Ramsden
Mrs. Daphne Bird

to the following for making serum samples available to the study

Dr. M.J. Denham
Dr. D.B. Grant
Dr. T.W. Meade
Mrs. Sylvia Chalkley
Mrs. Gloria James
Clinical Chemistry Department of N.P.H.

and to those volunteers who donated their own serum for the study.

to Mr. P. Princé for photographing the plates

to my wife Judith Drysdale for the unenviable task of typing the Thesis, and to Miss Valerie Nicholson for typing the references.

Finally to my parents Mr. & Mrs. D.B. Drysdale for their constant support of S.E.O.S.B.

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1.1.

HISTORICAL INTRODUCTION

Seaweed was possibly used empirically in the treatment of goitre as early as the 15th Century B.C. by the Chinese, and certainly by Hippocrates in the 4th and 5th Centuries B.C. The first major step in elucidating the relevant factors occurred in 1812 when a new element was discovered in seaweed by the French chemist Bernard Courtois, and was subsequently named Iodine, by a fellow chemist Gay-Lussac, due to its deep violet colour on vaporization.

The significance of this new element in relieving goitre was quickly appreciated and was clinically applied by Prout in 1816 and later by Coindet in 1821. Evidence which substantiated Prout's and Coindet's treatment followed in 1850, when Chatin conducted Geographical surveys on the iodine content of air, water and soil and found an inverse relationship between the environmental iodine content and the incidence of goitre, and on the basis of his lengthy research, Chatin advocated iodine supplementation to the diets of communities deficient in iodine as an effective preventative measure against goitre.

Glycerine extracts of sheep thyroid glands were first used for the treatment of myxoedema in 1891 by an English physician George Murray and soon after in 1896, Bauman postulated the direct association between iodine and the thyroid after demonstrating the gland's high iodine content. Bauman believed iodine was in organic combination within the gland and named the compound, Iodothyrim. Three years later in 1899, Bauman's postulate was substantiated by the work of Oswald who, using hydrolysates and enzyme digestions of the gland revealed a relationship between iodine and a globulin,

found only within the thyroid follicles, which was subsequently named Thyroglobulin.

The concept of an internal secretion by the thyroid gland had been postulated as early as 1836 by T.W. King and evidence supporting this occurred in 1900 when Gley and Bourcet demonstrated the presence of plasma protein bound iodine in human serum. The nature of this iodine complex was partly resolved in 1914 by E.C. Kendall using alkaline hydrolysates of the gland to isolate a molecule containing four iodine atoms which he named Thyroxine. The chemical analysis of the structure of thyroxine followed in 1926 by Harington and 1927 by Harington and Barger enabling the subsequent synthesis of thyroxine. In 1939, Trevorrow demonstrated that circulating iodine was not dialysable from serum and that its coprecipitation with the plasma proteins indicated a strong bonding between the iodine and plasma proteins. Subsequent investigations have shown that iodine can be dissociated from the plasma proteins when treated with organic solvents, such as butanol, which suggests a non-covalent bond between the iodine complex and its binding plasma proteins.

Once the nature of the thyroid hormone had apparently been established, work was stimulated in the field of hormone transport and in 1947 and 1948 Taurog and Chaikoff demonstrated that the iodine containing molecules transported in serum were identical with Thyroxine.

During the twenty years from 1930 to 1950, Thyroxine was assumed to be the thyroid hormone, but due to accumulating chemical evidence there was increasing speculation suggesting that a second hormone might be secreted from the gland. This speculation culminated in 1952 with the discovery by J. Gross

and Rosalind Pitt-Rivers of a molecule in human plasma which contained three iodine atoms. This molecule was shown to be physiologically active and to be largely bound to the plasma proteins, as is thyroxine, and it was named triiodothyronine. Molecule for molecule, Triiodothyronine has been shown to be more physiologically potent than thyroxine in terms of speed of action and degree of elicited response though the effects are of shorter duration. These properties have led to the postulate of a prohormone-hormone relationship between thyroxine and triiodothyronine though this remains, to date, a speculative area.

The current fields of interest are many though one central phenomenon remains crucial to the further understanding of thyroid physiology, the transport mechanism of the hormone in serum.

The serum hormones are currently accepted to be in two forms:-

- 1) bound to certain plasma proteins and
- 2) free or unbound hormone dissolved in the plasma fluid.

The free or unbound hormone is the favoured 'active' form, but the factors affecting the concentration of free hormone are not solely those connected with total plasma hormone level. The concentration of binding protein, the relative affinity of protein for hormone, and the clearance of hormone from serum, all affect the concentration of free hormone, and the study of these binding proteins has become an important area for the understanding of thyroid hormone metabolism.

1.2. THYROID HORMONES

1.2.1. CHARACTERISATION OF THYROID HORMONES

The thyroid hormones have two fundamentally inter-related properties, i) a chemical composition and ii) a physical structure.

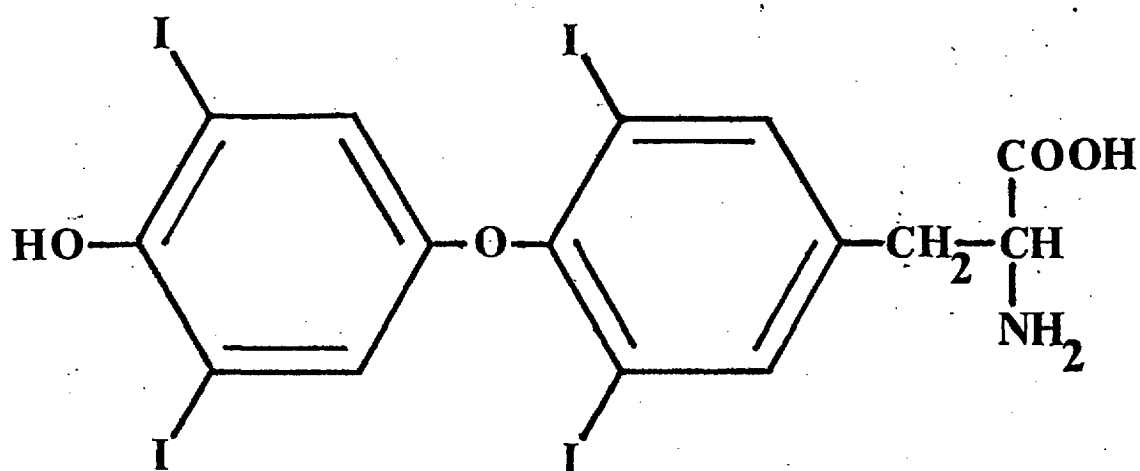
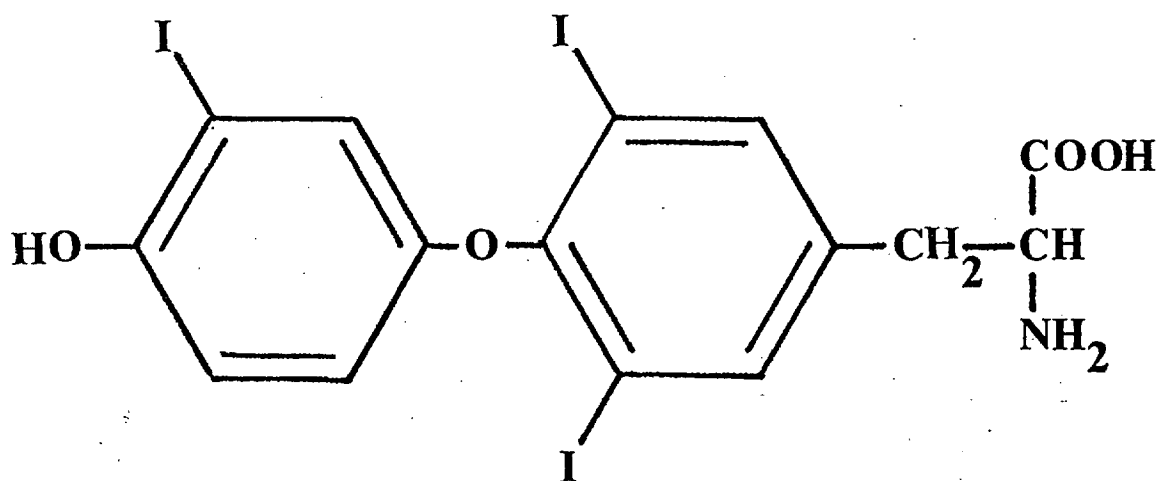
The chemical composition has been known since the second quarter of this century and is well established, but the physical structure is a more open field of research and has been only partly resolved.

i) Chemical Composition

The hormones are composed of two iodinated and condensed tyrosine moieties and differ only in the number of iodine atoms contained. The common elemental components are a diaryl ether with an alanine side chain in the 1 position, a phenol in the 4' position and iodine atoms at 3, 5 and 3'. Thyroxine possesses a further iodine at the 5' position, Figs. 1A & B.

The elemental composition of the naturally occurring hormone is not essential for some degree of biological activity and Jorgensen (Jorgensen 1970) has summarized the hormonal features necessary for a significant level of activity in mammals:-

- "1. a diaryl ether, sulphide or methane structure.
2. an aliphatic side chain containing a carboxyl group or its metabolic precursor in the 1 position, the L-alanine group giving maximal activity.
3. a phenolic group, amino group or a group capable of being metabolically transformed into a phenolic group in the 4' position.
4. Halogen atoms or methyl groups in the 3, 5 positions, the order of activity being $I > Br > Cl \approx CH_3$.
5. for maximal activity a variety of groups may be attached in the 3' or 3' and 5' positions with contributions to activity being in the order:-
isopropyl > I > ethyl > Br > methyl > tert-butyl > phenyl > Cl > H > F > Hydroxyl for 3' monosubstitution. It is found in general that 3' monosubstitution is more favourable than 3', 5' disubstitution."

Fig. 1a Thyroxine**Fig. 1b Triiodothyronine**

ii) Physical Structure

The chemical components of the thyroid hormones are arranged in specific positions with a stable three dimensional configuration. This spatial arrangement is essential for the hormone's action, as the biological receptor rejects a molecule of different structure.

The physical conformation of the hormone places the two benzene rings at right angles to one another through the long axis, and inclined at 110° through the ether oxygen link at right angles to the long axis. The ether link is a classic site for free rotation, and in the thyronine molecules, although rotation was thought to be inhibited by the angle of the ether link, and by the atomic size of the iodine atoms (Lehmann 1972), it has subsequently been shown that the rotation, though restricted, does occur (Emmett & Pepper, 1975).

1.2.2. BIOSYNTHESIS AND SECRETION
 OF THYROID HORMONES

Biosynthesis of thyroxine occurs entirely within the thyroid gland via four stages which form a continuous process. Thyroglobulin is synthesised to form the colloid protein and, since no transfer-RNA for iodotyrosine or thyronine has been characterized, iodination at specific sites presumably follows thyroglobulin synthesis. Various iodinated products are formed but only thyroxine and tri-iodothyronine have any significant hormonal activity. These hormones remain within the thyroglobulin structure until the protein is pinocytosed from the colloid by the apices of the follicle cells. These cells engulf the colloid (Deiss & Peake, 1968; Seljelid & Nakken, 1968) forming phagocytic vacuoles which migrate from the apex toward the base of the cell (Wetzel, Spicer & Wollman, 1965). After fusing with a lysosome these compound "lysosome-colloid droplet-phagosome" particles continue through the cell whilst the colloid is being hydrolysed, liberating the iodothyronines and iodo-tyrosines (Greer & Grimm, 1968; Green, 1968). It seems that the iodothyronines and companion substances are then released directly into the blood since iodothyronines are, at least partially, deiodinated on exposure to follicle cell protoplasm (Haibach, 1971), a biologically unfavourable event. Support is also given to the direct release hypothesis by the finding that thyroglobulin and its iodinated protein components may be isolated from blood and lymph leaving the gland (Roitt & Torrigiani, 1967; Daniel, Pratt, Roitt & Torrigiani, 1967).

Once liberated from the gland the hormones are bound by the carrying proteins in serum and are transported to the peripheral target organs.

1.3. THYROID HORMONE BINDING PROTEINS

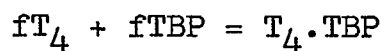
The Thyroid hormones are virtually insoluble in aqueous solutions and the majority (>99%) are carried in the plasma bound to proteins.

The circulating thyroid hormones thus occur in two forms:-

- i) in free solution
- ii) bound to carrier molecules.

Free Hormone

The protein carrier molecules, which transport the majority of hormone, interact with it to form a reversible relationship:



which conforms with the Law of Mass Action and may be written:

$$K = \frac{(T_4.TBP)}{(fT_4).(fTBP)}$$

where K = Equilibrium constant for the reaction and is unique for each binding protein (Table 1).

(T_4) = Concentration of free T_4

(TBP) = Total number of unoccupied sites on the binding proteins.

$(T_4.TBP)$ = Total number of occupied sites on the binding proteins.

Using this type of theoretical approach, free hormone has been described in terms of total hormone concentration, the concentration of the binding proteins and their association constants (Robbins & Rall, 1960; Distefano & Chang, 1971; Princé & Ramsden, 1977). In practice, the total

TABLE 1.

	M.W.	Serum Concentration	Plasma (?)	Number of Binding Sites	^a Affinity Constant for T ₄	Affinity Constant for T ₃ @ 25°C	% Binding of T ₄	% Binding of T ₃
TBG	36,500 to 63,000	SEE TABLE 2	5 ± 1.2. days	1	2.0 x 10 ¹⁰ M ⁻¹	^b 6 x 10 ⁸ M ⁻¹	70%	65%
(?) TBPA	50,000	4 - 5 mg/100 ml. <u>Report</u>	2 days	2	i) 5.5 x 10 ⁷ ii) 5.5 x 10 ⁵ M ⁻¹	^c 1.2 x 10 ⁷ to ^d 1.3 x 10 ⁶ M ⁻¹	15 - 30%	10%
ALB.	69,000	3.5 - 4.5 g/100 ml.	15 days	Multiple	i) 4.0 x 10 ⁵ ii) 3.0 x 10 ⁴ M ⁻¹	^e 16.7%K _{T₄}	10%	20 - 30%

- a. Princé & Ramsden (1977) for resume of literature regarding affinity constants.
b. Korcek & Tabachnick (1976).
c. Pages, Robbins & Edelhoeh (1973).
d. Nilsson & Peterson (1971).
e. Steiner, Roth & Robbins (1966).

TABLE 2

	Mean mg/100 ml.	Number	Range mg/100 ml.	Male (Mean) mg/100 ml.	Number	Female (Mean) mg/100 ml.	Number
Levy et al. 1971	3.4 ± 0.1 SE	51	2 - 4.8	-	45	-	6
Chopra et al. 1972	2.85 ± 0.08 SEM	59	1.6 - 4.2	3.01 ± 0.14 SEM	20	2.77 ± 0.09 SEM	39
Castro & Ugarte 1974	0.0166	POOL					
Bradwell et al. 1976	-	-	-	1.1 ± 0.28 SD ($0.63 - 1.61$) (Range)	24	1.21 ± 0.28 SD ($0.67 - 1.5$) (Range)	20
Hesch et al. 1977	0.98 ± 0.14 SD	34	-	-	-	-	-
Burr et al. 1977	-	-	-	1.12 ± 0.049 SE	29	1.25 ± 0.075 SE	13

concentration of hormones has been extensively studied and the normal ranges are now accepted as 60-150 nmol/l for thyroxine and 1.2 - 2.8 nmol/l for triiodothyronine (Hoffenberg, 1973). The usually accepted concentration of free T_4 is approximately 0.03% of total T_4 concentration, and free T_3 is approximately 0.3% of total. These figures were obtained by equilibrium dialysis or related techniques (Sterling, 1971; Uchimura, Nagataki, Tabuchi, Mizuno & Ingbar, 1976; Pedersen, 1974). However, one recently described direct assay reports somewhat different levels (Yeo, Lewis & Evered, 1977).

The free hormone is currently accepted as the metabolically active form, whilst the bound form represents a large circulating reservoir of potentially active hormone. From theoretical considerations free hormone concentration can be seen to be dependent on both total binding protein concentrations and total hormone concentrations. Individuals with congenital absence of TBG are usually euthyroid with very low total T_4 concentrations, but free hormone is within the normal range (Burr, Ramsden, Evans, Hogan & Hoffenberg, 1977). Also subjects with high plasma TBG concentration (either congenital or due to endogenous or exogenous oestrogens) have above normal total T_4 concentration, but again free hormone concentration is thought to be within the normal range and these subjects are clinically euthyroid.

The "free" thyroxine is the accepted metabolically active form of the hormone, and its concentration is dependent in part on the binding protein concentration. The further study of the transport protein picture, and the relationship between free and bound hormone, is therefore important in

the understanding of thyroid physiology.

Bound Hormone

Five main serum proteins have been identified as having the capacity to transport thyronines and in decreasing order of thyroxine binding they are:-

Thyroxine Binding Globulin (TBG)

Thyroxine Binding Prealbumin (TBPA)

Albumin

α_1 -Lipoprotein (α_1 -LP)

β -Lipoprotein (β -LP)

Each of these distinct proteins has its own characteristics. Their importance in thyroid hormone transport depends upon these characteristics and upon their relative concentrations in plasma. The primary T_4 binding protein was identified by three groups of workers (Gordon, Gross, O'Connor & Pitt-Rivers; 1952; Larson, Deiss & Albright, 1952; Robbins & Rall, 1952) who established that it was an inter- α -globulin and Robbins & Rall (1952) named it Thyroxine Binding Globulin (TBG). A second protein, Albumin, was also found to bind thyroxine, and subsequently a third plasma protein, Thyroxine Binding Prealbumin (TBPA), was identified (Ingbar, 1958; Tanaka & Starr, 1959). Using conventional immunoelectrophoresis in physiological buffer at pH 7.4, Miyai, Itoh, Abe & Kumahara (1968) identified the thyroxine binding proteins in human serum as TBG, TBPA, Albumin, α_1 -Lipoprotein and β -Lipoprotein in descending order of binding importance.

Protein binding of triiodothyronine has received far less attention due probably to the difficulty of carrying out studies at the very low physiological concentration of the hormone. However, Woeber, Hecker & Ingbar (1970) have shown using filter paper electrophoresis and glycine-acetate

buffer at pH 8.6 that triiodothyronine is bound to TBG and Albumin, and other studies have also suggested that TBPA does not bind triiodothyronine (Braverman & Ingbar, 1965; Ingbar, 1963), but more recently contrary evidence has been obtained (Davis, Handwerger & Gregerman, 1972).

The quantitative importance of the individual proteins in binding thyroid hormones has received much attention. The normal distribution of thyroxine in plasma is TBG 60%, TBPA 30% and Albumin 10% (Oppenheimer, Squief, Surks & Hauer, 1963; Ingbar & Freinkel, 1960) when determined by electrophoretic methods. More recently specific immunoprecipitation techniques have shown that the percentage bound by TBPA may be somewhat less (Woeber & Ingbar, 1968). Triiodothyronine is somewhat differently distributed. Again the major binding protein has been shown to be TBG 65-70%, whilst albumin was thought to carry the remainder (Woeber et al., 1970). However, Davis and his colleagues (1972) have shown that 7-9% of total T_3 in normal serum is bound to TBPA and this proportion rises to 30% in serum with a low TBG concentration.

It can be seen from the foregoing brief account that the binding proteins play a major role in the transport of thyroid hormones in serum. Therefore, a more detailed description of the binding proteins follows.

a) Thyroxine Binding Globulin (TBG)

Thyroxine Binding Globulin is the major thyroid hormone binding protein. Each molecule of TBG has been shown to possess one binding site that may accept either a T_4 or T_3 molecule (Seal & Doe, 1965; Sterling, Hamada, Takemura, Brenner, Newman & Inada, 1974) resulting in competitive binding (Thorson, McIntosh & Willms, 1969).

The serum concentration of TBG has been variously reported between 0.6 and 5 mg./100 ml. serum, using a wide range of techniques (Levy, Marshall & Velayo, 1971; Chopra, Solomon & Ho, 1972; Bradwell, Burnett, Ramsden, Burr & Hoffenberg, 1976) (Table 2). Thyroxine Binding Globulin is formed in the liver (Glinioer, Gershengorn & Robbins, 1976) and has a circulating half-life in humans of about 5 days $\pm$ 1.2 days (Cavalieri, McMahon & Castle, 1975).

Thyroxine Binding Globulin is an acid glycoprotein with a molecular weight of between 36,500 and 63,000 (Cavalieri et al., 1975; Refetoff, Fang & Marshall, 1975; Sterling et al., 1974; Giorgio & Tabachnick, 1968) and the high sialic acid content of 9 moles/mole of protein (Seal & Doe, 1965). When TBG is desialylated the binding affinity is unaffected, but the electrophoretic mobility is decreased (Woeber & Ingbar, 1974). Although desialylated TBG is not found in normal serum, significant amounts have been detected in patients with obesity and severe liver disease and it has been postulated that the desialylation is the first step in the catabolism of TBG (Refetoff et al., 1975). The plasma concentration of TBG is increased by administration of oestrogens and decreased by androgens.

b) Thyroxine Binding Prealbumin (TBPA)

Thyroxine Binding Prealbumin is the second major thyronine binding protein and has been variously reported to carry 15% - 30% of tracer thyroxine (Oppenheimer et al., 1963; Woeber & Ingbar, 1968; Davis & Gregerman, 1970; Lutz & Gregerman, 1969), and perhaps a small quantity of triiodothyronine (Woeber et al., 1970; Davis et al., 1972). The TBPA molecule contains two identical, but independent binding sites for thyroxine and these sites ex-

hibit negative co-operativity (Ferguson, Edelhoch, Saroff & Robbins, 1975).

The serum concentration of TBPA has been shown to be between 20 and 30 mg./100 ml. serum (Oppenheimer, Martinex & Bernstein, 1966; Ingbar, 1963). TBPA is probably synthesised in the liver as four equivalent sub-units each being formed by a single polypeptide chain, and rich in the amino acid tryptophan. These sub-units form a strong association to result in a protein of molecular weight of about 50,000 (Raz & Goodman, 1969; Blake, Geisow, Swan, Rerat & Rerat, 1974). Its plasma half life is about 2 days which shows it to be one of the most rapidly metabolised proteins in man (Woeber & Ingbar, 1974). About one third to one half of TBPA in normal circulation is associated with retinol binding protein (RBP) which transports the alcoholic form of Vitamin A. The RBP has a molecular weight of about 21,000 and a serum concentration of 4-5 mg./100 ml. serum (Smith, Raz & Goodman, 1970). This RBP/TBPA association gives a combined molecular weight of about 71,000 (Oppenheimer, Surks, Smith & Squeef, 1965; Seal & Doe, 1965). The binding of RBP to TBPA does not affect T_4 binding indicating different sites of attachment (Raz & Goodman, 1969).

In contrast to TBG, the serum concentration of TBPA is increased by administration of androgens (Braverman, Socolow, Woeber & Ingbar, 1968).

c) Albumin

The third major thyronine binding protein is albumin and it transports about 10% of serum T_4 and 20-30% of serum T_3 (Woeber et al., 1970). In contrast to the two other major binding proteins, TBG and TBPA, albumin has multiple thyronine binding sites and no survey has successfully managed to

saturate the protein with T_4 . The serum concentration in contrast to TBG and TBPA is extremely high and in normal serum is between 3.5 and 4.5 gm./100 ml. serum. Albumin is synthesised in the liver, and its biological half-life is about 15 days. Albumin has a molecular weight of about 69,000 and the cigar shaped molecule has multiple roles in serum which contrasts both TBG and TBPA.

d) The Lipoproteins

The lipoproteins (α_1 and β lipoprotein) are included as the first major secondary binding proteins. Their role in hormone binding has been largely overlooked, but their normally high lipid content makes them ideal carriers of the lipophilic hormones (Miyai et al., 1968). These higher molecular weight proteins may be shown to be significant carriers of T_3 particularly at levels of hormone above physiological concentration (Miyai et al., 1968, Ramsden, Drysdale & Hoffenberg, in Hoffenberg, 1973).

1.4. MEASUREMENT OF THYROID HORMONE

BINDING PROTEINS

1.4.1. HISTORICAL ASPECTS

The methods used for binding protein estimation date from the earliest protein separation technique of salting out. This relatively crude method was superceded by an electrophoretic method developed by Tiselius (1939-40) and modified by Longsworth (1939). Lewis and McCullagh (1944) used this electrophoretic method to study the plasma protein picture of hyper- and hypothyroid patients, and their survey indicated major changes in the α_1 -globulin and albumin regions inferring a connection between these electrophoretic protein regions and thyroid status.

Although the zone electrophoresis technique was adequate for the qualitative determination of the thyroxine binding proteins, the method could not be used quantitatively to determine either the amount of binding protein or the percentage binding of hormone by the individual proteins. In 1956, Robbins modified the zone electrophoresis technique to great advantage by opposing the phenomena of electrophoretic motion and flow of buffer medium. The system was named the Reverse-flow electrophoresis method and allowed sufficient resolution between the major binding proteins to enable effective quantitation of hormone carriage. The reverse-flow method has been used to indirectly estimate binding protein concentration using the Maximum Binding Capacity method (M.B.C.) (Perry, Hodgman & Starr, 1965; De Nayer, Malvaux, Van Den Schrieck, Beckers & De Visscher 1965; Harvey 1971; Wellby, Pharoah & Hetzel, 1974).

Other indirect methods of quantifying TBG are the Red Cell Uptake and Resin Uptake Tests. These methods are based

on the principle of estimating the unoccupied binding sites in the serum population of TBG molecules by making use of ^{125}I T_3 and its lower affinity for thyroxine binding sites. This estimate, together with the serum endogenous T_4 estimation, which indicates the size of the TBG population involved in T_4 binding, allows an indirect estimate of TBG concentration (Hamolsky, Stein & Freedberg, 1957; Hamolsky, 1971; Mitchell, 1958).

The method of crossed immunoelectrophoresis developed by Ressler (1960), modified by Laurell (1965) and further modified by Minchin-Clarke & Freeman (1968) has more recently been used for the direct quantitation of the binding proteins (Freeman & Pearson, 1969). Using this method the antigen proteins are precipitated by an antiserum, in the form of Gaussian or near-Gaussian peaks whose area is directly proportional to the antigen concentration. The binding proteins are identified by autoradiography.

A second method developed by Laurell (1966), known as the Laurell 'rocket' Technique has also been used to directly quantitate the binding proteins (Nielsen, Buus & Weeke, 1972; Kranz, Trautwein & Sieber, 1974). The technique involves migration of the antigen proteins directly into a gel impregnated with an antiserum and narrow peaks or 'rockets' are formed.

Polyacrylamide gel electrophoresis has also been used to study the binding proteins and percentage binding by individual proteins (Davis & Gregerman, 1970 & 1971; Davis, Handwerger & Gregerman, 1972). Binding proteins are estimated using the maximum binding capacity technique. A modification of the Polyacrylamide gel system is the "DISC" form, and Hagen & Elliott (1973) have used this method to study binding proteins in serum and cerebrospinal fluid. The "DISC" (or

discontinuous gel electrophoresis) uses a double buffer system and small rods of polyacrylamide gel, and estimation of protein quantity is by maximum binding capacity.

Recently a number of methods have been published which do not employ electrophoresis and are direct measures of serum binding proteins. Levy, Marshall & Velayo (1971) have developed a Radioimmunoassay for TBG, a method that requires absolutely pure TBG as an antigen so that a monospecific anti-serum may be raised, and an equally pure labelled TBG preparation (^{125}I TBG) made.

Chopra, Solomon & Ho (1972) developed a competitive ligand binding assay for TBG which utilizes the phenomenon of triiodothyronine partitioning between TBG and T_3 - binding antiserum. The assay requires skill and needs two antisera.

A process of diazotization, involving a solid phase assay using thyroxine bound to glass beads, has been developed by Castro & Ugarte (1974) to quantitate TBG. The assay involves covalently binding thyroxine to glass beads and extracting TBG by affinity chromatography, but again the process is arduous.

These methods represent the progression of thyroid hormone binding protein measurement from their inception in the first third of this Century to the current position (1974). The most widely used method is some variant of the Resin Uptake Test though it has, in common with each of the other methods, certain disadvantages which make the search for more direct, simple and efficient methods an important aim in thyroid research.

1.4.2. CRITICAL ANALYSIS OF CURRENT METHODS

As outlined in the previous section the current methods available for the measurement of TBG in serum include a range of methods which vary widely in their technique and often in their results. Each is critically considered below:-

ELECTROPHORETIC TECHNIQUES

The electrophoretic methods used to study the binding proteins are:-

- i) Filter Paper Electrophoresis
- ii) Polyacrylamide Gel Electrophoresis
- iii) Laurell Two Dimensional Immuno-electrophoresis
- iv) Laurell Rocket Immuno-electrophoresis
- i) Filter Paper Electrophoresis

This process has been used to measure the carrier protein and estimate the amount of hormone bound by the individual proteins.

This method of quantifying the carrier proteins, by Maximum Binding Capacity, has two major disadvantages, one, it is an indirect estimate of protein concentration, and two, it uses relatively large amounts of labelled hormone which is expensive and risks contamination. The actual concentration of the protein is not measured, but is estimated by determining the maximum amount of radio-labelled thyroxine that can be bound by the protein. Protein concentration is then expressed as the amount of hormone bound per unit volume of serum.

Electrophoresis is generally performed at pH 8.6 since at this pH electrophoretic migration is maximized and electro-endosmosis minimized. The buffer ions used are either glycine-acetate, Tris-maleate or barbitone buffer (Perry et al. 1965; De Nayer et al. 1965; Harvey 1971; Wellby et al. 1974), but the maximum binding capacities of TBG and TBPA have been found

to decrease in the order glycine-acetate, Tris maleate, and barbitone. Because these differences occur even when all tests are performed at pH 8.6 using the same technique (Robbins 1956; Ingbar & Fienkel 1960), the inference is that the choice of buffer ion affects binding of T_4 . Support is given to this conclusion by Blumberg, Farer, Rall & Robbins (1961) who found TBPA binding of thyroxine to be almost completely inhibited in barbitone buffer. None of the buffer ions used in these studies are comparable to biological systems, and may all imprecisely reproduce the physiological situation.

The pH used in these studies is also far from physiological and Davis & Gregerman (1970) have shown TBG to bind 50% more thyroxine at physiological pH while TBPA bound more thyroxine at pH 9.0. These results seem to indicate that the most logical conditions under which to study hormone binding are those most closely resembling the physiological.

ii) Polyacrylamide Gel Electrophoresis

Two variations of the polyacrylamide gel electrophoresis methods have been used. The first, by Davis & Gregerman (1971), used gel blocks, and the second used rods of gel and the "DISC" form of electrophoresis (Hagen & Elliott, 1973). This gel has the disadvantage of a relatively small pore size and thus inhibits migration of the essential, large lipoprotein molecules, which have been shown to bind thyroxine (Miyai, Itoh, Abe, & Kumahara, 1968). Both studies depend upon the indirect maximum binding capacity method to estimate protein concentration.

iii) Laurell Two Dimensional Immuno-electrophoresis

Direct estimation of the thyroxine binding proteins has been performed using the Laurell crossed immuno-electrophoresis system. Freeman & Pearson (1969) measured the area

under the carrier protein peaks on the autoradiographs which is directly proportional to the protein concentration. The electrophoresis was performed in a barbitone buffer at pH 8.6 and the labelled T_4 (^{125}I T_4) was bound to the serum sample after electrophoresis necessitating a large equilibration volume. This method is technically cumbersome, time consuming, extravagant in its use of materials, and technical skill is required throughout the process. Measurement of TBG from the autoradiograph is difficult and often inaccurate, particularly when the protein peak 'feet' on the autoradiograph are not well developed.

iv) Laurell Rocket Technique

The Rocket method (Laurell 1966) differs from the crossed technique in that only a one dimension separation occurs. Superimposed protein peaks are formed, the heights of which are virtually directly proportional to antigen concentration. The method is more conservative of antiserum than the crossed technique since many samples may be run on one plate and tracer hormone is equilibrated with serum before electrophoresis.

Two studies (Nielsen et al. 1972 and Kranz et al. 1974) have used this method to estimate serum TBG both using non-physiological electrophoresis systems of barbitone buffer at pH 8.6. The Kranz et al. (1974) study used a specific anti-TBG antiserum whilst the Nielsen et al. (1972) group used a semispecific antiserum. These antisera have the double disadvantage of being both expensive to prepare and allowing only one, or a small number of proteins to be measured simultaneously. The Nielsen et al. (1972) method included a radiolabelled thyroxine trace which necessitates the additional disadvantage of requiring expensive developing equipment

(R.P.X-Omat developer from Kodak). The Nielsen et al. (1972) paper draws attention to an implied binding anomaly when using a polyvalent antiserum, but the actual results using anti-whole human antiserum are not mentioned, and, perhaps significantly, no relevant discussion follows.

T₃ Uptake Method

The T₃ Uptake method for TBG estimation is indirect, but is the most commonly used method in routine departments (Hamolsky, et al., 1957; Mitchell, 1958; Hamolsky, 1971). To interpret the T₃ Uptake result, a second parallel estimation of total thyroxine content must be performed on an aliquot of the same serum sample and a TBG value is computed from the two results. The method involves not only the additional expense of another test for its interpretation, but also requires the accurate working of the totally unassociated method, therefore compounding any errors.

Recent Direct Methods for TBG Estimation

Three new methods have been published for the specific quantitation of TBG and are:-

- i) a Radioimmunoassay
- ii) a Competitive Ligand Binding Assay
- & iii) a Solid Phase Radioassay.

i) The Radioimmunoassay developed by Levy et al. (1971) has been reported as a sensitive and specific method, giving a normal range of 2.0 - 4.8 mg./100 ml. and a mean 3.4 mg./100 ml. $\pm$ 0.1 SE with 51 subjects. The method depends on the absolute purification of a TBG preparation and the use of this as a) a standard, b) an assay reagent after radiolabelling, and c) an antigen to raise antibodies. The isolation and purification of TBG is technically difficult and the subsequent raising of antibodies to the TBG, and its iodination requires skill which renders application to routine work difficult. The actual assay procedure is complex.

ii) Competitive Ligand Binding Assay

This assay, developed by Chopra et al. (1972), gives the TBG normal range of 1.6 - 4.2 mg/100 ml. with a mean of 2.85 mg/100 ml. $\pm$ 0.08SEM. and is comparable with the normal range of Levy et al. (1971). The competitive ligand binding assay involves a large number of steps, demands technical skill and requires expensive specific antisera.

iii) Solid Phase Radioassay

This radioassay, (Castro & Ugarte 1974) requires stringently pure TBG which is obtained by passing whole serum down an affinity column, just once, with apparently no verification of its purity. The presence of other proteins could interfere with the assay and affect the results.

The radioassay procedure, is performed in non-physiological barbitone buffer at pH 8.6, and the iodination for the radio-labelling of assay TBG requires four different buffers and a change of pH from 11 to 8.6.

The major thyroid hormone binding proteins are TBG, TBPA and Albumin, and though well established techniques are available for quantitation of TBPA and Albumin, this is not so for TBG. This, the most important thyroid hormone binding protein, is in such low serum concentration that normal protein staining techniques and isolation procedures fail to produce quantitative results for TBG estimation. The current search, therefore, is for a technique of direct, relatively simple, accurate and reproducible estimation of TBG.

The requirements of any new method should aim at the following:-

1. Inexpensive, but effective materials.
2. Widely available apparatus.

3. Technical simplicity in both a) Performance of Technique
b) Measurement of Results.
4. Approximation to physiological milieu using both:-
 - a) Physiological buffer ions and
 - b) Physiological pH.
5. The Method should be specific, sensitive, reproducible and accurate.

To the above fundamental requirements, it would be advantageous to have all thyroxine binding proteins in the same analysis, and incorporating directly related standards. If these requirements could be met, the technique would be of significant advantage in the field of thyroid physiology.

2.

AIMS

From the preceding discussion in the Introduction it may be seen that the current methods for determining serum TBG concentration suffer from one or more of the following disadvantages:-

- a) being indirect measurements
- b) being performed in non-physiological milieu
- c) being unable to simultaneously quantitate binding of hormone and concentration of protein.
- d) requiring pure or semi-pure TBG and specific anti-TBG antiserum.
- e) high cost

Two methods were developed to eliminate the above disadvantages and each method was used to perform three main tasks:-

- i) to reappraise known results of TBG concentration and hormone binding.
- ii) to examine controversial results from other studies involving TBG concentration and hormone binding.
- iii) to investigate the effect of different conditions on TBG concentration and hormone binding.

The two methods were differentially used to conform with their design purpose so that the first method, the Modified Rocket Technique, was used to directly estimate TBG concentration in different sera whilst the second method, the Modified Line Technique, was used for the direct and simultaneous estimation of the percentage binding of hormone to the carrying proteins, the characterization of these proteins and their serum concentration.

3. MATERIALS & STANDARD METHODS3.1. LIST OF MATERIALS & REAGENTSReagentsSuppliers

- | | |
|--|--|
| 1. All Chemicals | Duncan Flockhart & Co. Ltd.,
Birkbeck St., E2 6LA.
and
Hopkin & William,
Freshwater Rd., Chadwell Heath,
Essex. |
| 2. Agarose Type A37 | L'industrie Biologique
Francaise S.A.,
35-49 Quana du Moulin de Cage,
Gennevilliers (Seine), France.
Distributed in the U.K. by:-
Micro-Bio Laboratories Ltd.,
46 Pembridge Rd., W.11. |
| 3. Amido Black 10B | Hopkin & William,
Freshwater Rd., Chadwell Heath,
Essex. |
| 4. Aminex Resin | Bio Rad Laboratories Ltd.,
27 Homesdale Rd., Bromley, Kent. |
| 5. Antiserum (Anti Whole Human) | Burroughs Wellcome, Beckenham,
Beckenham, Kent.
and
Paines & Byrne,
Perivale, Middx.
and
Dakopatts from:-
Mercia Brocades Ltd.,
Brocades Hse., Pyrford Rd.,
W. Byfleet, Surrey, KT14 6RA. |
| 6. Antiserum (Specific) | Paines & Byrne (as above)
and
Dakopatts (as above)
and
Behringwerke,
Hoechst Pharmaceuticals
Hoechst U.K. Ltd.,
Hoechst Hse., Salisbury Rd.,
Hounslow, Middx. |
| 7. Buffer Pump (Model C15/50) | Charles Austin Pumps Ltd.,
100 Royston Rd., Byfleet, Sy. |
| 8. Constant Temperature | Grant Instruments (Cambridge)
Ltd.
Barrington, Cambridge. |
| 9. Cooler (Churchill Chiller Thermocirculator) | Churchill Instruments Co. Ltd.,
Walmgate Rd., Perivale, Middx. |
| 10. Electrophoresis Tanks | M.B.I. (Model M.B.I. No. 260)
(C-3740Z, Immunoelectrophoresis Tank), Scientific Suppliers & Bioengineering, Northwick Pk. Hospital, Harrow, Middx.
(M.B.I. now: Chem-Lab Inst. Ltd., Hornminster Hse., 129 Upminster Road, Hornchurch, Essex, RM11 3XJ.) |

ReagentsSuppliers

- | | |
|--|--|
| 11. Filter Paper Circles | Whatman: local agents e.g.
A. Gallenkamp & Co. Ltd.,
P.O. Box 290, Technico Hse.,
Christopher St., EC2P 2ER. |
| 12. Freeze Drier | Edwards High Vacuum,
Manor Royal, Crawley, Sussex. |
| 13. Gamma-Counter
(LKB - Wallac 80000) | LKB Instruments Ltd.,
LKB Hse., 232 Addington Rd.,
Sth. Croydon, Sy., CR2 8YD. |
| 14. Glass Plates | Chance Proppter Ltd.,
Spon Lane, Smethwick, Warley. |
| 15. Hamilton Syringes | V.A. Howe & Co. Ltd.,
88 Peterborough Rd., SW6 3EP. |
| 16. Heater/Stirrers | A. Gallenkamp & Co. Ltd.,
P.O. Box 290, Technico Hse.,
Christopher St., EC2P 2ER. |
| 17. Isomantle | Electrothermal,
London, E.7. |
| 18. LH20 Resin | Pharmacia (G.B.) Ltd.,
Paramount Hse., 75 Uxbridge Rd.,
W5 1BR. |
| 19. Power Packs
(Output 0-500 V; 500mA) | M.B.I. Scientific Supplies,
(MBI No. 264; Catalogue No.
3745Z) Chem-Lab. Inst. Ltd.,
129 Upminster Rd., Hornchurch,
Essex. |
| 20. Razor Blades
(Valet No. 7003) | Gillette Surgical,
Great West Rd., Isleworth, Mx. |
| 21. Safety Lamp
(Safelight Filter No. 6B) | Kodak Ltd.,
Imperial Hse., Rayners Lane,
N. Harrow, Middx. |
| 22. Scalpel Blades & Handles | Swan Morton Ltd.,
Sheffield. |
| 23. Thyroid Hormones
(Radiolabelled) | Radiochemical Centre,
Amersham, Bucks. |
| 24. TEAE | Bio Rad Laboratories Ltd.,
27 Homesdale Rd., Bromley, Kent. |
| 25. X-Ray Developer
"Polycon" | May & Baker Ltd.,
Dagenham, Essex. |
| 26. X-Ray Film Kodirex | Kodak Ltd.,
Imperial Hse., Rayners Lane,
N. Harrow, Middx. |
| 27. X-Ray Fixer "Perfix" | May & Baker Ltd.,
Dagenham, Essex. |
| 28. X-Ray Hardener "C" Type | May & Baker Ltd.
(as above) |

3.2.

WORKING SOLUTIONS1. Amido Black 10B Staining Solution

<u>Reagent</u>	<u>Ratio</u>	<u>Volume</u>
Methanol (G.P.R.)	5	500 ml.
Distilled Water	5	500 ml..
Glacial Acetic Acid	1	<u>100 ml.</u>
		<u>1,100 ml.</u>

To make 0.1% stain solution, dissolve 1.1 gm. of Amido Black 10B powder in the, mixed, 1,100 ml. of solution. Thoroughly stir and dissolve before using.

To make the "wash" for destaining, use the same 5:5:1 solution without adding the Amido Black 10B powder.

2. Acetate Buffer for LH 20 Column

<u>Reagent</u>	<u>Ratio</u>	<u>Volume</u>
Ethyl Acetate (Analar)	4	800 ml.
Methanol (Analar)	1	200 ml.
2M Ammonia	0.4	<u>80 ml.</u>
		<u>1,080 ml.</u>

Preparation of LH 20 packing for column:-

Allow LH 20 sephadex to swell for 3 hours in the buffer. Wash three times using Methanol - 2M Ammonia (9:1), stir, leave to settle and tip off supernatant each time. Then wash twice with ether and filter through a Buchner funnel. Finally, wash twice with the acetate : methanol : 2M ammonia buffer above. For each wash use twice the volume of buffer to that of sephadex.

The LH 20 sephadex with acetate buffer was used to purify the radiolabelled T_4 and T_3 (Williams, Freeman & Florsheim 1969).

3. Phosphate Buffer

<u>Reagent</u>	<u>Quantity</u>
Sodium Dihydrogen Orthophosphate ($\text{NaH}_2\text{PO}_4, 2\text{H}_2\text{O}$)	15.6 gm. }
Distilled Water	2 l. } 0.05M Soln.

Sodium Hydroxide Soln. 5M

Use for making gel as 0.05M solution.

Use for tank buffer as 0.025M solution.

Dissolve sodium salt in 1.5 l of distilled water then titrate to pH 7.4 using the 5M sodium hydroxide. When solution reaches pH 7.4 add remaining distilled water until total volume is 2 l.

4. Veronal Buffer

<u>Reagent</u>	<u>Quantity</u>
Sodium Barbitone	185.56 gm. }
Calcium Lactate	11.52 gm. } 0.12M Soln.
Sodium Azide	1.5 gm. }
Concentrated Hydrochloric Acid	21 ml

Make up to 15 l with distilled water.

Use for making gel as 0.06M solution.

Use for making tank buffer as 0.03M solution.

5. Tri-Sodium Citrate Buffer

<u>Reagent</u>	<u>Quantity</u>
Tri-Sodium Citrate dihydrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$)	14.71 gm.
Distilled Water	900 ml.
Sodium Hydroxide Soln. 2M	25 ml.

Concentrated Hydrochloric Acid

Dissolve the citrate in the distilled water, then add the

sodium hydroxide whilst stirring. Boil this solution for 10 minutes then allow to cool to room temperature. Titrate the solution to pH 2.875 using the concentrated hydrochloric acid.

Make up volume to 1 l using distilled water.

3.3.

STANDARD PROCEDURES

3.3.1.

HORMONE STRIPPING PROCEDURE

A 2 cm. long column of Aminex ion exchange resin in pH 2.875 Trisodium Citrate buffer was prepared. A 1 ml. aliquot of a serum sample (I.D.) was equilibrated with 1 μ l of a purified preparation of radiolabelled thyroxine ($^{131}\text{I T}_4$) for 2 hours at room temperature. Tracer hormone is believed to be uniformly distributed with the endogenous hormone after 30 minutes, but the increased time was allowed to ensure complete equilibration. The movement of the radiolabelled hormone was assumed to mirror that of the endogenous hormone (Taurog & Chaikoff, 1948; Freinkel, Dowling & Ingbar, 1955).

An aliquot (0.35 ml.) of the labelled serum was passed down the Aminex column twice and eluted with the citrate buffer. The volume of each elution was measured and the number of counts left on the sample estimated (Table 3). The final sample from the column was three times more dilute than the original serum and this factor of 3 was carried forward to the modified Line plate sample preparation. Three aliquots were equilibrated with the stripped serum so that sample one aliquot contained 20 nmol/l of radiolabelled thyroxine ($^{125}\text{I T}_4$), sample two contained 50 nmol/l and sample three contained 100 nmol/l. These samples were equilibrated for 1 hour at room temperature and then used to produce the modified Line sample. These samples were also run against unstripped serum to determine that no TBG was lost during stripping.

TABLE 3

STRIPPING OF THYROXINE FROM SERUM PROTEINS
USING AMINEX RESIN

SERUM VOLUME	ELUANT VOLUME	COUNTS/10 SECS.	PERCENTAGE STRIPPED
0.35 ml.	-	13,000	-
	0.65 ml.	4,600	64.6
0.65 ml.	-	4,600	-
	1.00 ml.	600	95.4
1.00 ml.	-	600	95.4

3.3.2. PREPARATION OF AGAROSE GEL

The agarose used during this study was a 2% mixture of agarose Type A37 powder in distilled water. The stock agarose was made up, usually in 2 litre batches, as a 4% solution to give the required 2% gel on the plate after dilution with buffer or antiserum/buffer mixture.

The agarose powder (80 gm.) is weighed and mixed with about 200 ml. of cold distilled water using a mortar and pestle so that a relatively even mix is made. Of the remaining 1800 ml. of distilled water, 1300 ml. is poured into a large (2500 ml.) conical flask which is placed on a heater/stirrer and a magnetic bar stirrer is placed in the flask. The heater thermostat is set at about 65°C and the stirrer started. The agarose mixture is then poured into the flask and the mortar contents washed into the flask with the remaining 500 ml. of distilled water. The mixture is kept stirring until the agarose has melted and formed an homogenous clear mixture.

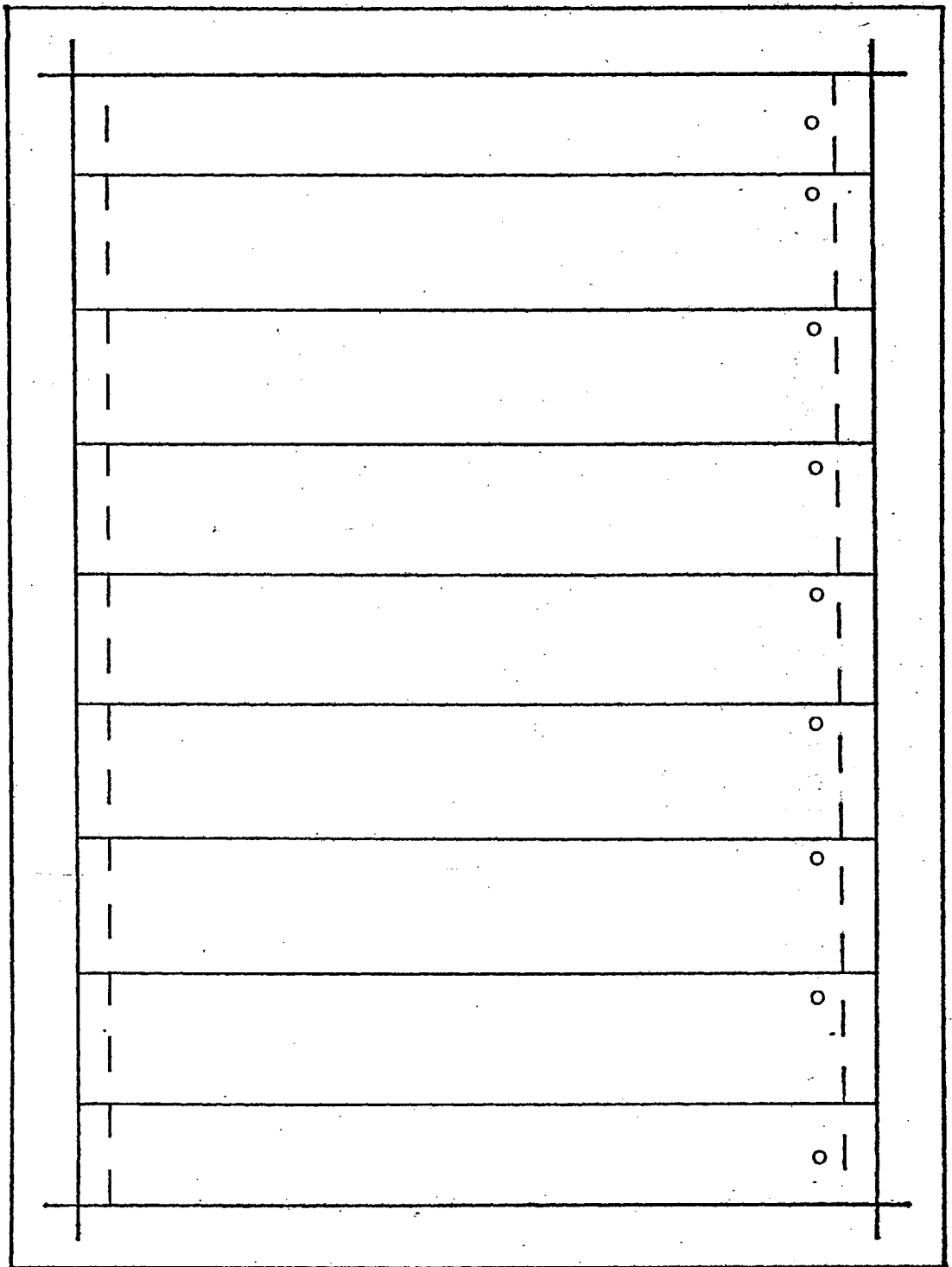
The 4% agarose mixture is poured through course muslin into 200 ml. medical flat bottles for storage at room temperature, until used. Before use, the bottles of agarose are placed in a boiling water bath until the agarose has melted when it may be pipetted in the required volumes into test-tubes.

3.4.

STANDARD METHODS3.4.1. LAURELL CROSSED IMMUNOELECTROPHORESISTECHNIQUE

A 20 ml. aliquot of 0.06M, pH 8.6 Barbitone buffer is pipetted into a sterile glass bottle while 20 ml. of molten 4% agarose is pipetted into a similar bottle. The two solutions are allowed to equilibrate to 48°C in a water bath and the contents are then thoroughly intermixed. The mixture is then carefully poured on to a cleaned 17 cm. x 12 cm. glass plate placed on a levelled surface. (The volumes used are calculated to give a 0.2 cm. thick gel on the glass plate. Equal volumes of buffer and gel are always used and may be adjusted for plate size.) The gel is allowed to set and is then "matured" for 1-2 hours in a damp box. Once matured, the plate is transferred to a pattern with gel sections and hole positions marked (Fig. 2). Nine holes 2 mm. in diameter, 2 cm. apart and 1.5 cm. from, and parallel to one long edge of the plate, are punched in the gel. The punch used is a 2 mm. outside diameter stainless steel tube sharpened to the outside rim and with a rubber teat attached for sucking out the gel plug.

A marker sample is made up of acetylated albumin in 1% Evan's Blue Dye and 2 µl of this is pipetted into each of the extreme holes in the gel. Acetylated albumin is used as it migrates faster than serum albumin, and by monitoring its progress one may ensure that fast travelling proteins, e.g. albumin and pre-albumin, remain on the plate. The remaining five holes in the gel are filled with 2-5 µl aliquots of test sera using a 5 µl Hamilton Syringe. Between each pipetting the syringe is thoroughly washed with distilled water. Care is taken to note the hole number, sample and volume.

FIGURE 2LAURELL PLATE FIRST DIMENSION GEL PATTERN.

The loaded plate is placed on a cooled level plate across two unconnected 6 l buffer tanks each containing 4 l of 0.03 M, pH 8.6 barbitone buffer. The cooled plate is maintained between 3°C and 4°C by an antifreeze solution circulated by a Churchill chiller. Two lint wicks are cut and folded double so that the folded edge is exactly the same width as the long edge of the plate. The wicks are soaked in the buffer and laid gently along each edge of the plate so that one wick hangs into each of the buffer tanks and overlaps the gel by 0.5 - 1 cm. The two buffer tanks are then connected via the two wicks and the agarose gel.

Each buffer tank contains two connected compartments one of which receives the wick while the other contains a platinum wire submerged in the buffer and running the whole length of the tank. One end of the wire is attached to a pole of a constant voltage D.C. supply and is so arranged that the cathode is attached to the tank proximal to the sample holes. The whole tank system is contained in a large sealed perspex box to maintain a constant high humidity around the agarose plate.

A voltage of 10v/cm. is passed across the plate, moving the proteins from the cathode to the anode until the acetylated albumin marker just reaches into the anode wick (1 - 1½ hours) when the current is turned off and the plate removed and repositioned on the pattern. The gel is then cut so that both marker gel sections and a 0.5 cm. strip from top and bottom of the plate may be discarded. The remaining gel is cut into sections 11 cm. x 2 cm. with the sample hole nearest to one edge. A long (15 cm.) razor blade is wetted in distilled water and pushed gently under the first gel section which is then lifted on to the blade and transferred to a second glass plate 11 cm. x 11 cm. so that the gel lies along one edge with the hole at the right-

hand side nearer to the centre of the plate (Fig. 3). The plate is numbered to indicate sample number for later identification.

The antibody, or second dimension, gel is then made up using a known standard volume of antiserum, found by trial and error (in this study 2.5 ml. of Burroughs Wellcome (B.W.) antiwhole human antiserum are used) and determined by the peak heights on the final plate. The volume of antiserum is made up to 9 ml. using 0.06M, pH 8.6 barbitone and placed in a 48°C bath with a second test tube containing 9 ml. of molten 4% agarose. After equilibration the contents of the two tubes are thoroughly mixed and carefully poured on to the plate containing the gel from the first dimension (Fig. 4). This is repeated for each of the first dimension gels. The gels are allowed to set and are then placed on a second electrophoresis system employing larger buffer tanks and a buffer circulating system with reservoir. Lint wicks are cut, soaked in 0.03M, pH 8.6 barbitone buffer and laid over perspex bridges which cross the gap between anodal and cathodal buffer tanks. The plate is inverted on to the wicks, and between two side pieces on the bridge, so that the plate traps a small saturated air volume on the gel side of the plate. All the gels are treated in the same manner and when all are in position a lid is placed on the tank and a current passed across the plate, using 2-3 v/cm., so that the antigen molecules at the cathode migrate into the antibody containing gel, i.e. the antigens now run at right angles to their first separation. The run continues for 22 hours.

On completion of the run, the plates are removed and placed, gel surface uppermost, in perspex holders in a 0.9% sodium chloride solution for 12 hours to remove excess

FIGURE 3

LAURELL FIRST DIMENSION GEL ON SECOND
DIMENSION PLATE.

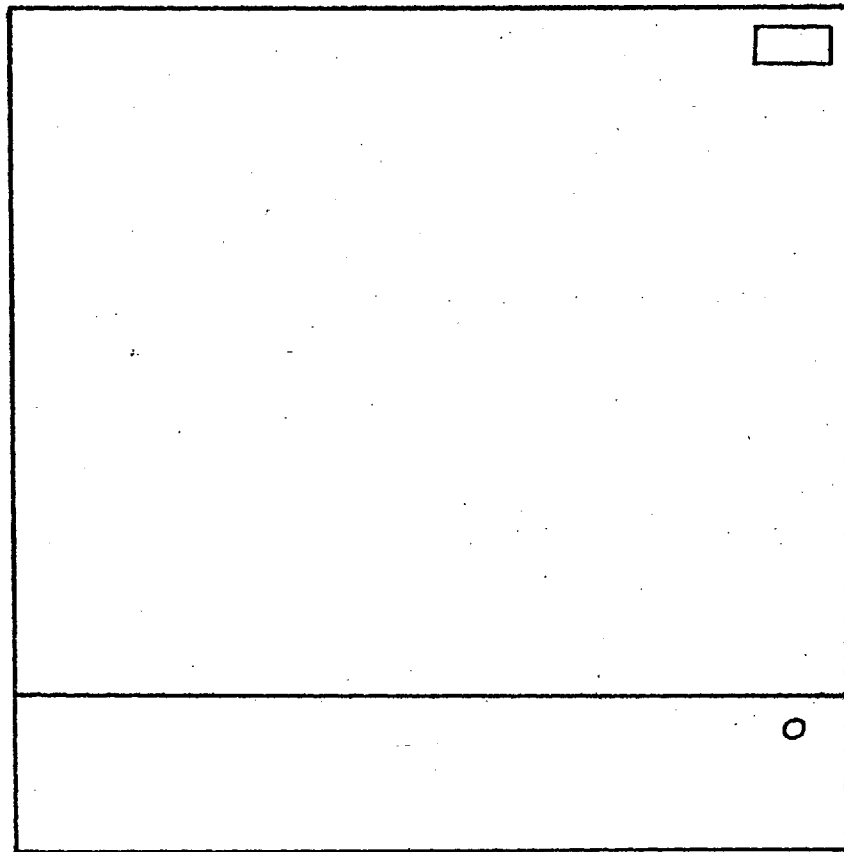
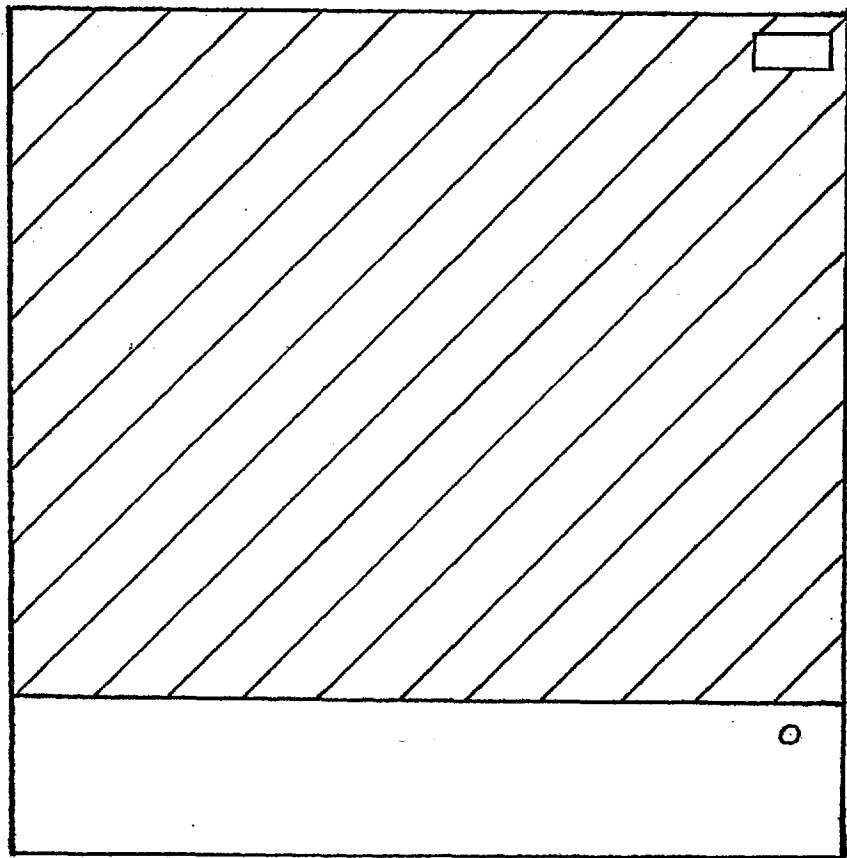


FIGURE 4

LAURELL SECOND DIMENSION PLATE READY
FOR IMMUNOELECTROPHORESIS.



antibody. The plates are then transferred to a water bath for 1 hour to wash out the saline, which, if allowed to remain would tend to lift the gel off the glass during the drying procedure. After washing, a 15 cm. ring of Whatman filter paper is laid carefully on the gel which is then dried in a warm air stream. Once dry the filter paper may be easily removed.

Staining

A non-specific protein stain, Amido Black 10B, is made up as a 0.1% solution in Methanol : Water : Glacial Acetic acid (in ratio of 5:5:1) and used to stain the plates.

The dried plates are carefully washed in water to remove any loose fibres of filter paper and then placed in the 0.1% stain. After about 30 minutes the plates are removed and placed in "wash" (methanol : water : glacial acetic acid, 5:5:1) for 30 minutes and then into methanol for 5 minutes. The plates are then removed and allowed to air dry (Fig. 5).

Autoradiography

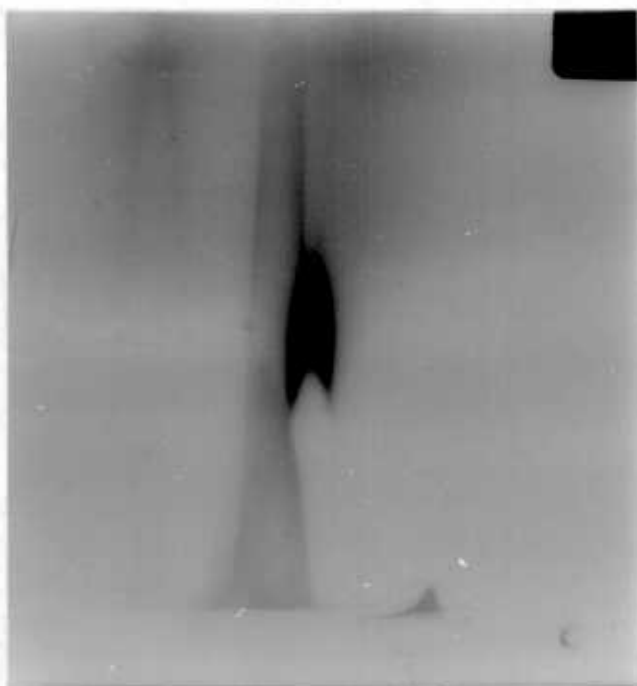
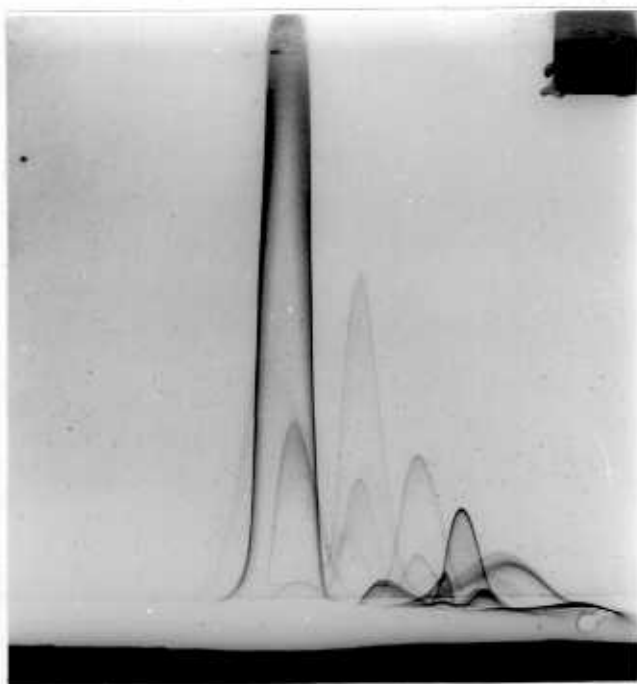
When a gamma emitting isotope has been used, $^{125}\text{I T}_4$ or $^{131}\text{I T}_4$, the process of autoradiography may be employed to determine the distribution of radioactivity.

The washed, dried, but unstained electrophoretogram, is mounted in a dark room on to an X-ray sensitive film (Kodirex). A thick piece of cellophane, with the same dimensions as the glass plate, is placed on the film, to prevent chemical images forming, and the glass plate, gel side downwards, is placed on the cellophane. Two pieces of 1 inch Sellotape are used to stick the glass plate firmly to the film to prevent movement. The film is marked with the plate number and returned, with attached plate to its envelope and is kept in the dark to

allow exposure. The exposure time depends on the concentration of radioactivity added, and its half-life, though normally plates are left to expose for between 2 days and 2 months. On completion of exposure the films are developed and fixed. On the final dry autoradiograph the activity shows up as black peaks. The autoradiograph and its subsequently stained plate may then be compared (Fig. 5).

FIGURE 5

PHOTOGRAPH OF A STAINED LAURELL PLATE AND
THE CORRESPONDING AUTORADIOGRAPH



3.4.2. LAURELL ROCKET TECHNIQUE

A known quantity of undiluted antiserum, to give final peaks of desired height, is made up in a test tube to 12 ml. with 0.06M, pH 8.6 Barbitone buffer. Into a second test-tube 12 ml. of molten agarose is measured and the two tubes are placed in a 48°C water bath to equilibrate. On equilibration, the contents of the two tubes are thoroughly inter-mixed and the mixture poured on to an 11 cm. x 11 cm. cleaned glass plate. (The volumes of antiserum buffer and molten gel are calculated to give a final thickness of gel on the plate of 0.2 cm.). The gel is allowed to set and is marked for identification.

The gel is matured for 1-2 hours in a damp box and then placed on a pattern (Fig. 6) and eight holes punched in the gel 0.5 cm. from one edge and parallel to it, and about 1.2 cm. apart. The centre six holes are filled with 2-5 μ l of serum sample, the two extreme holes usually being reserved for standard serum. Once loaded, the plates are run on the same system and by the same method as the Laurell second dimension, with 2-3 v/cm. measured between the wicks, for 22 hours.

On completion of the run the plates are washed, dried and stained as previously described. The superimposed protein rocket peaks may then be studied on the stained plate (Fig. 7).

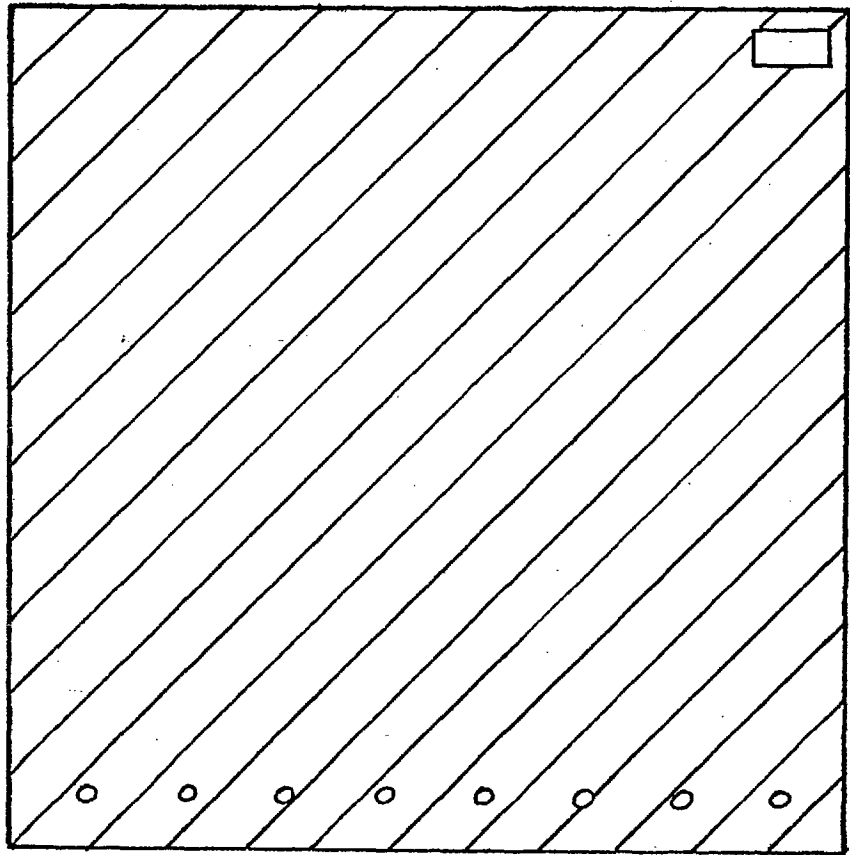
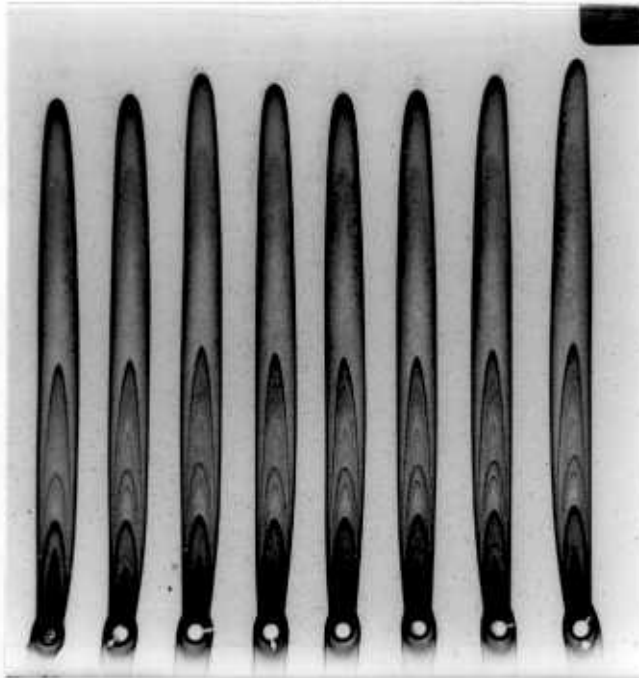
FIGURE 6STANDARD ROCKET PLATE.

FIGURE 7

PHOTOGRAPH OF A STAINED STANDARD

ROCKET PLATE



3.4.3.

LINE TECHNIQUE

The line technique has been published for use in protein determinations (Kroll, 1968) and has been modified for this study.

A 0.1 ml. aliquot of test serum is pipetted into a test tube and mixed with 1.8 ml. of 0.06M, pH 8.6 barbitone buffer. A second tube receives 1.9 ml. of molten agarose and the two tubes are allowed to equilibrate to 48°C. On equilibration the contents of the two tubes are thoroughly inter-mixed and the mixture poured on to a standard microscope slide giving a final gel thickness of 0.2 cm. When set, the gel is cut into strips 7 cm. x 0.2 cm. (and 0.2 cm. thick) using a double bladed scalpel designed for the operation. The outside edges of the gel are discarded and the first sample gel strip is lifted using a long razor blade and placed on an 11 cm. x 11 cm. glass plate so that the long axis of the gel is parallel to one edge and about 1.5 cm. from it. The plate is numbered for identification. The antiserum gel, composed of a known volume of antiserum made up to 12 ml. with 0.06M, pH 8.6 barbitone buffer, and 12 ml. of molten agarose, equilibrated to 48°C and thoroughly mixed, is poured carefully round the sample gel (Fig. 8). Once set, the plate is run on the electrophoresis system in exactly the same way as the second dimension of the Laurell using 2-3 V/cm. for 22 hours.

When the run is complete the plate is removed, washed, dried and stained with Amido Black 10B.

The stained plate shows precipitin lines parallel to each other and to the sample gel, each line representing a unique protein (Fig. 9).

FIGURE 8

STANDARD LINE PLATE.

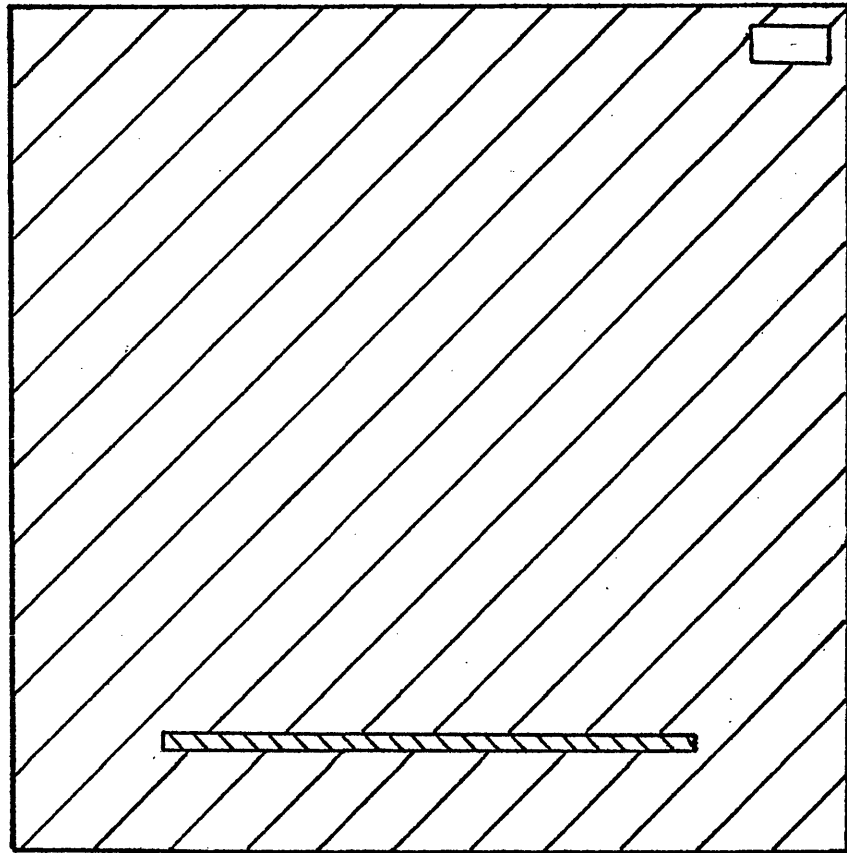
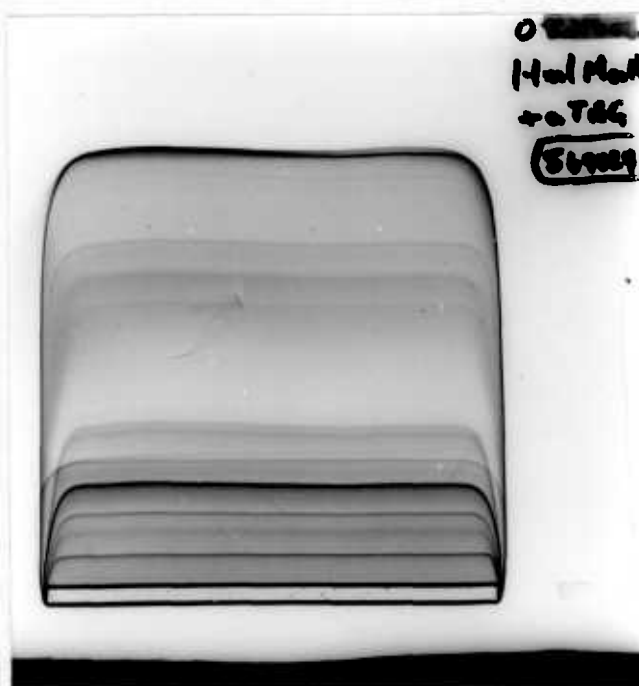


FIGURE 9

PHOTOGRAPH OF A STAINED STANDARD
LINE PLATE



4.

RESULTS & DISCUSSION

4.1.

INTRODUCTION

This section is divided into two broad sub-sections which deal respectively with:-

1. the Methodological innovations developed during the period of study, and
2. the Quantitative results from investigations of normal and sick individuals' sera using the technical developments.

During the period of study two closely related, but quite distinct methods were developed which have individual advantages and applications. The second subsection relating the Quantitative results is therefore further divided to show results using the two methods.

- a) the Modified Rocket Technique and
- b) the Modified Line Technique.

The results from these sub-sections have been discussed immediately following the individual Results so that the Result and Discussion may be easily related and stand as separate units.

A general conclusion follows each of the main sections and a final Conclusion completes the Thesis.

4.2.

METHODOLOGICAL RESULTS

The Results in this Section are related to the technical developments involved in designing modified methods. The actual results are not necessarily in chronological order, but are slotted into the most logical position regarding the structure of the Sections. Thus the relationship between the buffer composition and hormone binding was investigated initially using the Laurell cross immunoelectrophoresis system since, at the outset of the work, only this method was available, but once the superior Modified Line Technique had been developed it was used to quantitatively investigate hormone binding in the different buffers. The two results therefore appear together even though the Modified Line Technique development is not given until the following section.

The Methodological Results section follows the development of the methods and concludes with a general synopsis of the modified methods used for data collection during the remaining part of the Thesis.

4.2.1. INTRODUCTION TO MODIFICATION

The primary reason for modifying the standard methods was that the unidirectional techniques of Laurell Rocket and Line, during electrophoresis, resulted in dissociation of radiolabelled thyroxine from the thyroxine binding proteins. To establish a working method using these unidirection methods, investigations were made into this dissociation phenomenon.

Secondly, it was considered that an approximation to the in vivo environment might give a more accurate reflection of thyroid hormone distribution. Therefore, a pH 7.4 physiological buffer was used in place of the usual barbitone pH 8.6 system.

The buffer composition was modified before the dissociation phenomenon was investigated because, a) the changes could be implemented immediately, b) the changes were potentially more straightforward, and, c) they could possibly affect binding.

4.2.2. MODIFICATION OF BUFFER MEDIA

Before changing the buffer system from the established 0.03M barbitone buffer at pH 8.6 to a new phosphate buffer, the buffering characteristics of the phosphate were investigated.

A sodium dihydrogen orthophosphate solution was examined by titrating 200 ml. of the 0.025M phosphate against 2.5M sodium hydroxide and plotting the results graphically as pH against volume of sodium hydroxide. A definite plateau in the pH 7.4 region indicates a pronounced buffering capacity and to effect a change in pH from 7.3 to 7.5 the phosphate required a volume of 0.2 ml. of 2.5M sodium hydroxide.

The ionic strength of the phosphate buffer was determined by experiment, and systems of ionic strength between 0.5M and 0.00625M (0.5, 0.25, 0.1, 0.05, 0.025, 0.0125 and 0.00625M) phosphate were investigated. By trial and error the best total result was achieved using a 0.025M buffer which gave fastest migration and best resolution of the serum proteins with minimum heating and distortion of the agarose gel.

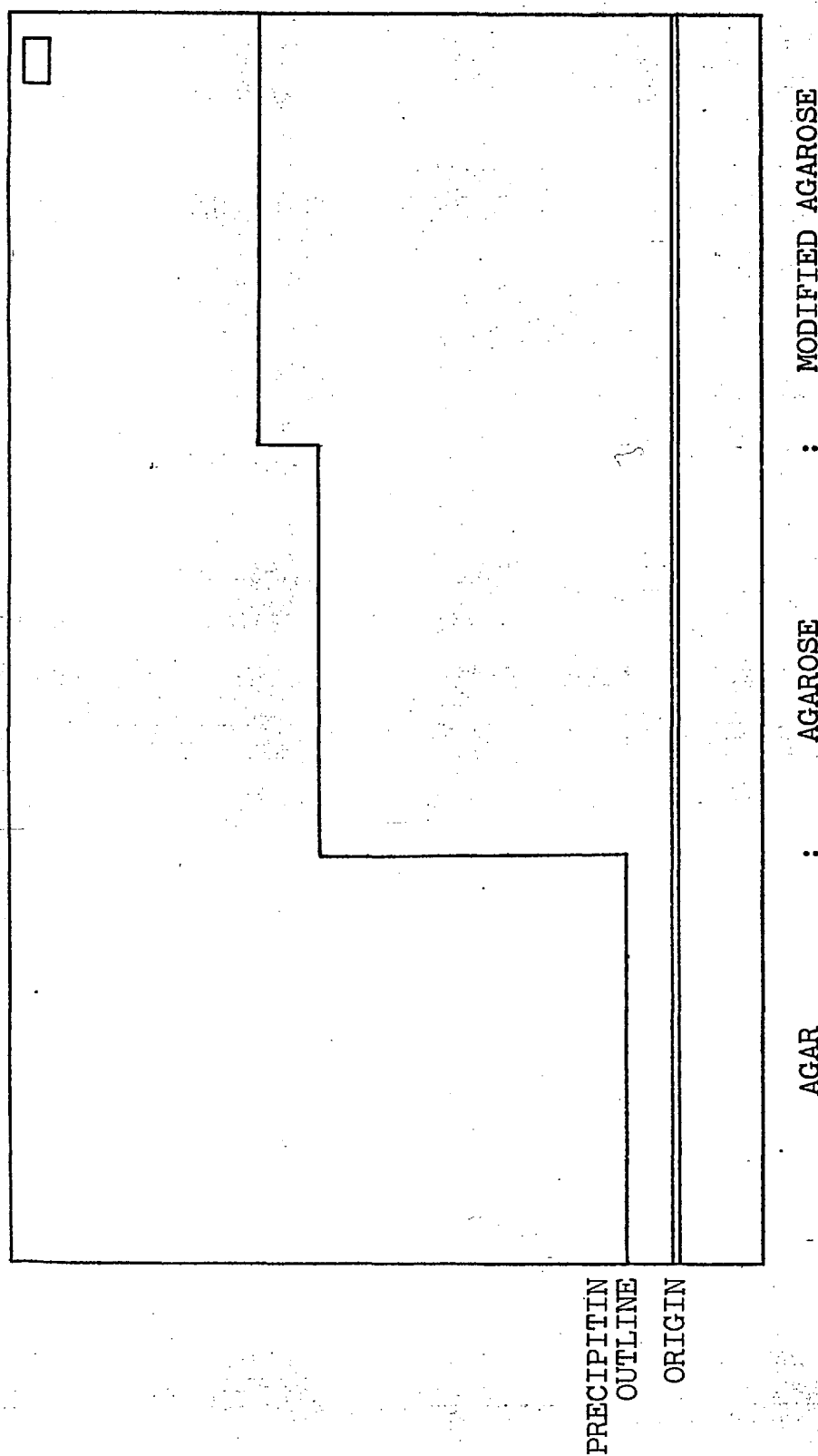
The reduction of electroendosmosis that occurs in agarose at pH 8.6 may also be achieved at the more acidic physiological pH of 7.4 by removal of part of the ionisable sulphate and carboxyl groups by simple purification. An ion exchange resin, Triethyl Aminoethyl Cellulose (TEAE), was mixed with molten agarose during preparation of the gel. Before mixing TEAE with the agarose, the exchange resin was swelled in pH 7.4 phosphate, and to determine the optimum concentration of the buffer for this purpose, buffer concentrations between 0.1M and zero concentration i.e. distilled water (0.1, 0.05, 0.025, 0.0125, 0.00625M) were used. When the 0.1M buffer was used the resulting gel was not demonstrably different from

the untreated agarose, but conversely when distilled water was used the resulting gel lost all "body" and completely failed to set on cooling. The best results were obtained when the TEAE was swelled in 0.025M phosphate buffer. The probable reason for the different affects of buffer concentration on TEAE efficacy is that the concentration of phosphate masks the full potency of ion exchange so that not all the charged groups are removed and sufficient remain to allow maintenance of the gel substance.

The result of the electroendosmotic affect on electrophoresis was demonstrated by running one long Line sample through three adjacent gels of agar, agarose, and purified agarose, each with the same antiserum concentration. The antigen/antibody precipitin lines were found to run progressively further from the origin in direct relation to agarose purity (Fig. 10).

FIGURE 10

DIAGRAM OF A LONG LINE PLATE USING AGAR,
AGAROSE AND MODIFIED AGAROSE ANTIBODY GELS



4.2.2.1. QUALITATIVE VALIDATION OF THE
MORE PHYSIOLOGICAL SYSTEM

The effects of pH and ionic composition on hormone binding by the serum proteins was investigated by running the same samples in both phosphate pH 7.4 and barbitone pH 8.6 buffers and tracing the binding pattern by autoradiography. The Laurell crossed immunoelectrophoresis technique was employed because of the wide experience of its use in the laboratory.

Radiolabelled thyroxine was equilibrated with aliquots of three untreated individual sera (D.P., D.B.R., R.W.) to give nine additions of hormone between 25 and 2,400 nmol/l (i.e. 0.25 to 24 times the normal physiological concentration, (normal taken as 100 nmol thyroxine/l of serum). These radio-labelled sera were divided into two equivalent sets, one being run in the phosphate pH 7.4 system and the other in barbitone at pH 8.6.

Two antisera were used for the samples with exogenous hormone levels below 300 nmol/l. One was a polyvalent commercial antiserum from Burroughs Wellcome (3 ml./plate) and the other an antiserum raised in goats (2 ml./plate) by Drs. Clarke & Freeman. The goat antiserum contained a higher titre of antibodies to the serum proteins, especially α_1 lipoprotein, but was used only at the lower end of the exogenous hormone scale due to its short supply.

Thus, using the same serum sample, four plates were run with exogenous hormone concentration below 300 nmol/l (a, b, c & d) while only two plates were run with exogenous hormone concentration above 300 nmol/l (a & b).

- a) B.W. antiserum in phosphate pH 7.4 buffer.
- b) B.W. antiserum in barbitone pH 8.6 buffer.

- c) Goat (G.P.19) antiserum in phosphate pH 7.4 buffer.
- d) Goat (G.P.19) antiserum in barbitone pH 8.6 buffer.

Excepting these buffer and antiserum differences, the experimental conditions were identical. The electrophoresed plates were autoradiographed for 2-8 weeks depending on the level of radiolabelled hormone added, and those plates with equal amounts of added hormone were autoradiographed for the same time. The extent of hormone binding was assessed subjectively by the relative degree of blackening of the X-ray film after autoradiographic exposure (Table 4).

In the pH 7.4 phosphate system using goat antiserum and exogenous hormone concentrations of 25, 50 and 75 nmol/l, five binding proteins were found on the exposed autoradiograph. TBG was found to be the major carrier and bound more radioactivity than the other binding proteins (Table 4). Because the commercial (B.W.) antiserum contained a low titre of anti α_1 lipoprotein, the α_1 lipoprotein peak was diffusely precipitated and was therefore not visible on the autoradiograph. Otherwise, however, the results were identical to those using the goat antiserum, which showed up the α_1 lipoprotein peak. At pH 8.6 in barbitone using either the goat or B.W. antiserum, binding of exogenous hormone at sub-physiological additions was so reduced that autoradiography failed to result in exposure of the X-ray film.

In both buffers at exogenous hormone concentrations of 300, 600 and 1,100 nmol/l the qualitative distribution of thyroxine was similar, with TBG as the major binding protein. However, the quantity of hormone bound to the proteins was greater in the phosphate system, particularly to TBG as indicated by the relative blackening of the X-ray film.

At exogenous hormone concentrations of 1600, 2000 and

TABLE 4

DISTRIBUTION OF RADIOLABELLED THYROXINE ON HUMAN SERUM PROTEINS

ESTABLISHED USING LAURELL CROSSED IMMUNOELECTROPHORESIS

ELECTROPHORESIS AT pH 7.4 IN PHOSPHATE BUFFER USING BURROUGHS WELLCOME ANTISERUM									ELECTROPHORESIS AT pH 8.6 IN BARBITONE BUFFER USING BURROUGHS WELLCOME ANTISERUM						
0.25 ²	0.5	0.75	3.0	5.7	10.9	15.9	20.0	24.0	PROTEINS	3.0	5.7	10.9	15.9	20.0	24.0
*** ¹	***	***	****	****	****	****	****	****	T.B.G.	***	***	****	****	**	*
*	**	**	**	***	****	****	****	****	ALBUMIN	**	**	****	****	****	****
*	*	**	**	***	***	****	****	****	T.B.P.A.	**	**	**	***	***	***
3	*	*	*	**	**	***	***	***	α_1 LIPO.	*	*	**	**	**	***
*	*	**	**	***	***	***	***	***	β LIPO.	*	**	***	***	***	***

1. Asterisks refer to the relative blackening, and therefore binding of radiolabelled Thyroxine ($^{125}\text{I.T}_4$), of proteins on the autoradiographs.
2. Figures denote the concentration of Thyroid Hormone present as multiples of physiological concentration.
3. α_1 Lipoprotein does show binding when the stronger G.P.19 Antiserum is used.

2400 nmol/l, the autoradiograph peaks at pH 7.4 of TBPA, Albumin, α_1 and β lipoprotein were virtually as equally developed as the TBG peak indicating high levels of hormone binding by each protein. However, in the pH 8.6 system, a striking difference was apparent as the TBG peak apparently disappeared while the other binding proteins displayed developed peaks. This apparent disappearance of TBG at pH 8.6 was presumably due to the compound effect of the postulated reduction in binding in the unphysiological buffer, and the necessity to reduce exposure time to prevent over-exposure of the X-ray film in the regions of the other binding proteins.

4.2.2.2. QUANTITATIVE VALIDATION OF THE MORE PHYSIOLOGICAL SYSTEM

The Modified Line Technique was used to quantitatively investigate the effect of buffer and pH on the percentage binding of radiolabelled hormone to TBG.

Serum from an hypothyroid patient which contained 24 nmol/l of thyroxine was used so that additions of exogenous thyroxine ($T_4^{125}I$) could be equilibrated with the serum without exceeding the normal hormone level found in euthyroid individuals (circa 100 nmol/l). Both sub-physiological to supra-physiological levels of hormone distribution were investigated.

The hypothyroid serum (24 nmol/l) was divided into three aliquots and into each was pipetted radiolabelled thyroxine ($T_4^{125}I$) so that the total hormone concentrations became 75, 125 and 525 nmol/l. Three sample gels were made up using 0.9% saline and unpurified agarose. These gels were cut up and used in both buffer systems so that as far as possible the corresponding gels in the two buffers were identical. The same antiserum type, batch and concentration were used in both systems. After electrophoresis in the respective buffers for the same time and under similar electrophoretic conditions of current and voltage, the plates were cut up and counted.

The radioactivity counts were converted to percentages of the total counts and plotted against gel section numbers for each of the three hormone concentrations and the pH 7.4 and pH 8.6 results of the same sample were superimposed for comparison (Figs. 11, 12 & 13).

At the total hormone concentration of 75 nmol/l, the total radioactivity recovered was 100% from both buffer systems, but the distribution of the exogenous hormone was different in the two buffers. In the physiological buffer,

TBG bound more than 80% of the radiolabelled hormone (81% and 81.5%), whilst at pH 8.6 in barbitone buffer, TBG carried only 50% of the tracer hormone (Table 5). Also in the phosphate pH 7.4 buffer the activity was located between the TBG and the albumin precipitin lines, but in the barbitone this second peak was anodal to albumin (Fig. 11).

At the total hormone concentration of 125 nmol/l the percentage recovery was 100% in both buffers, but the radiolabelled hormone was again differently distributed. TBG in the physiological buffer bound 63% (63% and 63%) of the label whilst at pH 8.6 in barbitone, only 8% of the exogenous hormone was bound by this protein. The remainder of the hormone in the phosphate buffer was located between the TBG peak and albumin precipitin line in contrast to the barbitone buffer, where more than 70% of the total hormone was found in a region anodal to albumin and midway between the albumin precipitin line and the anodal edge of the plate where no binding protein has ever been characterised (Fig. 12).

At the total hormone concentration of 525 nmol/l only the plates run in the phosphate pH 7.4 system displayed a percentage recovery of 100%. In contrast, the same sample in barbitone displayed a recovery of only 86%. In the phosphate buffer, TBG bound 35% of the labelled hormone which was in marked contrast to the barbitone result where only 7% was associated with TBG. The remaining 65% of hormone on the plate run in phosphate buffer was distributed among at least five more proteins which could be either definitely distinguished as radioactive peaks or could be deduced from the change in slope of the activity graph (Fig. 13). The next largest peak after TBG lay immediately anodal to albumin in the TBPA position. The remaining proteins were found between TBG and albumin and a small β lipoprotein peak was located just cathodal

FIGURE 11

DISTRIBUTION OF THYROXINE AT A TOTAL CONCENTRATION
OF 75 nmol/l IN PHOSPHATE pH 7.4 (—), AND BARBITONE
pH 8.6 (-----) BUFFERS.

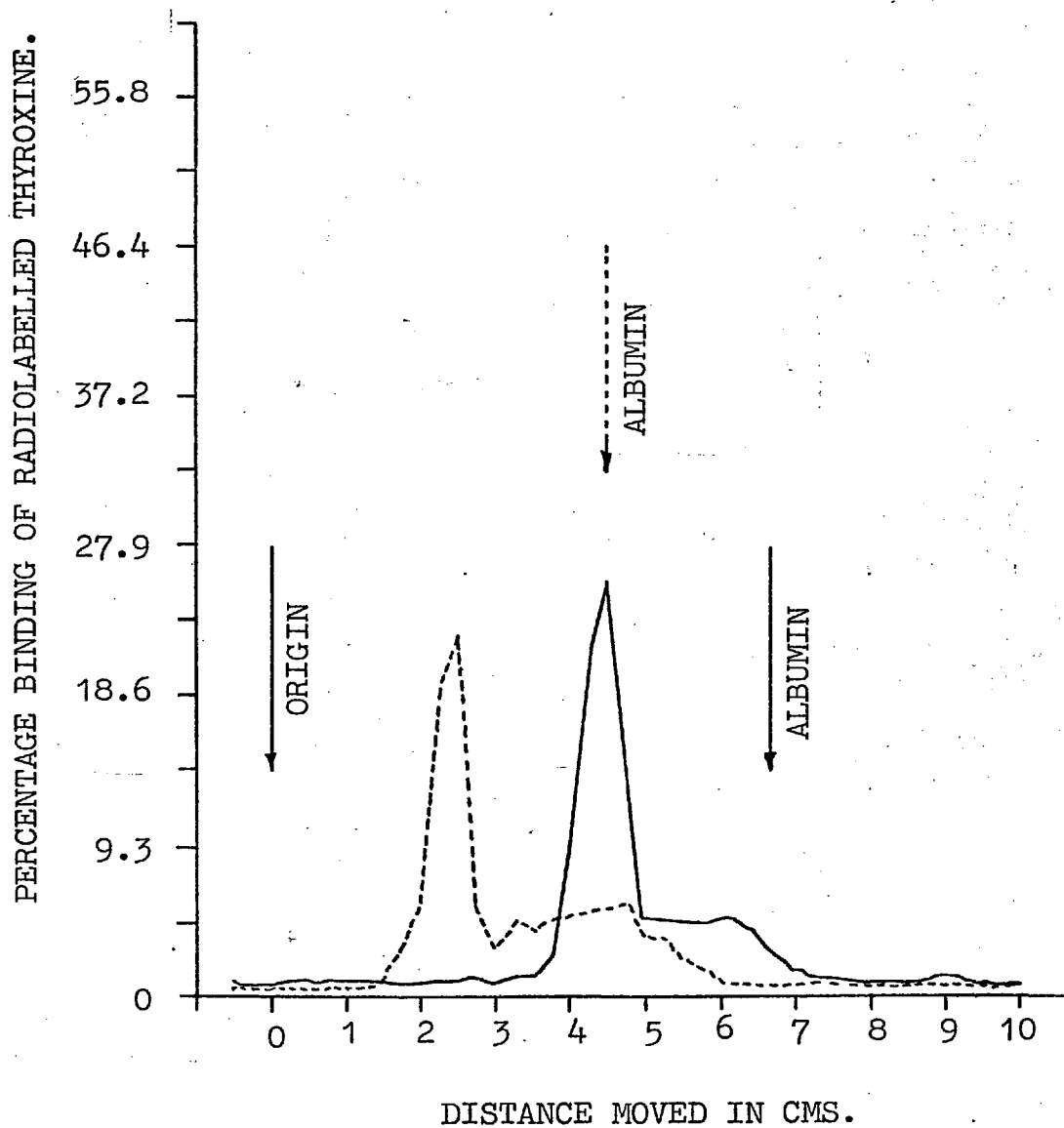


FIGURE 12

DISTRIBUTION OF THYROXINE AT A TOTAL CONCENTRATION
OF 125 nmol/l IN PHOSPHATE pH 7.4 (—), AND BARBITONE
pH 8.6 (----) BUFFERS.

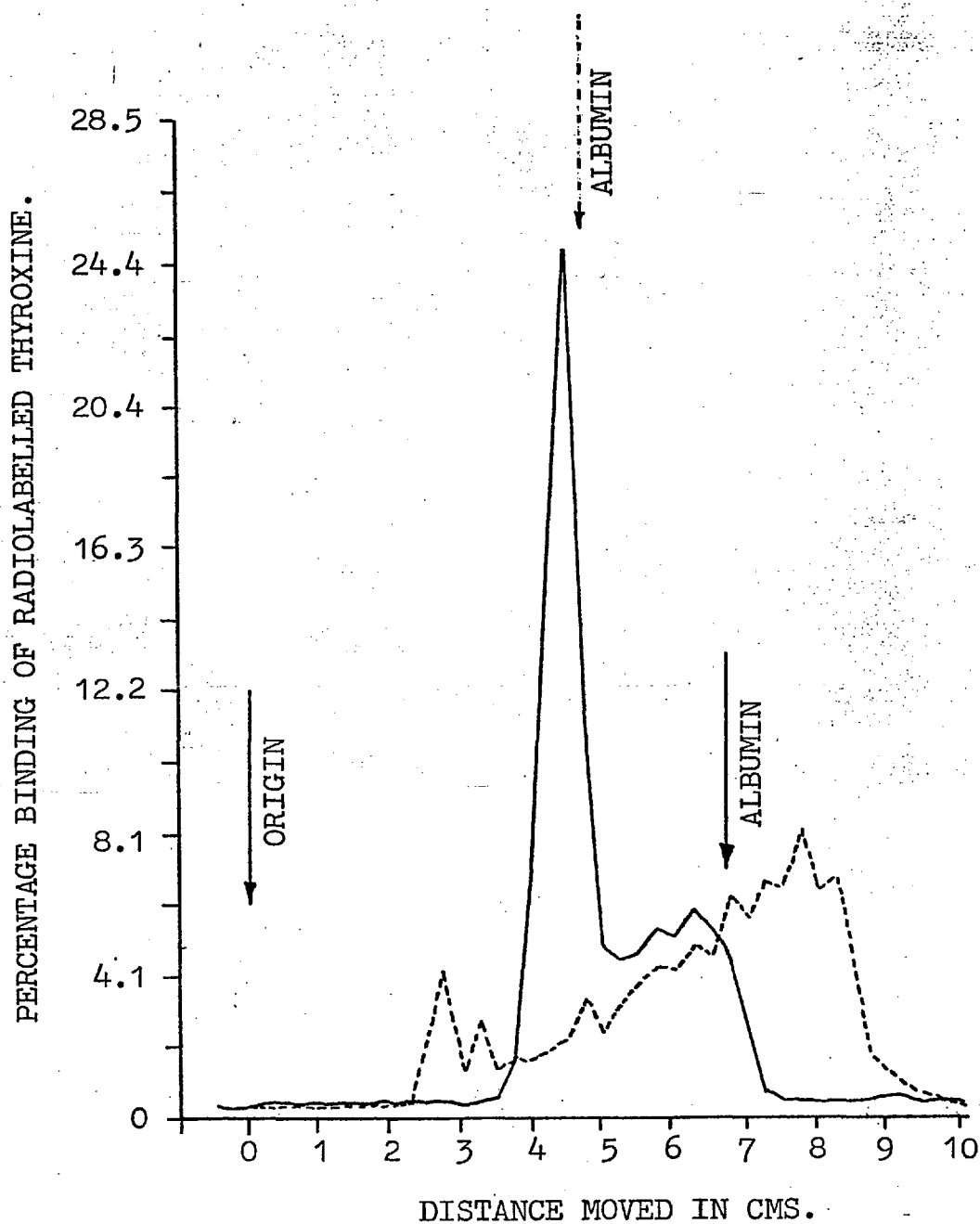


FIGURE 13

DISTRIBUTION OF THYROXINE AT A TOTAL
CONCENTRATION OF 525 nmol/l IN PHOSPHATE
pH 7.4. (—), AND BARBITONE pH 8.6. (---)
BUFFERS.

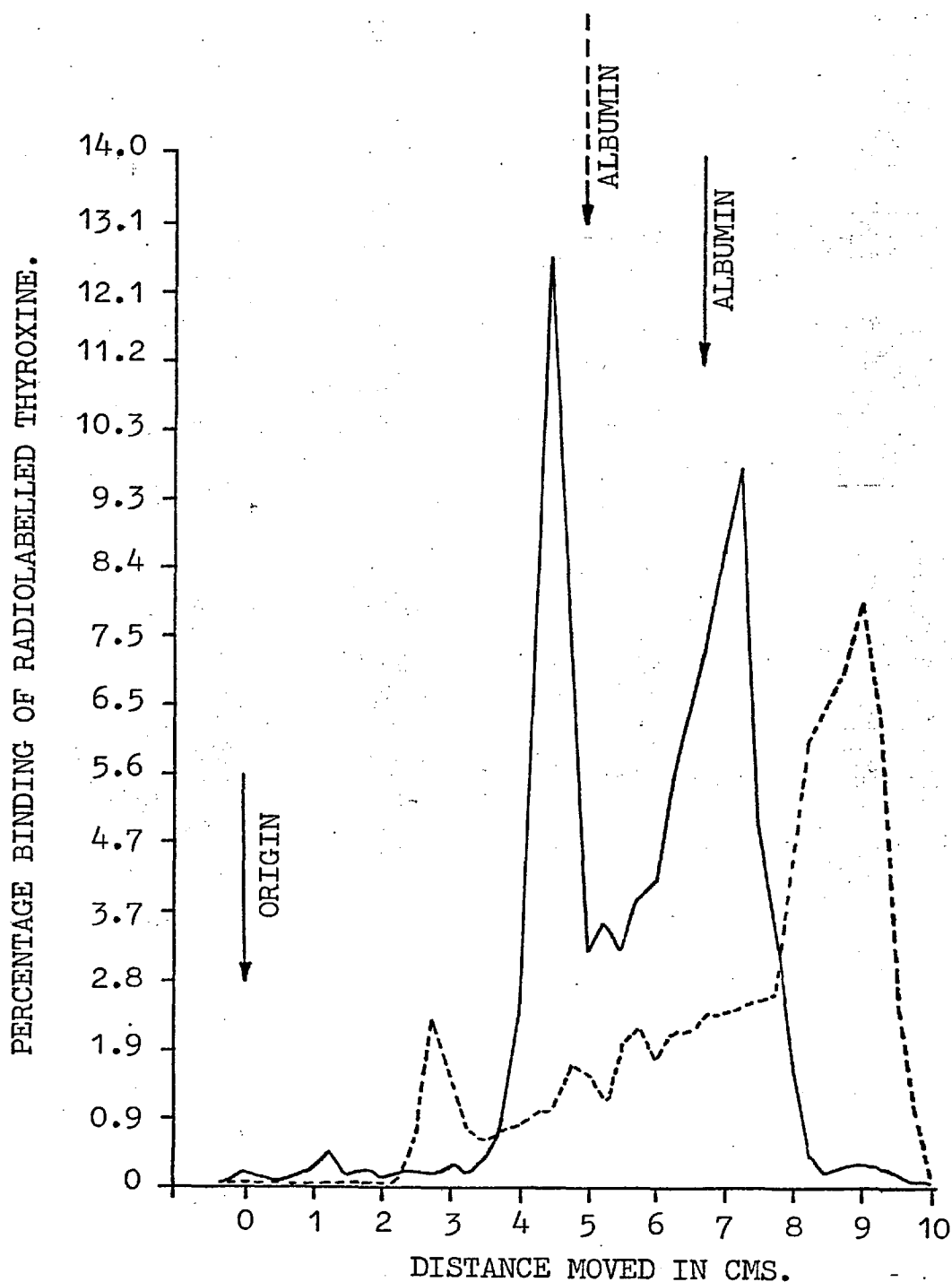


TABLE 5

COMPARISON OF RADIOLABELLED THYROXINE BINDING IN PHOSPHATE pH 7.4 AND BARBITONE
pH 8.6 BUFFERS USING THE QUANTITATIVE MODIFIED LINE TECHNIQUE.

PHOSPHATE pH 7.4 BUFFER			BARBITONE pH 8.6 BUFFER	
PERCENTAGE RECOVERY	PERCENTAGE BINDING TO TBG	TOTAL THYROXINE CONCENTRATION	PERCENTAGE RECOVERY	PERCENTAGE BINDING TO TBG
100%	81%	75 nmol/l	100%	50%
100%	63%	125 nmol/l	100%	8%
100%	35%	525 nmol/l	86%	7%

to the origin. In the barbitone buffer, more than 85% of the hormone on the plate was found considerably anodal to albumin and proximal to the anodal edge of the plate where no binding protein is known to precipitate (Fig. 13).

In addition to the hormone distribution, it was observed that the precipitin lines of the individual proteins were further apart in the phosphate buffer even though the concentrations of antigen and antiserum were identical on each plate. This indicated a better resolution of the precipitin lines in the phosphate system, presumably due to the reduction of electroendosmosis in the purified agarose.

Qualitative Confirmation of Buffer and pH Effects

Parallel plates to those above using the same samples were run using the Laurell crossed immunoelectrophoresis system. The results were similar to those found using the Modified Line Technique and confirmed a difference in hormone binding between the two buffers.

4.2.2.3. DISCUSSION OF THE DESIRABILITY OF USING A MORE PHYSIOLOGICAL SYSTEM

The system designed for immunoelectrophoresis in this study included the modifications set out in the introduction to render it more physiologically valid. Although the system is deductively more representative of the *in vivo* situation, it should be demonstrably different from other, non-physiological systems if it is to be considered as an advantage. Should no difference be found between systems of altered pH and ionic composition, then the validity of changing to the more physiological 'milieu' carries little weight. Therefore, the above experiments were designed to investigate any differences of hormone binding between the physiological and non-physiological buffers.

The results of the modified Line and Laurell Techniques using these buffers showed a significant increase in Thyroxine binding at pH 7.4 over the results at pH 8.6.

Using the Modified Line Technique, the percentage recovery and TBG percentage binding were calculated. In each case the percentage binding of radiolabelled thyroxine to TBG was significantly higher in the more physiological buffer. The smallest difference in TBG binding occurred using a total thyroxine concentration of 75 nmol/l when TBG bound 37% more hormone in phosphate than in barbitone. In the experiments using total thyroxine concentrations of 125 and 525 nmol/l, TBG bound more than 80% more hormone in the phosphate buffer than in the barbitone. Not only was the percentage binding to TBG different in the two buffers, but the total distribution of hormone was also markedly different. In the phosphate buffer the radiolabel not bound to TBG was distributed among the other binding proteins, TBPA, Albumin α_1 and β lipoprotein

whilst in the barbitone buffer the majority of the remaining hormone not bound to TBG was found anodal to albumin and in a position not associated with a binding protein (see Section 4.2.5.1.).

The reason for the binding difference between duplicate plates run in phosphate and barbitone buffers is conjectural, although the barbitone ion is known to inhibit thyroxine binding to TBPA, and buffers more alkaline than pH 7.4 have been shown to result in decreased binding by TBG (Davis & Gregerman 1970 & 1971).

Although Davis & Gregerman (1970 & 1971) used non-physiological buffers, they did study thyroxine binding to TBG at physiological and non-physiological (pH 9.0) pH and their results are consistent with the present study. They found that at pH 7.4 in PIPES buffer (Piperazine-N,N'-bis - (2-ethane sulphonic acid) the binding capacity of TBG was 30% higher than in Tris-Borate buffer at pH 9.0, and similarly that trace binding levels of hormone were 50% higher at the physiological pH.

Hagen & Elliott (1973) also studied binding, using two different buffers at pH 7.4. One contained the physiological buffer ion, phosphate (0.1M), while the other contained the non-physiological PIPES. Using sub-physiological additions of radiolabelled thyroxine (5.43 $\mu\text{g}/100\text{ ml. serum}$) these workers found that TBG bound $53\% \pm 1.6\%$ of the hormone in the phosphate, while in the PIPES TBG bound only 37%, indicating a 29% decrease of binding in the non-physiological buffer, even though both buffers were maintained at pH 7.4. The results of thyroxine binding in the 0.1M phosphate at pH 7.4 are consistent with the results of the present study with similar total hormone levels.

4.2.3. EVOLUTION OF THE "BUFFER" GEL

Radiolabelled thyroxine is almost completely dissociated from the serum binding proteins during electrophoresis, if the standard forms of the one dimensional immunoelectrophoretic techniques are used. This phenomenon of hormone dissociation was repeatedly demonstrated under controlled experimental conditions. Fifty standard Line and Laurell Rocket plates were run using new reagents for each set of five plates to minimise the possibility of an impurity interfering with binding. Dissociation of hormone occurred on every set of plates. Hormone remains bound to its carrier proteins under the conditions of the Laurell crossed immunoelectrophoresis method. Deductively, therefore, the hormone dissociation results from the differences between the crossed and unidirectional techniques. On considering the methods, there were two obvious differences; firstly, in the two dimensional method, an initial separation of the antigen proteins occurs in a plain gel before meeting their respective antibodies in the antibody impregnated gel; secondly, in the Line Technique, a gel sample was formed by impregnating the radiolabelled serum into agarose. This second factor of gel impregnation was eliminated as a causative factor because the similar unidirectional Laurell Rocket Technique did not include this gel, yet dissociation occurred. The initial slight protein separation that occurs during the initial phase of electrophoresis into a non-antibody containing gel was therefore considered as a possible preventive against dissociation.

4.2.3.1. QUALITATIVE EFFECT OF THE
"BUFFER" GEL

To investigate the possibility of the separation of antigens from antibodies being necessary for maintained binding, two Laurell Rocket plates were run, one with the samples being run through a plain gel before reaching the antibody impregnated gel, and the other with the samples being run immediately into the antibody gel (Figs. 6 & 14). Results from ten repeated experiments showed that binding only persisted when the plain gel was included.

To eliminate the chance of a factor occurring on individual plates, a single Laurell Rocket plate was run which started half the samples in a plain gel, and half in the antibody gel (Fig. 15). The results again showed that binding was only maintained when the samples ran through the plain gel (Fig. 16).

To find the optimum width of this plain gel, a wedge shaped gel was used, and a line of sample holes was punched 0.5 cm. from one edge of the plate and passed across the antibody and plain gels (Figs. 17 & 18). After electrophoresis and subsequent autoradiography, it was found that the samples surrounded by antibody gel, but with part of the wedge gel behind, i.e. cathodal to, the sample hole, retained some activity in proportion to the amount of plain gel cathodal to the sample hole. Those samples with increasing amounts of plain gel around the sample holes and ahead, i.e. anodal to the sample, continued to increase retention of hormone binding up to an optimum plain gel width of 1.1 cm. Because the expected result of hormone dissociation did not occur when the sample holes were surrounded by antibody gel, a second plate with a wedge gel was run, but the plain gel was cut short so that it did not reach across the whole width of the plate (Figs. 19 & 20).

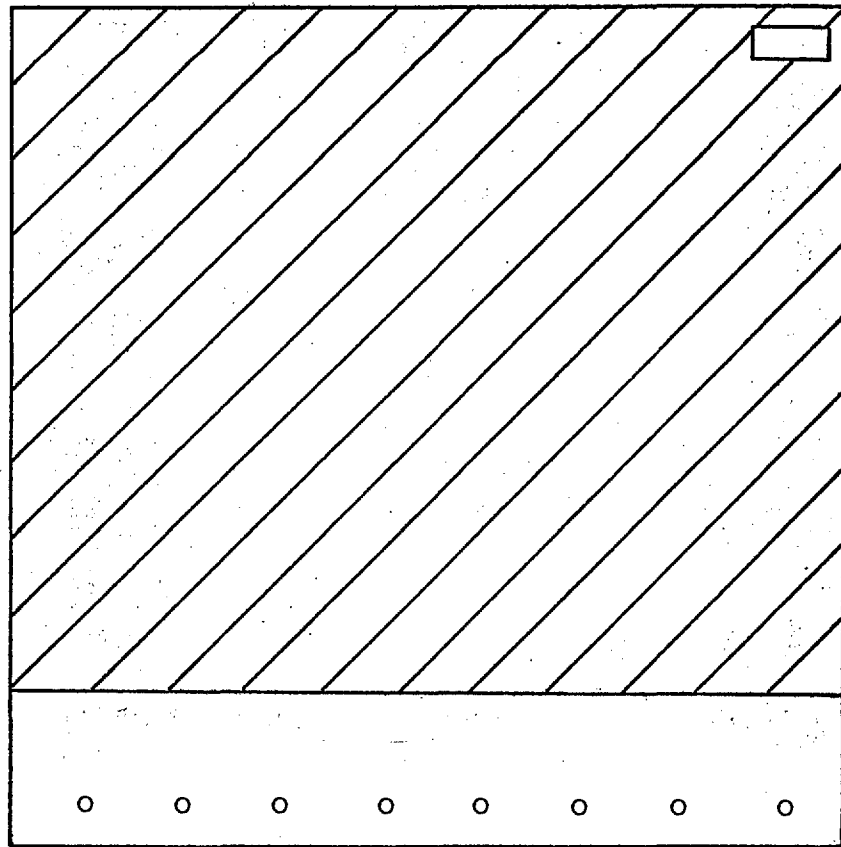
FIGURE 14ROCKET PLATE WITH "BUFFER" GEL ORIGIN

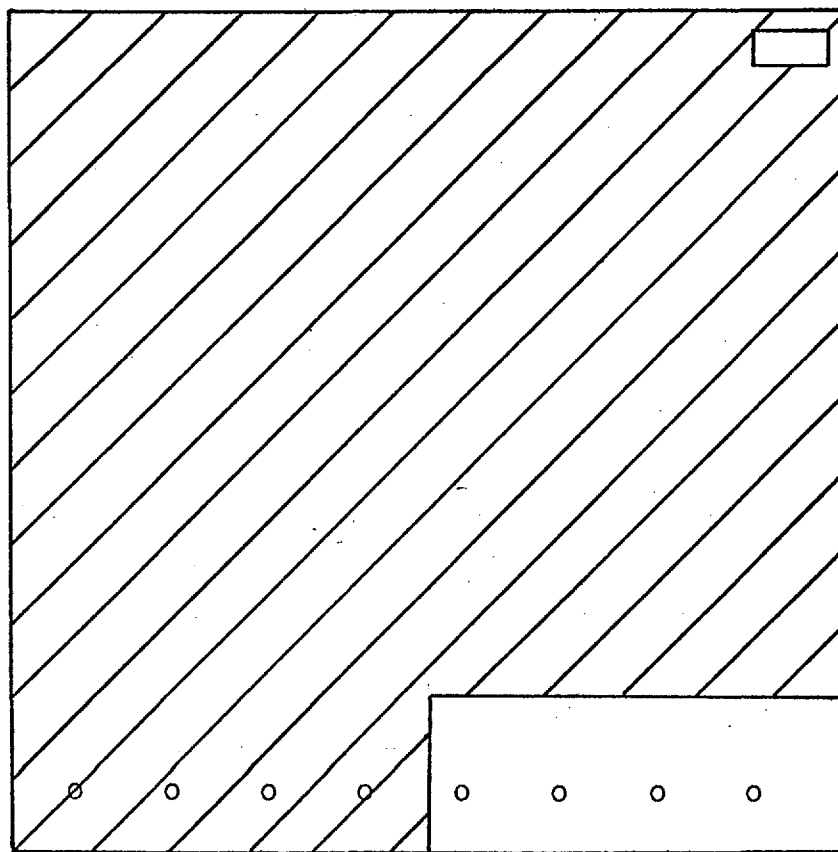
FIGURE 15ROCKET PLATE WITH STANDARD AND "BUFFER" GEL ORIGINS.

FIGURE 16

PHOTOGRAPH OF A STAINED PLATE AND THE CORRESPONDING
AUTORADIOGRAPH WITH STANDARD AND "BUFFER" GEL ORIGINS

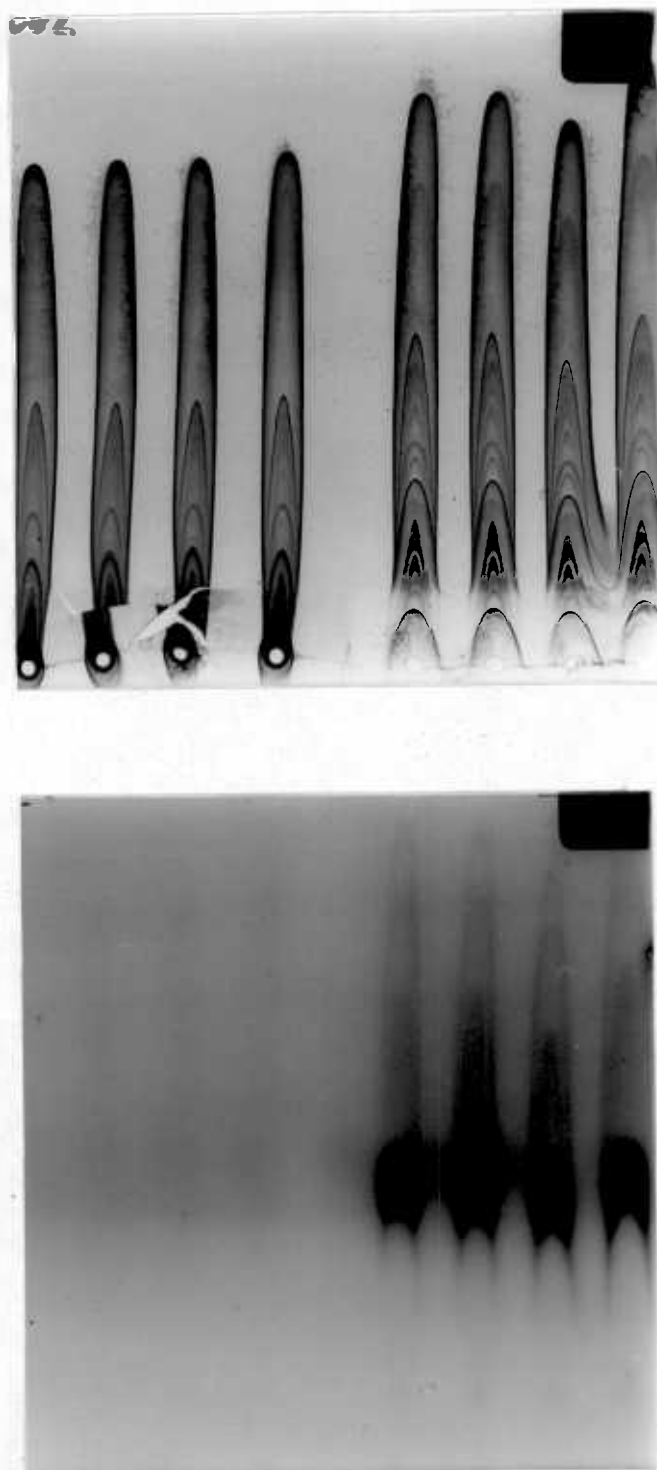


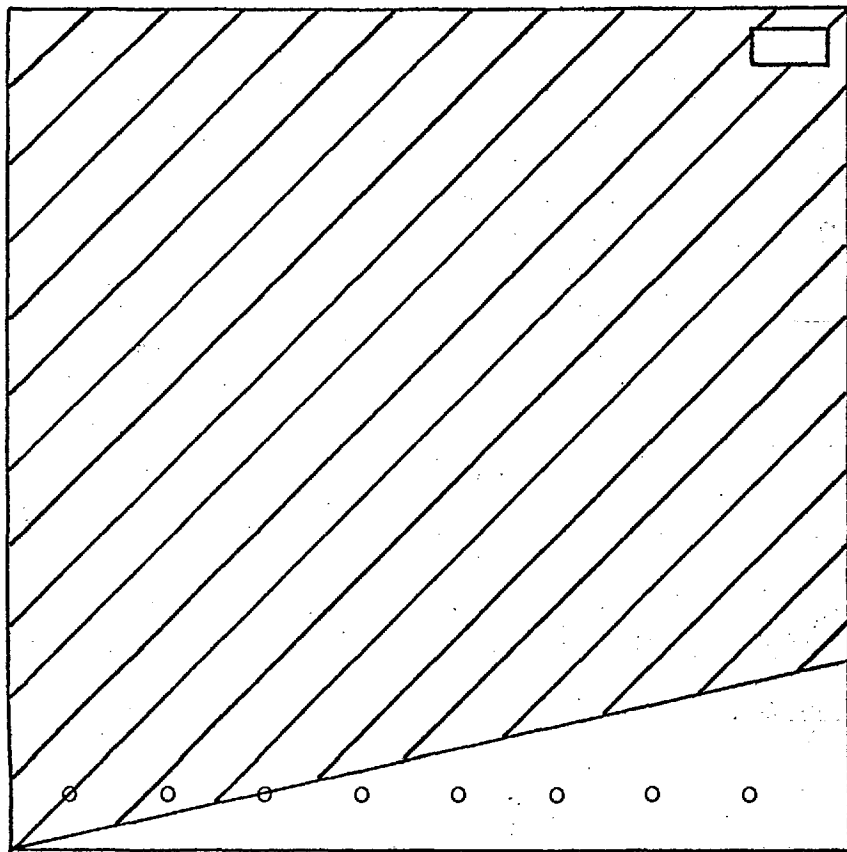
FIGURE 17ROCKET PLATE WITH FULL WIDTH WEDGE GEL.

FIGURE 18

PHOTOGRAPH OF A STAINED PLATE AND THE CORRESPONDING
AUTORADIOGRAPH WITH A FULL WIDTH WEDGE "BUFFER" GEL

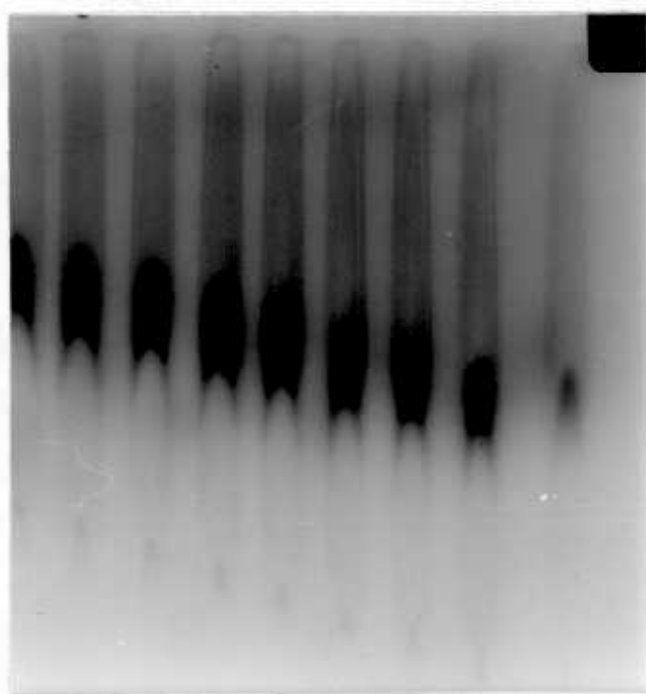
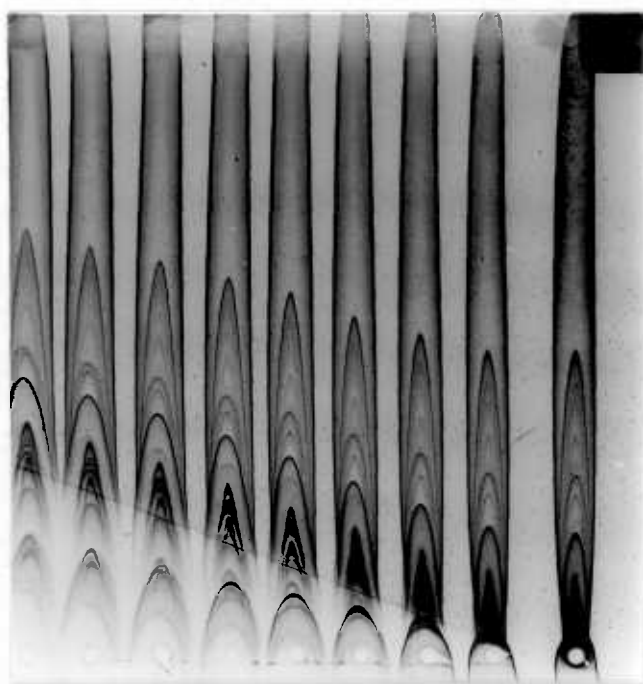


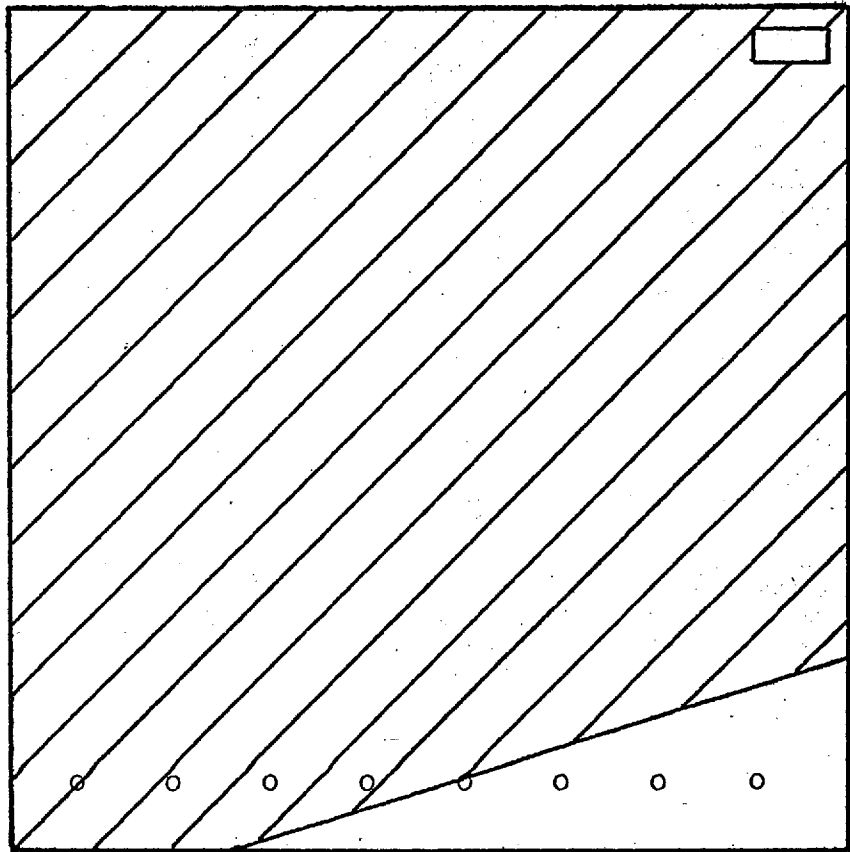
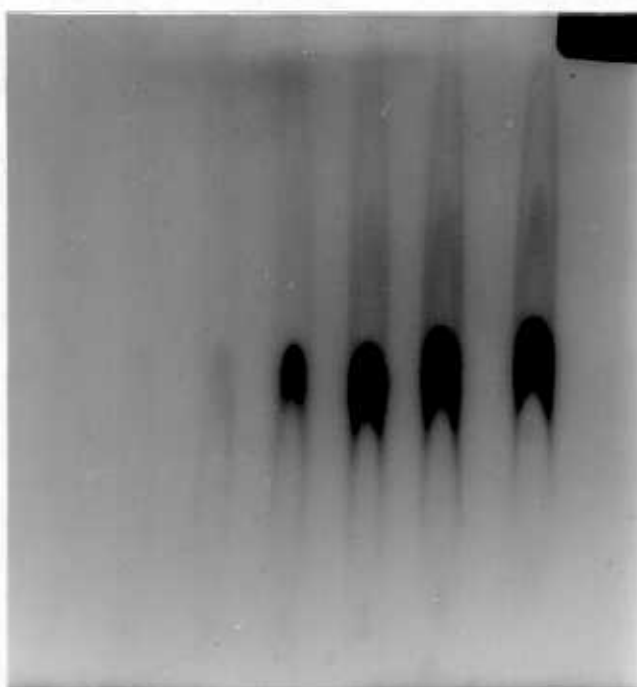
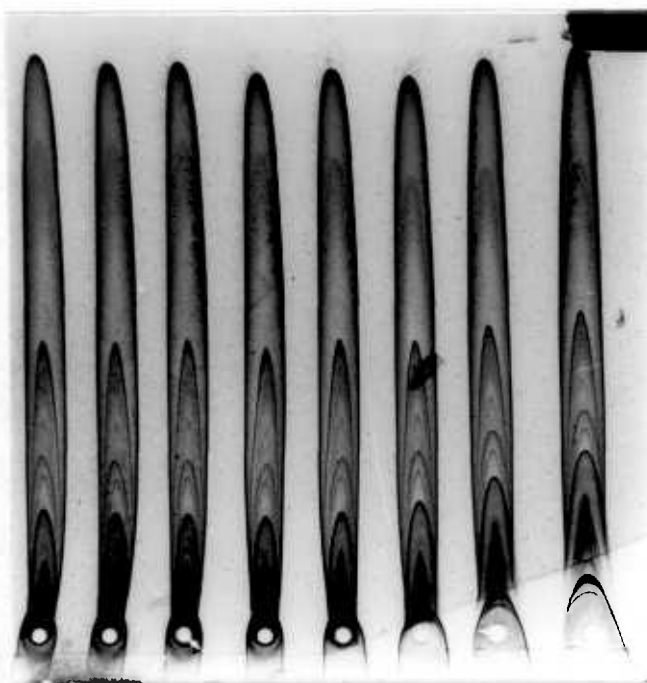
FIGURE 19ROCKET PLATE WITH HALF WIDTH WEDGE GEL.

FIGURE 20

PHOTOGRAPH OF A STAINED PLATE AND THE CORRESPONDING
AUTORADIOGRAPH WITH A HALF WIDTH WEDGE "BUFFER" GEL



On this second plate, therefore, the first two sample holes were surrounded by antibody gel, with no plain gel cathodal to them. The subsequent autoradiograph showed complete dissociation of hormone from the two samples without the cathodal plain gel. The samples with some cathodal plain gel showed considerable, but not complete dissociation, and the samples which ran through the plain gel again showed binding up to a maximum at, and above, the optimum gel width of 1.1 cm. from the leading edge of the sample hole to the plain/antibody gel boundary.

This plain gel was named the "buffer" gel due to its assumed function of buffering the interaction of the antigens and antibodies.

4.2.3.2. QUANTITATIVE EFFECT OF THE
"BUFFER" GEL

The quantitative effect on hormone binding of the "buffer" gel was investigated using the Line Technique. Three plates, with the same euthyroid serum sample equilibrated with radio-labelled thyroxine to give a total concentration of 150 nmol/l, were run using whole, half and no "buffer" gel respectively. The half "buffer" gel plate used this gel only cathodal to the gel sample (Fig. 21). After electrophoresis each plate was cut up and counted. The counts, as percentages of total hormone recovery, were plotted against sample number (Fig. 22).

The plate with the whole "buffer" gel retained 100% of the sample radioactivity and showed a TBG peak which bound 35% of the total radiolabelled hormone, with a second, more diffuse peak cathodal to and around albumin (Fig. 22a).

Using the half "buffer" gel the activity on the TBG peak decreased to 14% and the total radio-activity recovered fell to 90% of the original sample activity, but the diffuse peak cathodal to albumin remained (Fig. 22b).

With no "buffer" gel, radioactivity was completely lost from TBG, and only 40% of the original activity was recovered, all of which was anodal to albumin where no binding protein is known to precipitate (Fig. 22c).

FIGURE 21

MODIFIED LINE PLATE WITH HALF "BUFFER" GEL.

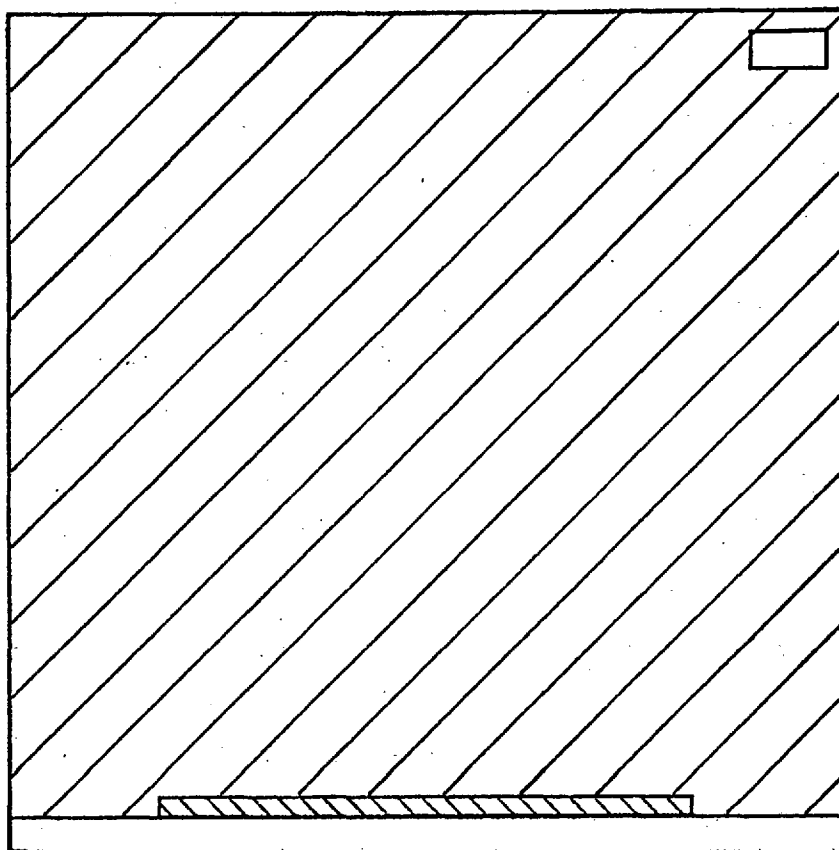


FIGURE 22a.

GRAPH OF RADIOACTIVITY DISTRIBUTION USING THE
MODIFIED LINE TECHNIQUE.

a) Distribution When Whole "Buffer" Gel Used.

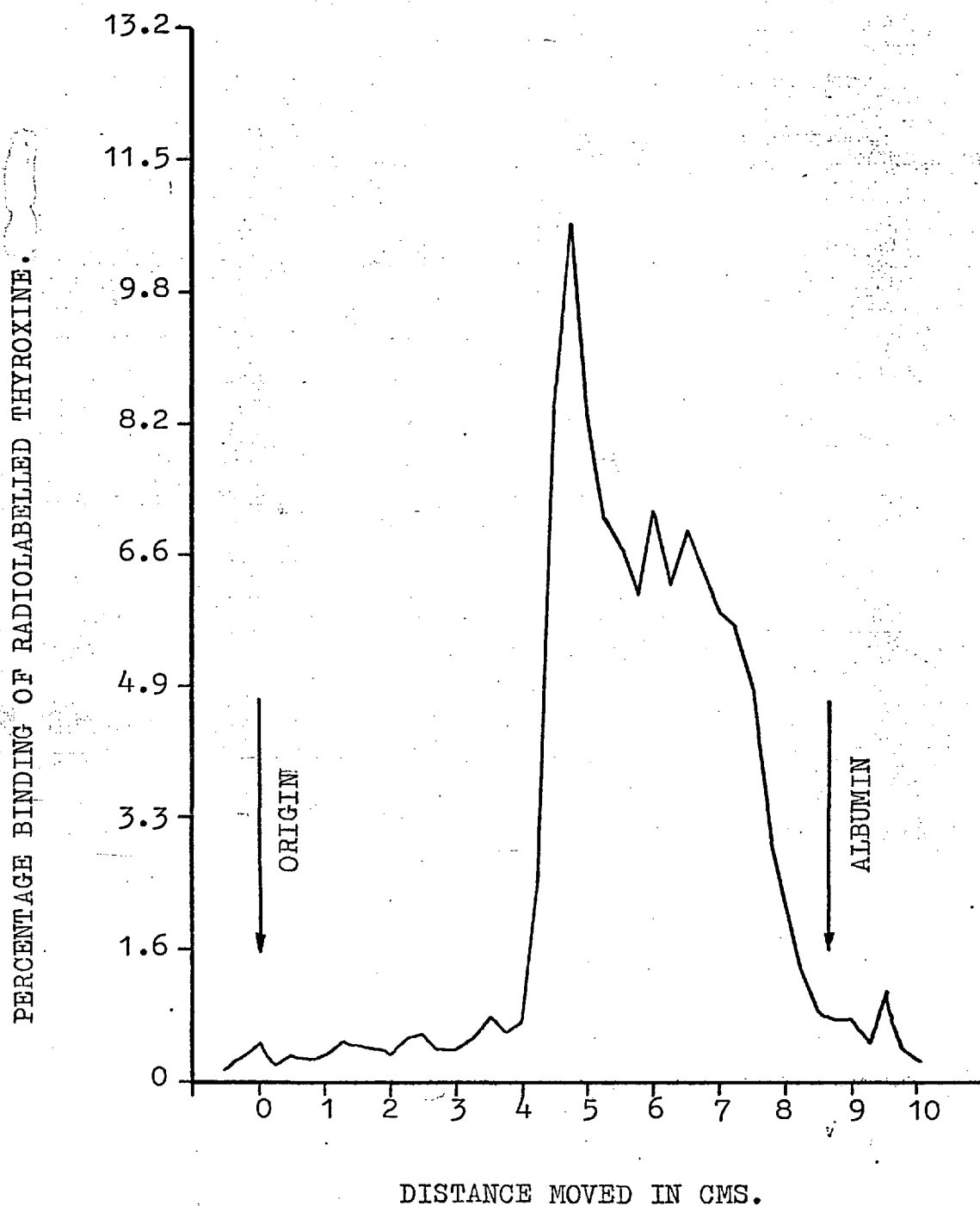


FIGURE 22b

GRAPH OF RADIOACTIVITY DISTRIBUTION USING THE
MODIFIED LINE TECHNIQUE.

b) Distribution when Half "Buffer" Gel Used.

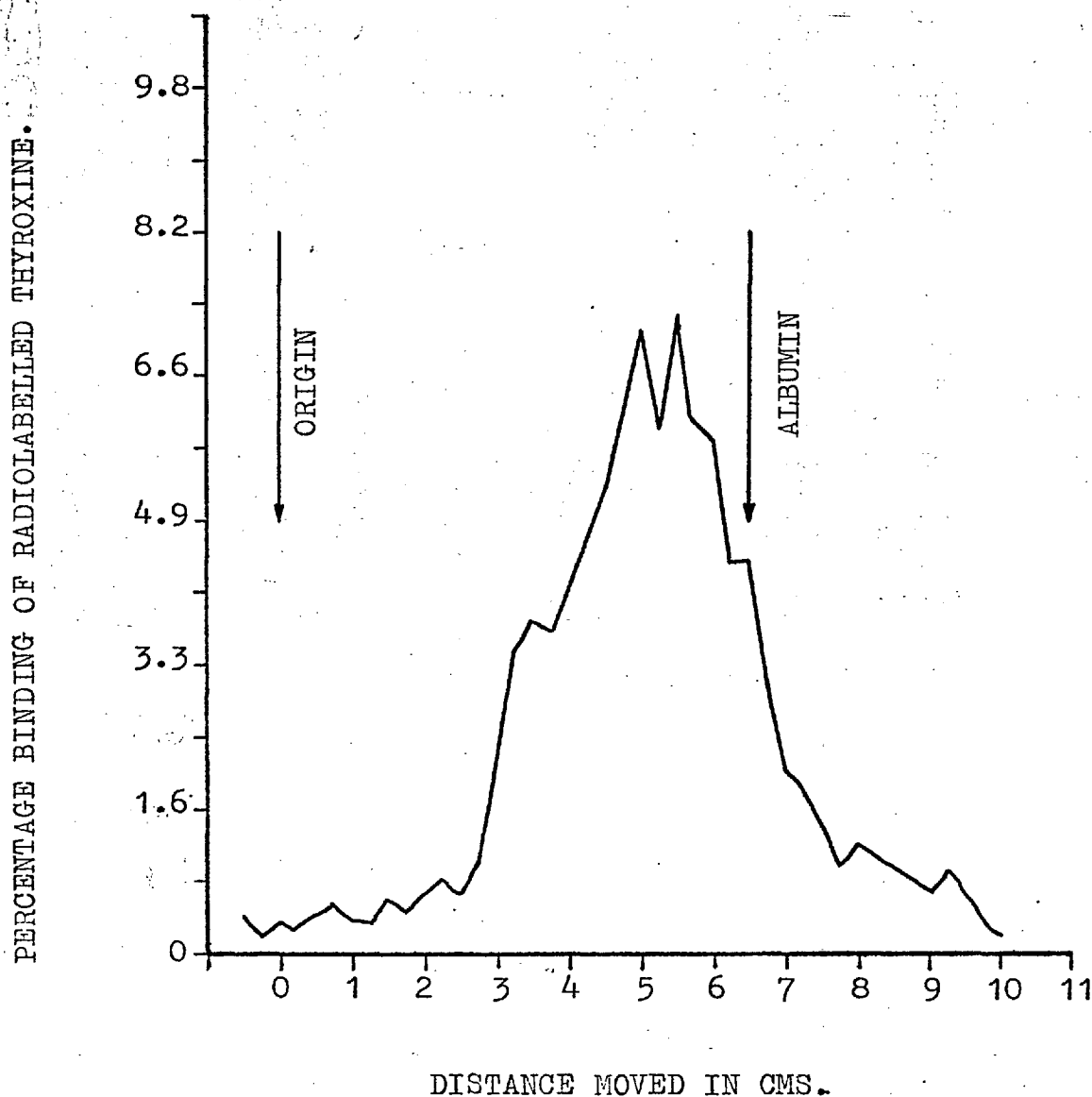
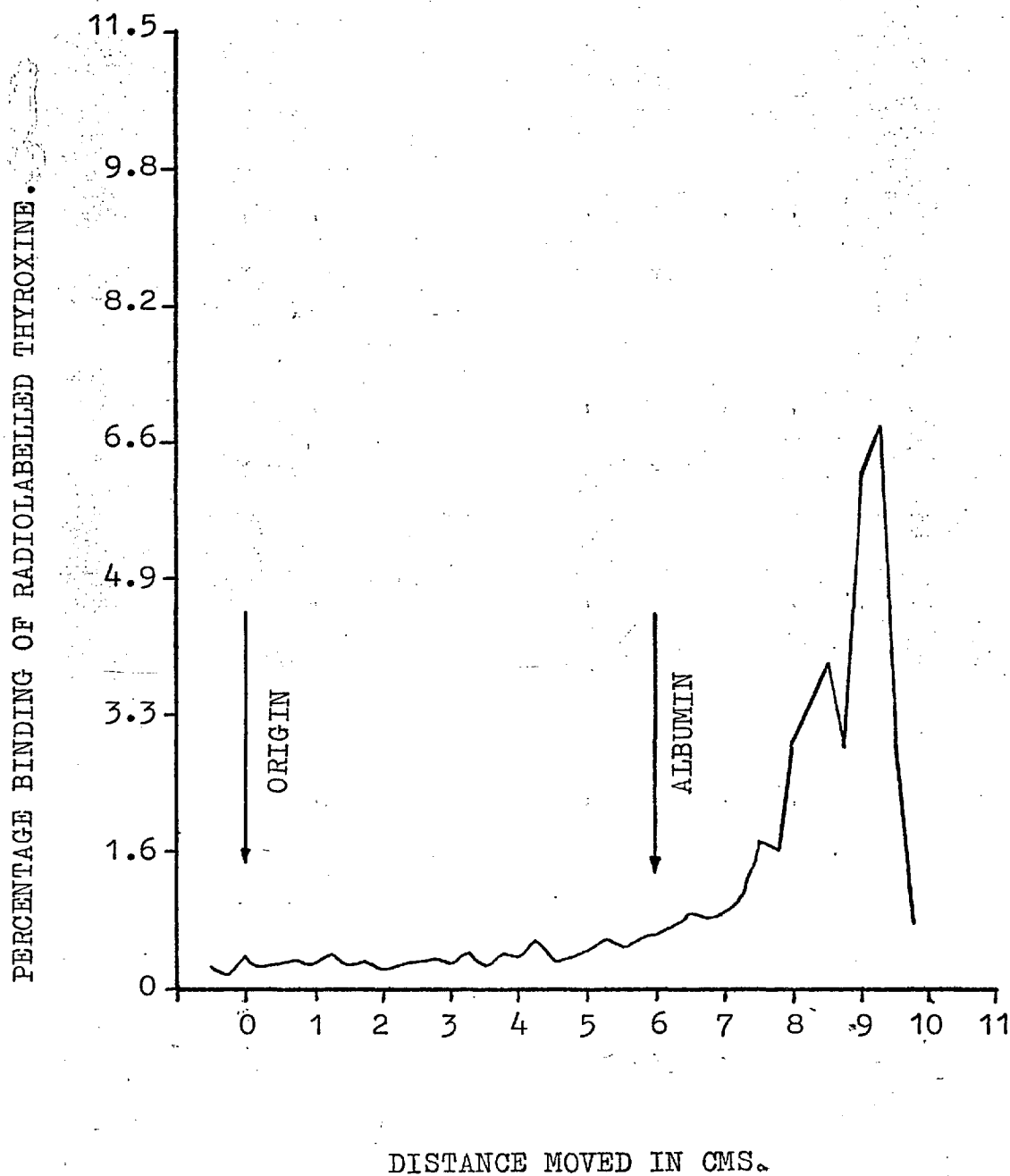


FIGURE 22c

GRAPH OF RADIOACTIVITY DISTRIBUTION USING THE
MODIFIED LINE TECHNIQUE.

c) Distribution when No "Buffer" Gel Used.



4.2.3.3. SPECIFIC EFFECT OF ANTIBODY IN BINDING DISSOCIATION

The absence of antibody around the serum sample appears crucial to hormone binding during electrophoresis. To investigate this factor further, two series of experiments were performed, one to determine whether the type of antiserum was critical, the second to determine whether a specific antibody was responsible or if a group of specific antibodies were necessary to dissociate hormone from binding proteins.

1. Antiserum Type

To investigate the affect of antisera on hormone binding, four different antisera (B.W., and Meath from sheep; Dakopatts from rabbits; Goat G.P. 19 from goats) were used against aliquots from the same radiolabelled serum sample. Chloroform was thought to be present in one of the antisera (Meath) from the strong smell of this compound in the serum. The thyroid hormone is known to be soluble in organic solvents of this type and the antiserum was dialysed for 24 hours against saline at 4°C to remove it. The dialysed and untreated Meath antisera were both used so that five plates were run against the different antisera under identical conditions on Line plates without "buffer" gel. All resulting plates failed to show any measurable radiolabelled hormone binding irrespective of the antiserum used.

2. Specific Antibody

To investigate the possibility of whether all, some or one of the antibodies in the polyvalent antisera discussed above were responsible for the dissociation, two 17 hole Rocket plates were run using the identical sample against a series of available monospecific antisera.

Two long (17 hole) plates were prepared using a "buffer"

gel and polyvalent B.W. antiserum. Fourteen monospecific antisera were available for the study and these were divided into two equal sets. Each "buffer" gel on the two long plates was divided into eight sections so that seven of the sections could be removed and replaced by a gel containing a monospecific antiserum, but leaving one unreplaced "buffer" gel section as a control. This control section was slightly wider than the other gel sections, due to the dimensions of the plate being unequally divisible, allowing three sample holes to be punched in this gel. All the holes on each plate were filled with 2 μ l of the same serum sample equilibrated with radiolabelled thyroxine ($^{125}\text{I T}_4$). Both plates were run in the modified pH 7.4 electrophoresis system, then dried and autoradiographed.

Where the "buffer" gel was used, well defined TBG peaks were found on the autoradiographs, but where this gel had been substituted by a gel containing a mono-valent antiserum, dissociation of binding occurred. Dissociation occurred to a different extent with all antisera used and the estimated degree of dissociation ranged from 40% to 95%. The degree of dissociation was estimated from the reduction of X-ray film blackening compared with the TBG rockets starting in the "buffer" gel. The monospecific antisera used and their estimated degree of dissociation are shown in Table 6, and the plates and corresponding autoradiographs in Figs. 23 & 24.

TABLE 6

ESTIMATED DISSOCIATION OF RADIOLABELLED
THYROXINE FROM TBG WHEN USING A RANGE OF
SPECIFIC ANTISERA AND THE MODIFIED ROCKET
TECHNIQUE.

ANTISERUM	ESTIMATED DISSOCIATION FROM TBG.
Anti- α_1 Antitrypsin	75%
Anti- α_1 Chymotrypsin	90%
Anti- α_1 Acid Glycoprotein	80%
Anti- α_1 Lipoprotein	60%
Anti- α_2 Macroglobulin	60%
Anti-Caeruloplasmin	70%
Anti-Haemopexin	75%
Anti-Transferrin	80%
Anti-IgA	40%
Anti-IgD	75%
Anti-IgE	99%
Anti-IgG	90%
Anti-IgM	90%
Anti- α_2 HS	75%

FIGURE 23

PHOTOGRAPH OF A STAINED PLATE AND THE CORRESPONDING
AUTORADIOGRAPH OF A LONG PLATE WITH SPECIFIC
ANTISERA IN THE "BUFFER" GEL

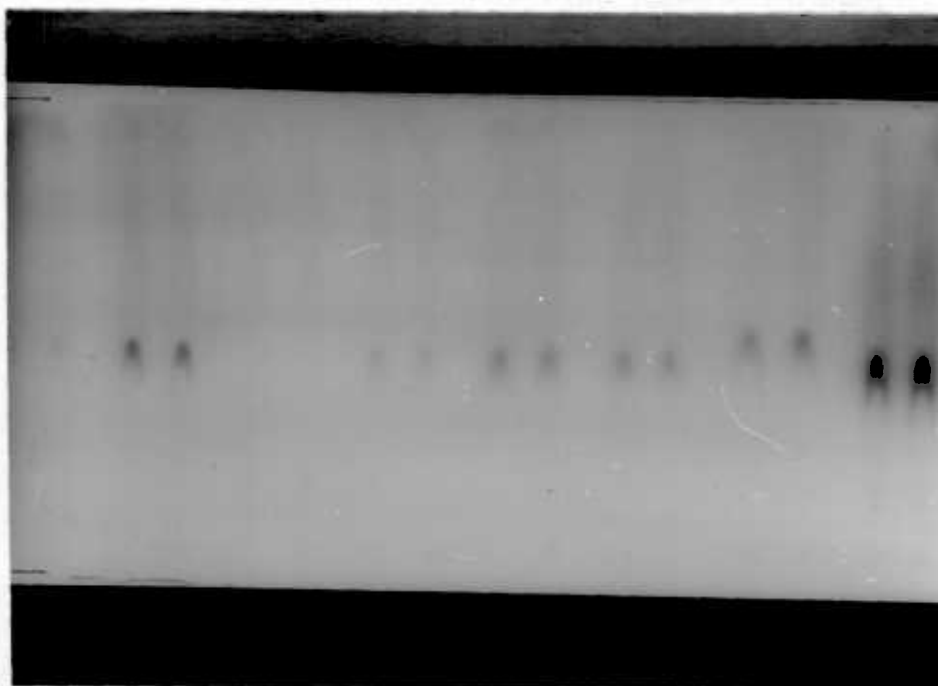
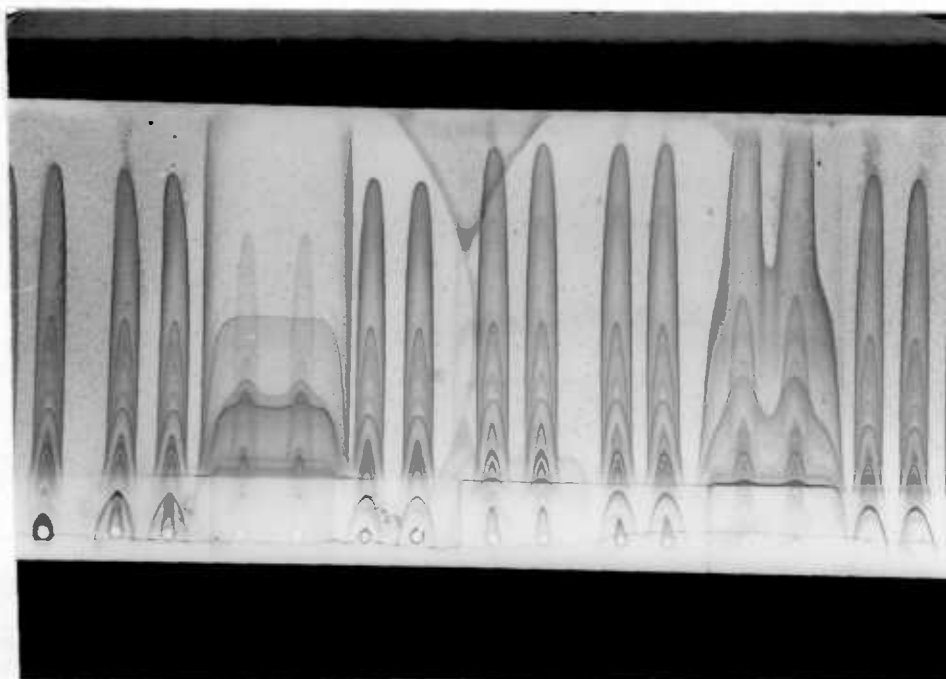
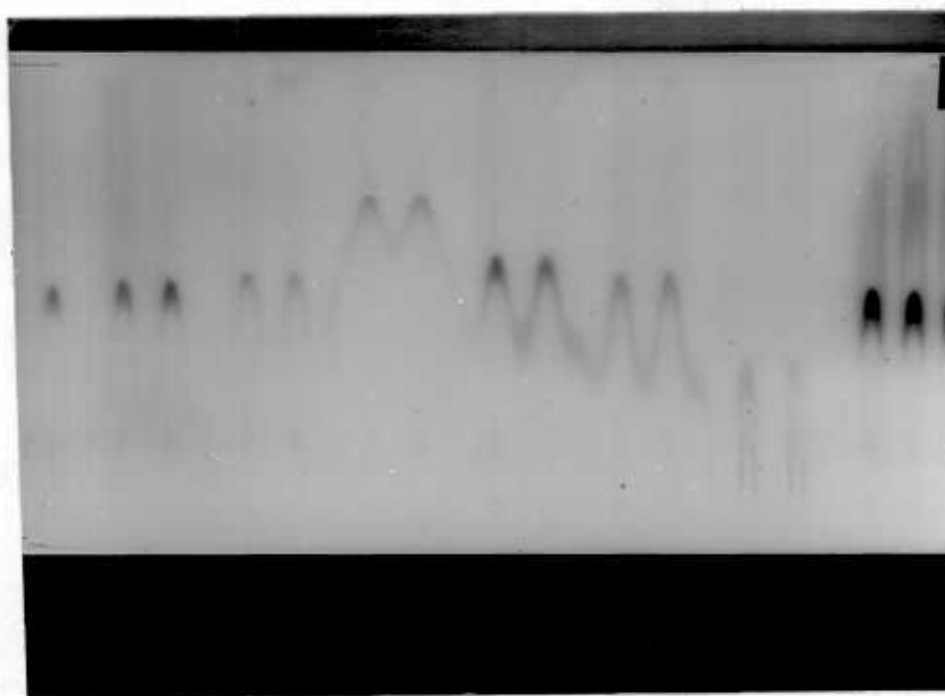
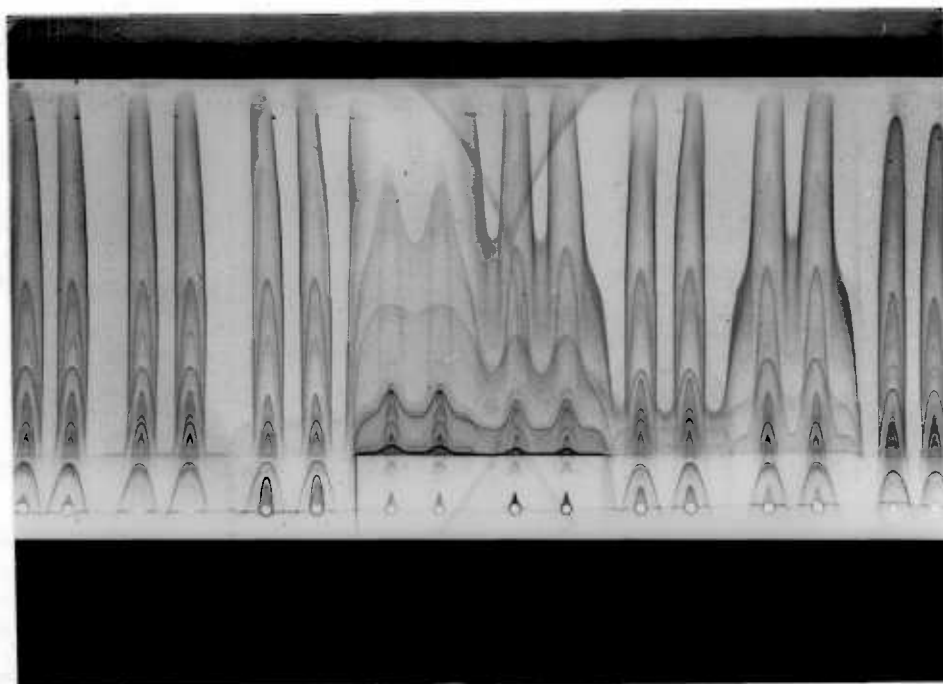


FIGURE 24

PHOTOGRAPH OF A STAINED PLATE AND THE CORRESPONDING
AUTORADIOGRAPH OF A LONG PLATE WITH SPECIFIC
ANTISERA IN THE "BUFFER" GEL



4.2.3.4. SUMMARY OF MODIFIED UNIDIRECTIONAL IMMUNOELECTROPHORETIC TECHNIQUES

The standard techniques described in Sections 3.4.2. and 3.4.3. were modified to include the changes evolved from the experiments of Sections 4.2.2. to 4.2.3. Both the Modified Line Technique and Modified Rocket Technique were altered to be used in phosphate buffer at pH 7.4 and a "buffer" gel of 2 cm. was included, into which the samples were applied (Figs. 25, 26, 27 & 28).

The Modified Line plates after electrophoresis were cut up into 0.25 cm. strips for counting using the plan shown in Fig. 29 . Each gel section was placed in a numbered tube with 2 ml. of 0.9% NaCl, stoppered and counted using a Wallac auto-gamma counter. The counts were then related to gel section number and calculated as a percentage of the total counts recovered. The percentage recovery of counts were then plotted against the distance moved by the activity i.e. the gel section number in 0.25 cm. units (Fig. 30a & b).

FIGURE 25

MODIFIED ROCKET PLATE.

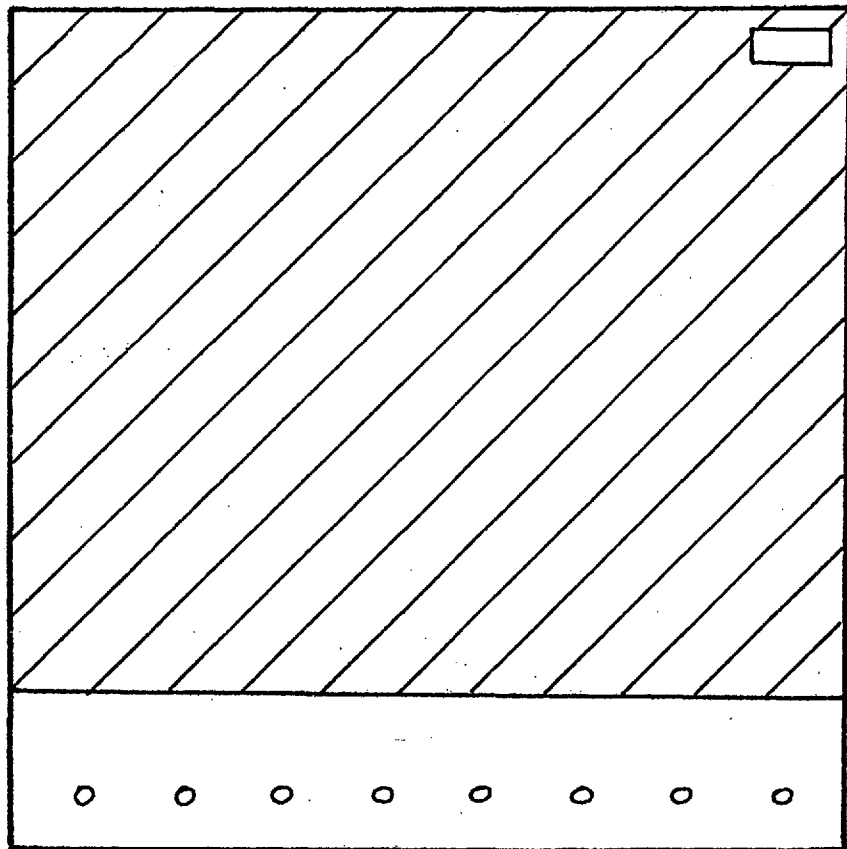


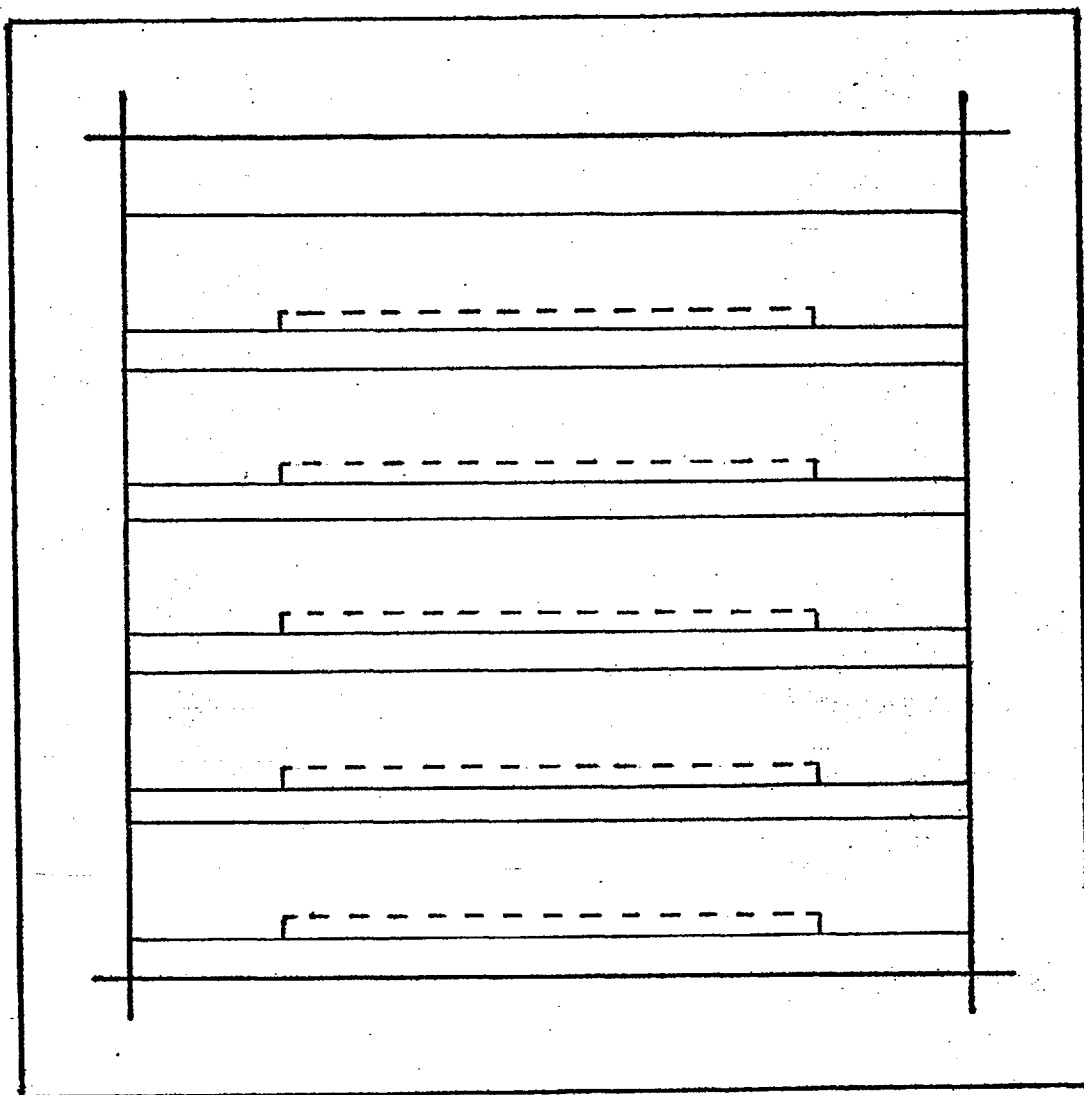
FIGURE 26PLAN FOR MODIFIED LINE TECHNIQUE SAMPLES..

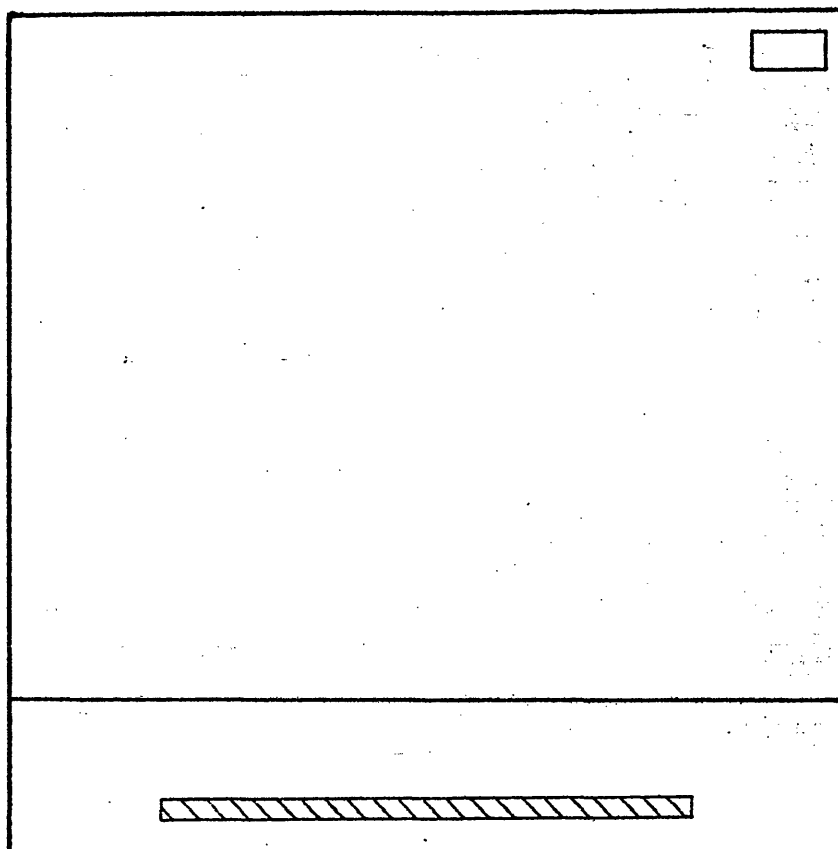
FIGURE 27MODIFIED LINE TECHNIQUE SAMPLE WITH "BUFFER"GEL ON PLATE.

FIGURE 28

MODIFIED LINE TECHNIQUE PLATE READY FOR
IMMUNOELECTROPHORESIS.

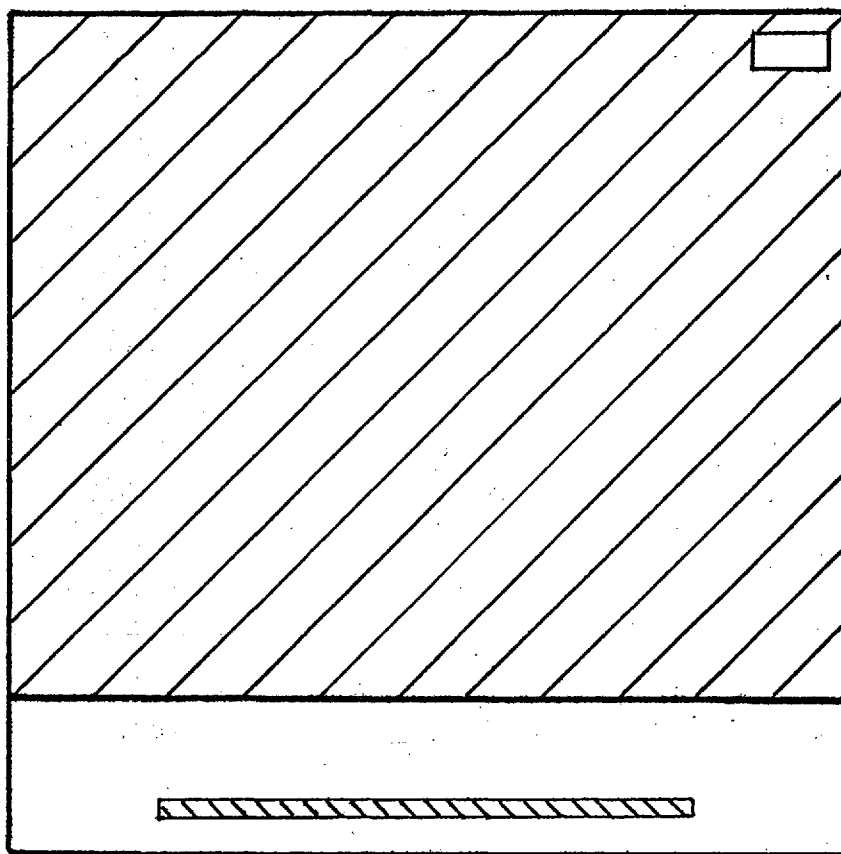


FIGURE 29

PLAN FOR CUTTING UP MODIFIED LINE TECHNIQUE PLATE
FOR COUNTING.

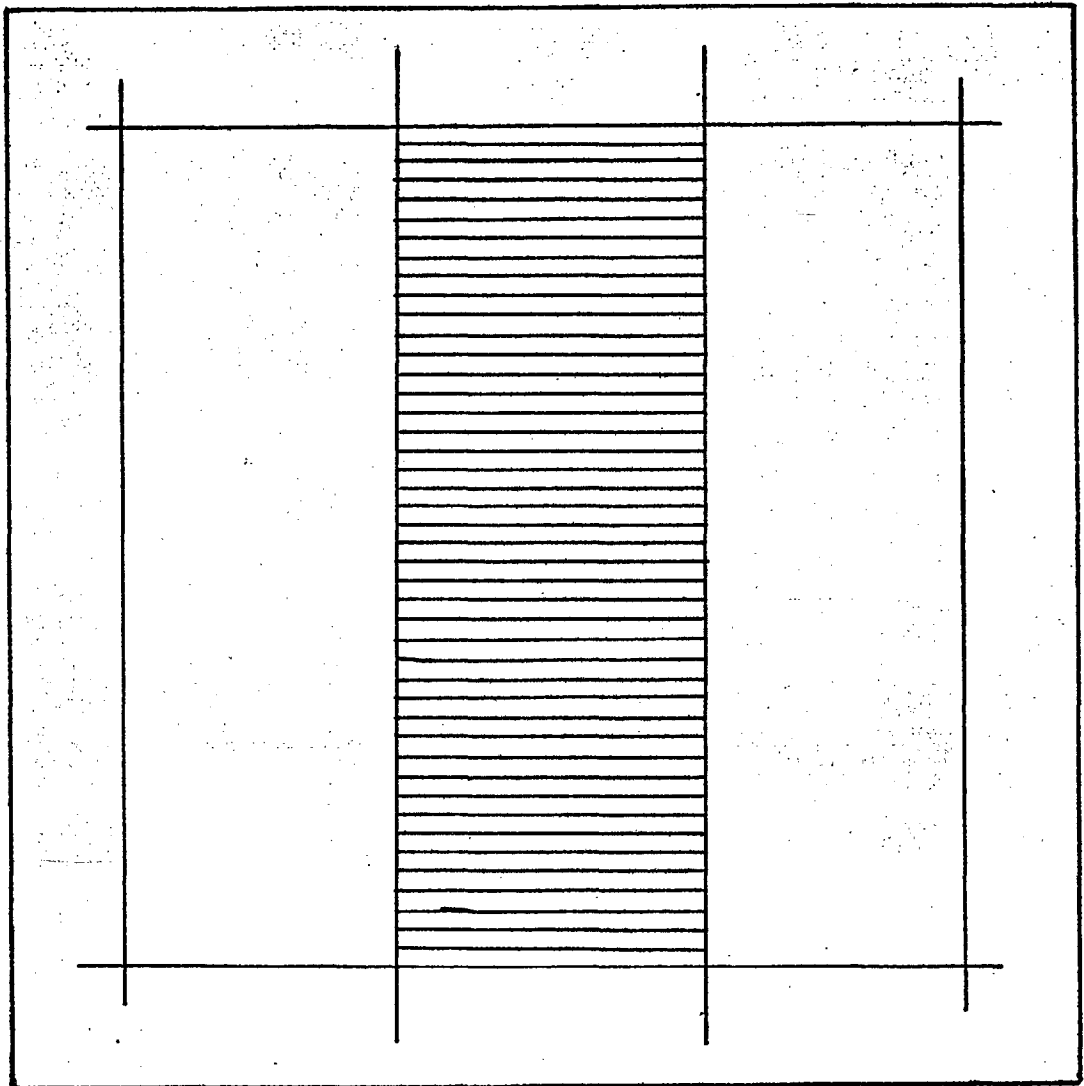


FIGURE 30a

EXAMPLE OF A MODIFIED LINE TECHNIQUE
COUNTING GRAPH.

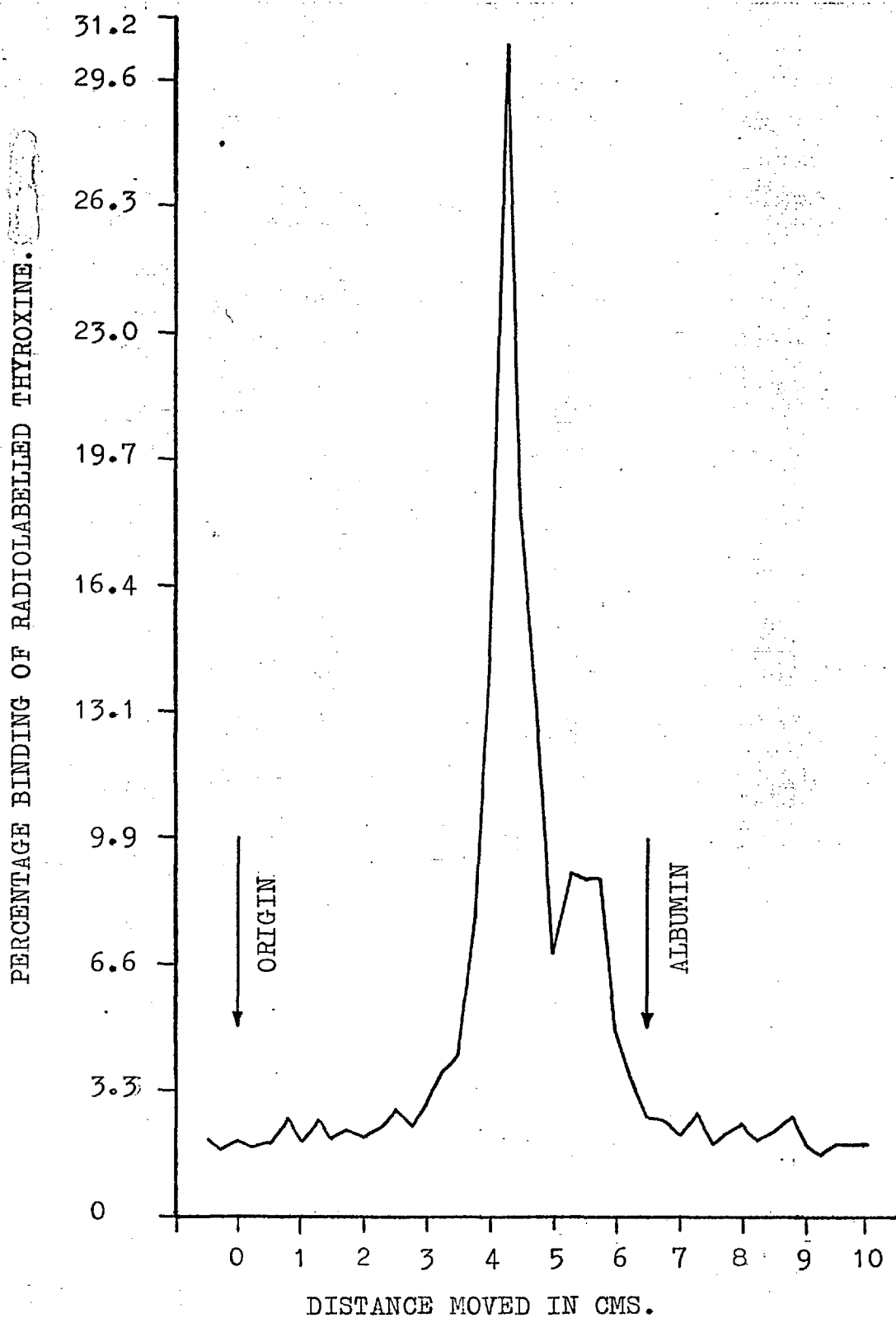
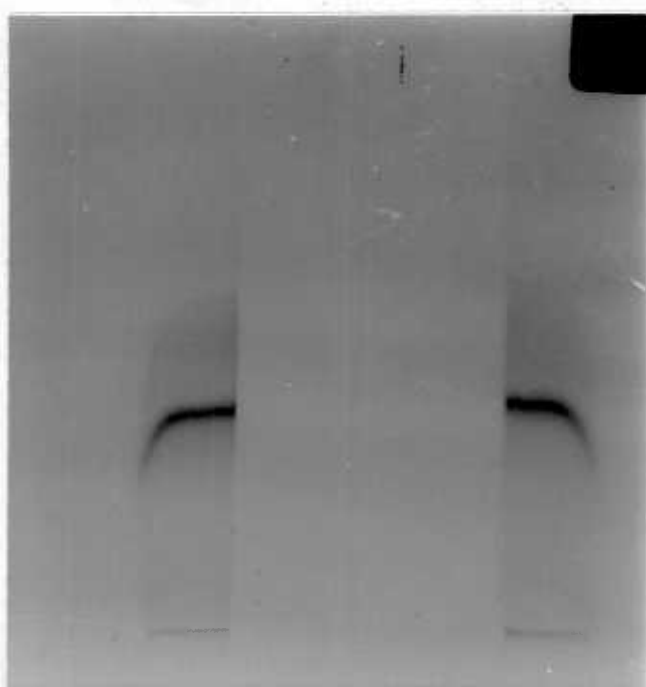
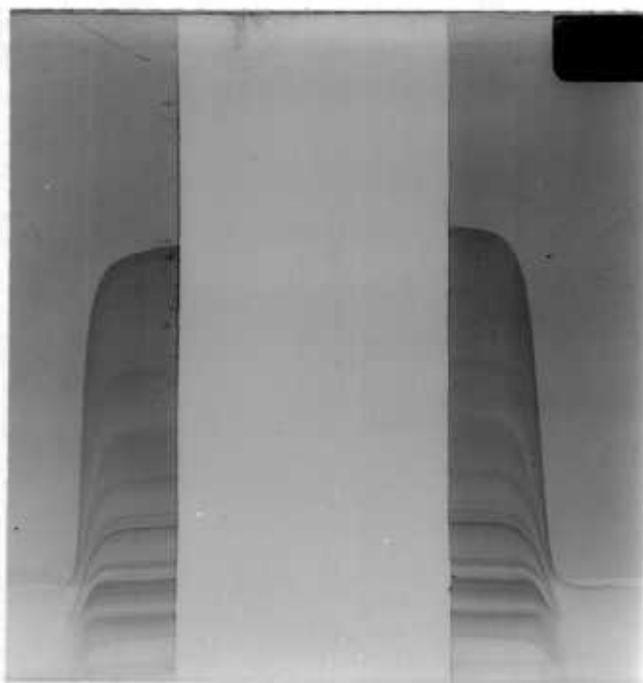


FIGURE 30b

PHOTOGRAPH OF A STAINED PLATE AND THE CORRESPONDING
AUTORADIOGRAPH OF A MODIFIED LINE PLATE AFTER
CUTTING UP FOR COUNTING



4.2.3.5. DISCUSSION OF "BUFFER" GEL

INVESTIGATION

The low serum TBG concentration necessitates the use of a marker which may be attached to the protein. The most convenient and easily applied marker is Radiolabelled thyroxine, (T_4 I¹²⁵), but when the protein is so marked and estimated using the standard forms of the Laurell Rocket (Laurell, 1966) and Line (Kroll, 1968) techniques, this label is dissociated from the protein and the marker is lost. An antigen/antibody phenomenon was deduced and subsequently proven to be the dissociating factor by using a series of methods to either eliminate the antiserum or change its composition so that binding was maintained. During the course of the investigation, using four polyvalent and fourteen monovalent antisera, it became clear that a non-specific factor was involved in the dissociation and that the phenomenon was only operable whilst the serum proteins were found as an integrated group as normally occurs in vivo. However, once the individual antigen proteins were teased apart, by the initial electrophoretic separation through a plain, non-antibody containing or "buffer" gel, the dissociating factor or factors become ineffective.

The use of different shapes and thicknesses of "buffer" gel has shown that a minimum physical separation of antigens from their antibodies is necessary if dissociation is to be avoided. If this minimum separation is reduced or absent then hormone binding is quantitatively reduced until it is totally eliminated.

These phenomena have been fully confirmed by an independent laboratory (Ramsden - personal communication; Burr & Ramsden, 1977) using both a commercial polyvalent antiserum and a specific anti-TBG serum with the modified Rocket

techniques.

Nielsen, et al. (1972) have also implied a binding anomaly when an antiwhole human antiserum is used with the Laurell Rocket technique, but they omitted mention of the relevant results. However, when they used semispecific antisera normal binding was apparently maintained though no quantitative evidence was quoted. The results from Nielsen et al. (1972) are therefore in complete contrast to results obtained in this laboratory and from Ramsden (personal communication) where binding dissociation occurred in the absence of a "buffer" gel.

Kranz et al. (1974) also used the Laurell Rocket Technique to study TBG and, in common with Nielsen et al. (1972), failed to appreciate dissociation of radiolabelled hormone without a "buffer" gel and reported normal thyroxine binding.

Apart from the different antisera used in these studies (Nielsen et al. 1972; Kranz et al. 1974) and the present work, the only difference was the buffer involved. This study used the developed phosphate 0.025M pH 7.4 system whilst both the other studies (Nielsen et al., 1972; Kranz et al., 1974) used barbital buffer at pH 8.6. However, Ramsden (personal communication) found in common with this present study that binding dissociation also occurred when he used barbitone buffer at pH 8.6. It is difficult to find an explanation for this fundamental difference between the results as both Nielsen et al. (1972) and Kranz et al. (1974) used (i) a barbital buffer at pH 8.6, (ii) washed the plates after electrophoresis (see Section 4.2.4.1.) and (iii) placed the serum sample wells in the antibody gel. Each of these factors has repeatedly been shown in this study to result in a decreased, if not complete dissociation of radiolabelled hormone from the binding proteins.

How the "buffer" gel works

The "buffer" gel acts as a physical separator of the antigen (An) and antibody (Ab) proteins, but it does not affect the interaction of the individual An and Ab molecules. The An/Ab interaction has, apparently, no effect on hormone binding as may be seen from studies using the Modified Line and Rocket Techniques when the interaction occurs, but binding persists. Also it has been demonstrated that the thyroxine binding sites on the TBG molecule ^{are} ~~is~~ different from the antibody binding site (Refetoff et al., 1975; Hamada, Takemura & Sterling, 1971).

The partition between the serum and its antiserum is considered necessary to separate the test serum TBG from any antiserum contained TBG, which due to its higher concentration competes with the test serum TBG and removes the bound thyroxine (Drysedale, Ramsden & Hoffenberg, 1975). The antiserum contained TBG then runs off the plate with the consequent loss of radiolabelled hormone. With the "buffer" gel in position, however, the animal TBG in the antiserum would always be anodal to the test serum TBG and could not therefore compete for the hormone and interfere with binding.

Rabbit antiserum (Dakopatts), however, apparently contains no TBG but it is usually contaminated with albumin which could similarly compete with the test serum TBG and dissociate bound thyroxine. Although some antisera are separated to concentrate the immunoglobulins, the TBG and/or albumin impurities are almost impossible to remove without extremely careful attention and at the cost of much of the immunoglobulin component (Ramsden - personal communication). This attention is not usually required in commercial antisera.

The impurity competition postulate is the favoured

mechanism for hormone dissociation, but a second mechanism is also advanced since the antisera without TBG pose a slight question on the validity of the mechanism, even though an albumin impurity may be present. The second postulate is related to the antigen proteins and not the antibodies. When the "buffer" gel is employed the antigen proteins are teased apart by the electrophoretic field and therefore meet their respective antibodies as individual units rather than integrated groups of proteins. This physical separation of antigens could be involved in the dissociation phenomenon so that if for example TBG were associated with albumin, as has been postulated (Inada & Sterling, 1970), and with thyroxine, then the TBG and albumin antibodies when they combined with the protein-hormone complex could result in physical contortions within the molecule too disruptive for the thyroxine-TBG complex, resulting in hormone dissociation. Once dissociated, the free hormone would come under the influence of the electric field and would quickly move away from the slower moving protein molecules making reassociation impossible. In this regard it is interesting that the dissociation phenomenon was stimulated by such a wide range of specific antisera (4.2.3.3. & Table 6).

4.2.4.

WASHING EFFECT ON THYROXINEBINDING TO TBG

To determine autoradiographic exposure times and percentage binding calculations, maximum recovery of radiolabel from the test sample is essential. Therefore the washing of electrophoresed plates was investigated to determine whether any hormone is lost during this process.

4.2.4.1. INVESTIGATION OF WASHING ON

THYROXINE BINDING TO SERUM PROTEINS

Three pairs of modified Line plates were run using a hypothyroid serum (24 nmol/l) equilibrated with radiolabelled thyroxine ($^{125}\text{I T}_4$) to give total hormone concentrations of 75, 125 and 525 nmol/l. The six plates were run on the modified Line system which contained 0.025M pH 7.4 phosphate buffer. After electrophoresis the plates were divided into two sets so that each contained one plate from the three hormone addition groups. One set was washed first in 0.9% saline for 12 hours, and then in distilled water for 1 hour, then both sets were cut up for counting. The two sets of tubes were counted on the same day to eliminate difference in isotopic decay. The results from the two sets of plates were compared (see Table 7 and Figs. 31a, 31b, & 31c).

On all of the unwashed places, 100% of the radiolabelled hormone was recovered. However, on the washed plates a significant difference was found with the recovery being 54%, 56% and 42% for the increasing exogenous hormone levels.

Percentage binding of radiolabelled thyroxine to TBG was calculated at each of the three hormone levels, by determining the total counts on the plate and then those on the TBG precipitin line, and computing the percentage binding (Table 7). Although the percentage binding to TBG was similar at the physiological level of added hormone, i.e. 66% for the washed and 64% for the unwashed plate TBG, the actual number of counts on the unwashed plate TBG, and therefore recovery, was 80% more than on the washed plate. On the plates containing 75 nmol/l total thyroxine concentration, the percentage binding to TBG was 70% for the unwashed and 59% for the washed plate, but the number of counts on the unwashed

TABLE 7

EFFECT OF WASHING ON PERCENTAGE RECOVERY AND TBG PERCENTAGE BINDING OF THYROXINE

UNWASHED				WASHED		
a	b	c	Hormone Concentration (nmol/l)	a	b	c
75	125	525		75	125	525
21778	38707	204058	Total Counts on Sample/100 sec.	21778	38707	204058
14059	25698	130879	Total Counts Recovered/100 sec.	7594	13896	55244
9850	16496	40899	Total Counts on TBG/100 sec.	4498	9174	22269
100	100	100	Percentage Recovery of Hormone	54	56	42
70	64	31	Percentage Binding to TBG	59	66	40
219	180	184	Relative Number of Counts = $\frac{\text{Unwashed}}{\text{Washed}} \times 100\%$			

Whole gel sample = 6.2 cms.

Counted gel section = 4.0 cms.

Each recovered radioactivity count is therefore multiplied by a FACTOR of $\frac{6.2}{4} = 1.55$

FIGURE 31a

GRAPH OF MODIFIED LINE TECHNIQUE PLATE GRAPHS
BEFORE AND AFTER WASHING THE PLATES.

a) Total Hormone Concentration on the Plates

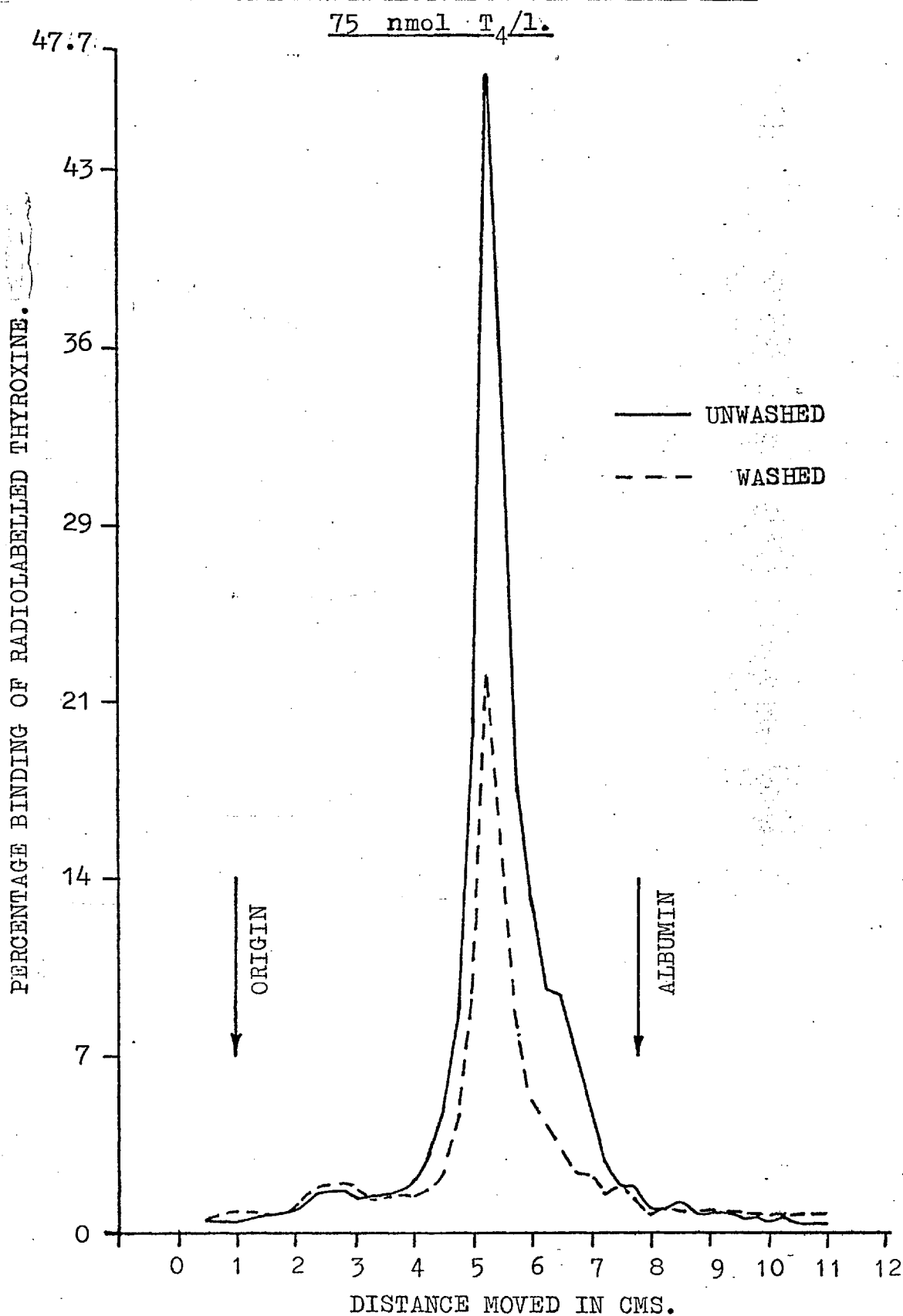


FIGURE 31b

GRAPHS OF MODIFIED LINE TECHNIQUE PLATE GRAPHS
BEFORE AND AFTER WASHING THE PLATES.

b) Total Hormone concentration on the Plates

125 nmol T_4 /l.

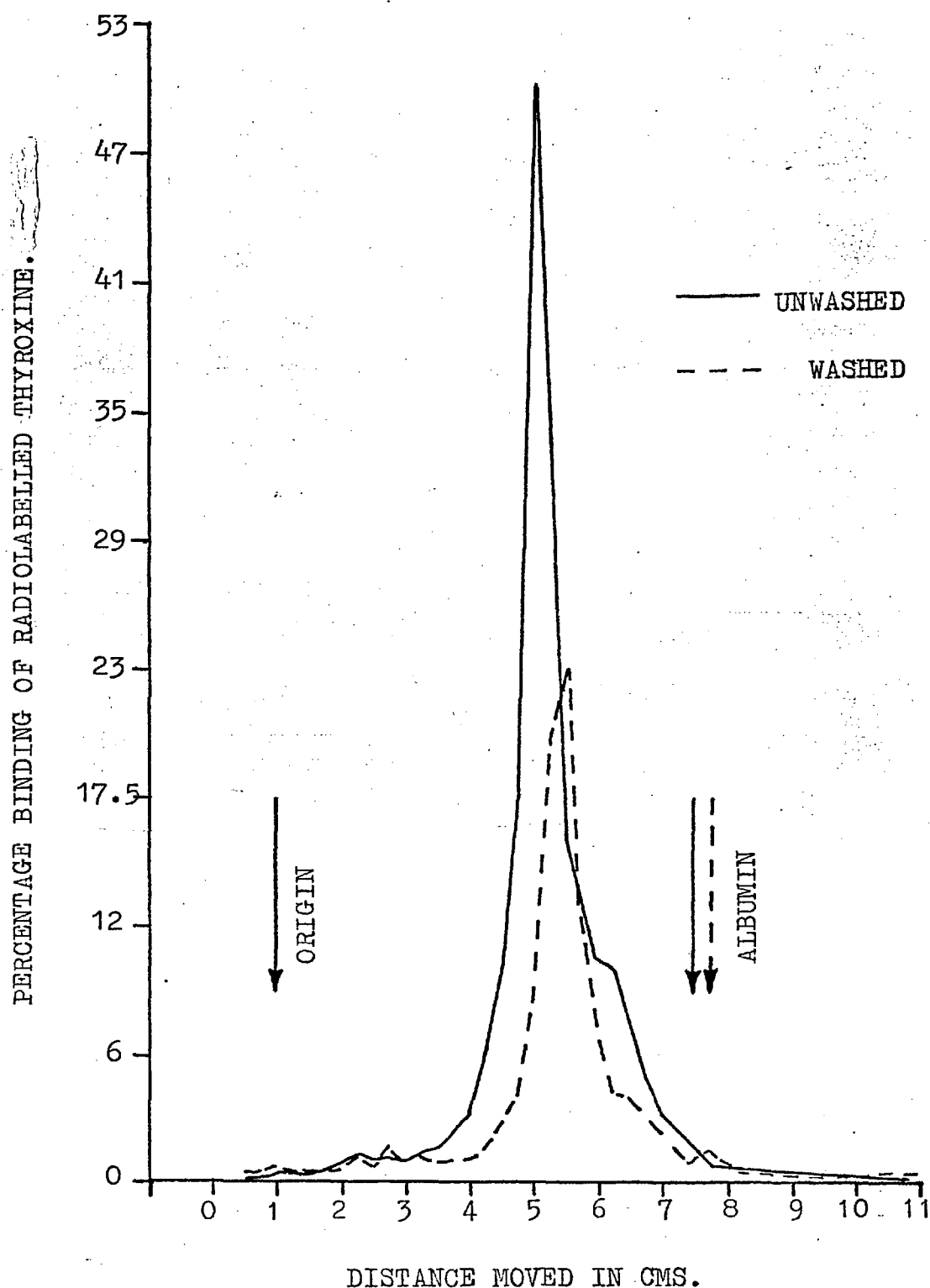


FIGURE 31c

GRAPHS OF MODIFIED LINE TECHNIQUE PLATE GRAPHS
BEFORE AND AFTER WASHING THE PLATES.

C) Total Hormone Concentration on the plates
525 nmol T_4 /l.

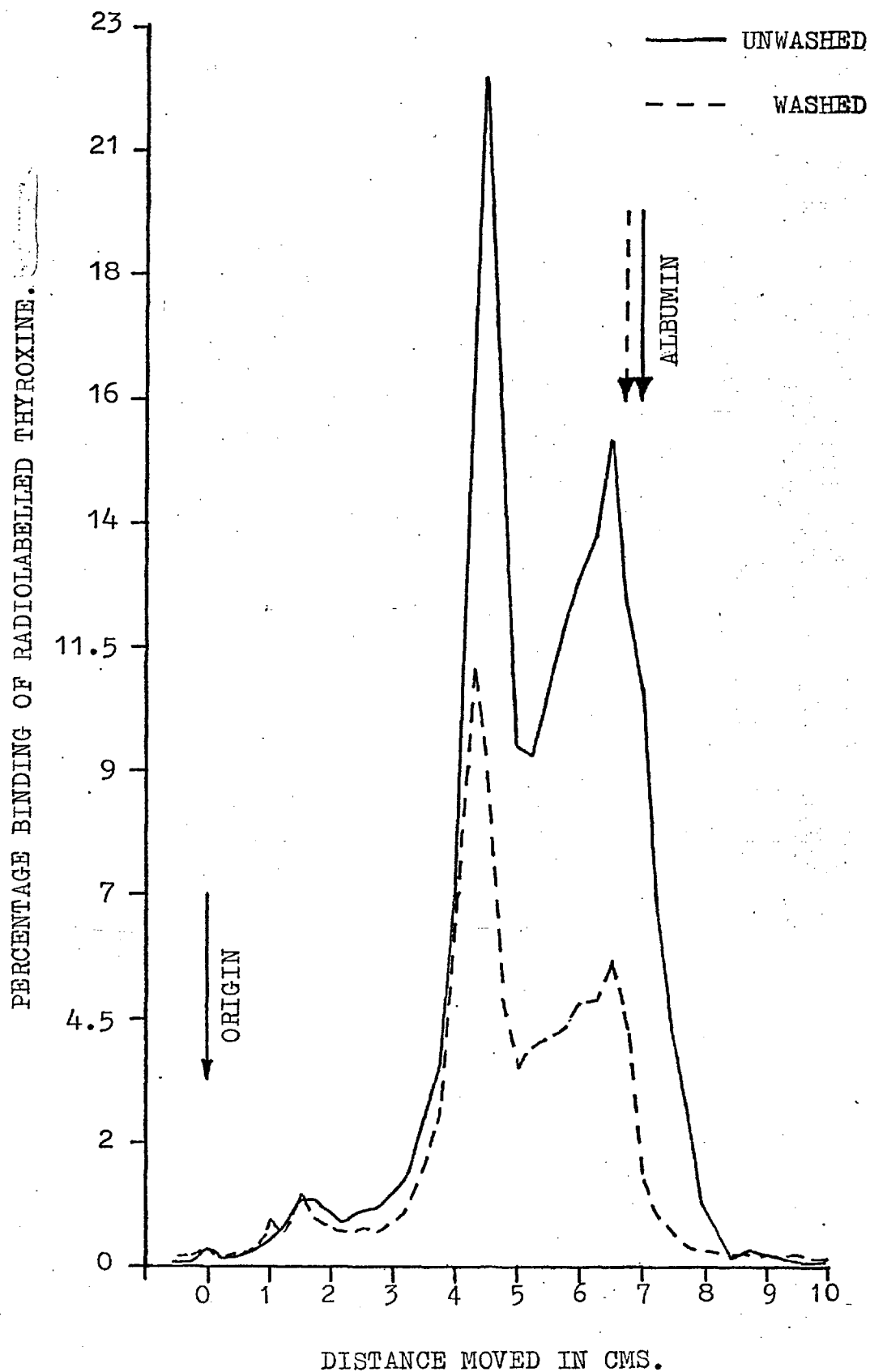


plate TBG was 119% more than the washed plate value. On the plates containing a total hormone concentration of 525 nmol/l, the percentage binding to TBG was higher on the washed plate, which bound 40% of the radioactivity, than on the unwashed which bound 31% of the total, though the total number of counts on the unwashed plate TBG was again significantly higher at 84% more than on the washed plate.

4.2.4.2.

DISCUSSION OF WASHINGTECHNIQUE

From the results it is apparent that while the percentage binding of radiolabelled hormone varies inconsistently between the washed and unwashed plate TBG, the number of radioactivity counts on this protein, and therefore the amount of hormone, was consistently 50% (46% - 56%) lower on the washed plate TBG. The difference between these results is probably due to different binding affinities of the individual thyroxine binding proteins. The reservoir size was identical in each case, negating a volume effect.

The greater hormone recovery on the washed plate with an initial total hormone concentration of 525 nmol/l was probably due to the larger amount of hormone being attached to the lower affinity proteins α_1 lipoprotein, albumin and TBPA. The hormone would be preferentially washed off these secondary proteins leaving the major protein, TBG, with an apparently higher hormone recovery. At the lower hormone concentrations the reverse would tend to occur, leaving TBG with a lower hormone recovery after washing, as is shown in the Results.

The consistent feature at all three total concentrations of thyroxine is that the washed plates lost 50% of the radio-labelled hormone.

4.2.5.

INTRODUCTION TO THEIDENTIFICATION OF LINE PLATEPROTEINS

During immunoelectrophoresis using the standard forms of the unidirectional techniques the radiolabelled thyroxine appears at characteristic positions on the electrophoretogram. To investigate the nature of this hormone so that the position of the binding proteins could be distinguished from free or unbound hormone, a series of experiments were designed.

The position of radiolabelled hormone after electrophoresis was determined and the results were compared with those from experiments run under conditions expected to result in maintained hormone binding. Two types of unbound hormone were considered to potentially exist on the standard Line plates; I) free hormone which had never been associated with the serum proteins, and II) dissociated hormone which had initially been bound to the serum proteins but had become dissociated during electrophoresis.

4.2.5.1. CHARACTERISATION OF RADIOLABELLED THYROXINE AFTER IMMUNOELECTROPHORESIS

The following four plate experiment was set up and run using the Line system.

- a) Radiolabelled thyroxine ($^{125}\text{I T}_4$) run through agarose.
- b) Radiolabelled thyroxine equilibrated with serum and run through agarose.
- c) Radiolabelled thyroxine run through agarose containing antiserum to human serum proteins.
- d) Radiolabelled thyroxine equilibrated with serum and run through agarose containing antiserum to human serum proteins.

On both plates without antiserum, a) and b), the radioactivity was completely lost by running off the anodal end of the plate (Figs. 32 & 33). During a subsequent experiment to investigate hormone binding, duplicate plates similar to b) were run and stopped after 6 hours as opposed to 15 hours for a standard Line plate. After the 6 hour separation two major radioactivity peaks were obvious after cutting up half the gel on the plates, and when the remaining half was stained for protein, the major peak was found to be in the TBG position while the second peak was in the TBPA position (Fig. 34) indicating continued association of binding protein and hormone.

On the plates with antiserum, c) and d), a proportion of the radioactivity remained on the plate, but was differently distributed. Plate c) which involved running radiolabelled hormone alone through antiserum, approximately 61% of the original radiolabelled hormone was lost with the remainder being distributed from a peak at the origin near the cathodal end of the plate and tailing off toward the anodal edge of the

FIGURE 32

DISTRIBUTION OF ^{125}I T_4 AFTER A 15 HOUR
ELECTROPHORETIC SEPARATION THROUGH AGAROSE.

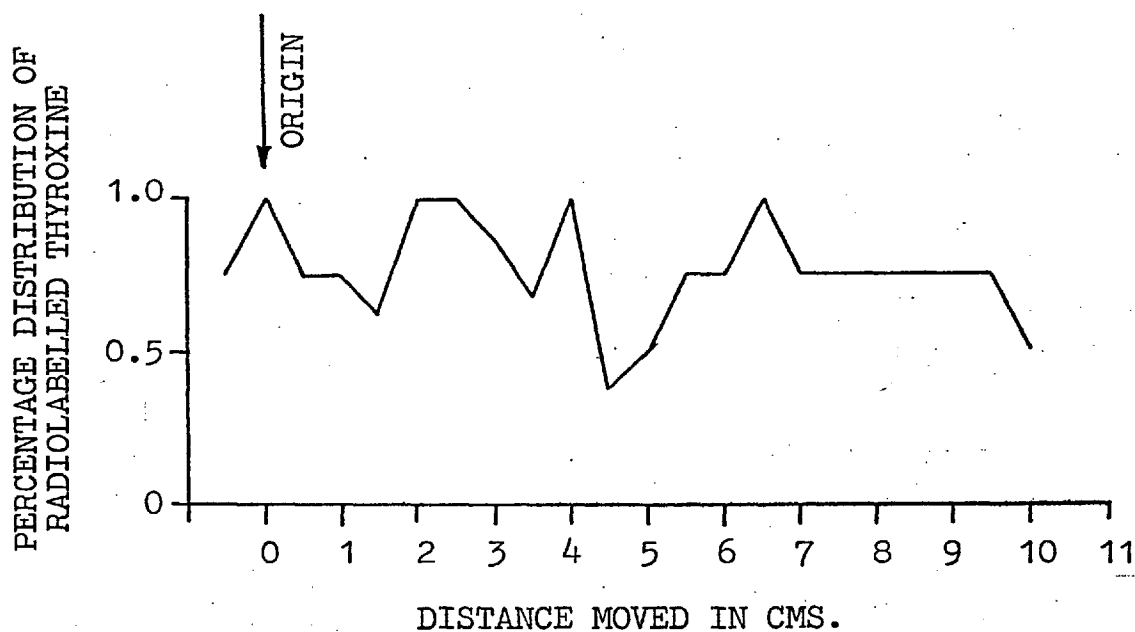


FIGURE 33

DISTRIBUTION OF SERUM EQUILIBRATED WITH ^{125}I T_4
AFTER A 15 HOUR ELECTROPHORETIC SEPARATION
THROUGH AGAROSE.

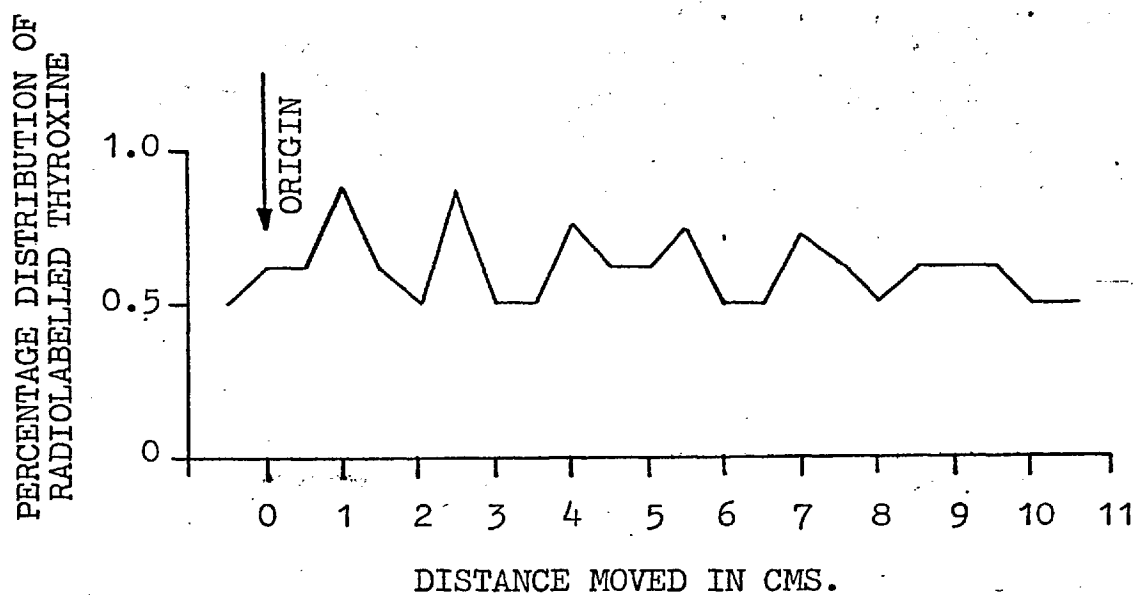


FIGURE 34
GRAPH OF RADIOACTIVITY DISTRIBUTION ON A PLATE
WITHOUT ANTISERUM AFTER A 6 HOUR ELECTROPHORETIC
SEPARATION.

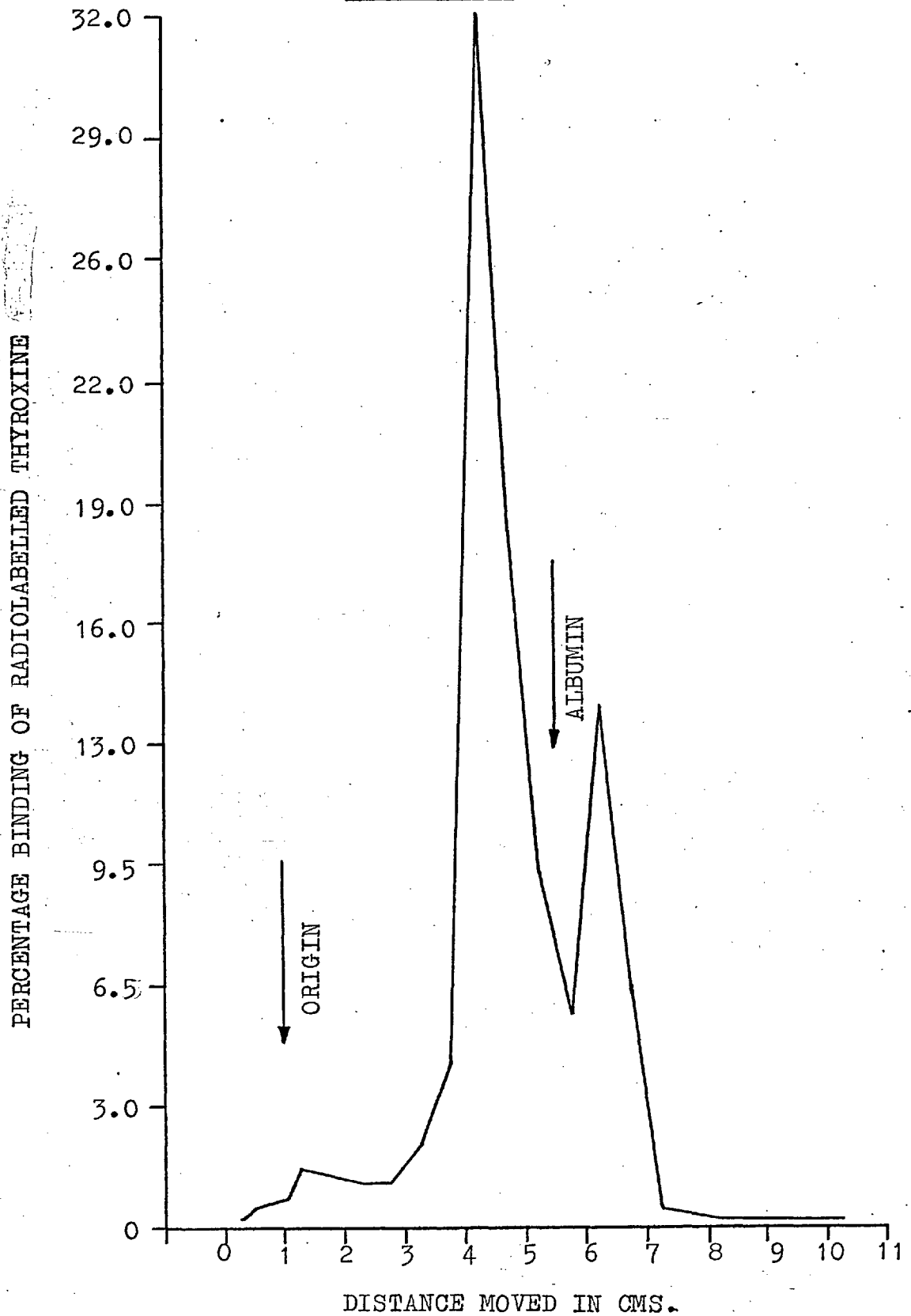


plate (Fig. 35). In contrast to this the plate d) which involved equilibrating hormone with serum before running through antiserum, the total loss of hormone was approximately 68%, but the distribution of the remainder was centred at the anodal edge of the plate far to the anodal side of the albumin precipitate (Fig. 36). This relative region anodal to albumin is in normal serum devoid of activity at the ranges of hormone used in this study. A time course experiment using the same procedure as d) was subsequently performed to determine the progressive migration of dissociated hormone. Ten identical standard Line plates were run, but were taken off at time intervals between 1 and 64 hours. (1, 2, 5, 12, 16, 20, 24, 26, 48 and 64 hours running time), cut up and counted (Table 8). Between 1 and 5 hours, two peaks became distinguishable, the first was a small fast running peak (1.5 cm/hr.) which ran off the plate after 5 hours and which was probably formed by free iodide (^{125}I). The second peak was much larger than the first and was still on the plate at 64 hours, close to the anodal edge. Near the normal running time of 15 hours, i.e. 12 and 16 hours, the major peak was found at the anodal end of the plate, and anodal to the albumin precipitin line. This main peak moved considerably slower (1 cm./4-5 hrs.) than the small iodide peak and also displayed reduced mobility as it moved toward the anode so that the final 3 cms of the plate took longer to traverse than the first 3 cms.

In comparison to these plates involving dissociation of hormone, a plate was run incorporating the "buffer" gel to maintain binding. This resulted in 100% recovery of activity from the plate and more than 90% of this radioactivity was in the centre of the plate cathodal to the albumin precipitin line, and at the position shown to be occupied by TBG (Section 4.2.5.2.).

FIGURE 35

DISTRIBUTION OF ^{125}I T_4 AFTER A 15 HOUR
ELECTROPHORETIC SEPARATION THROUGH ANTISERUM.

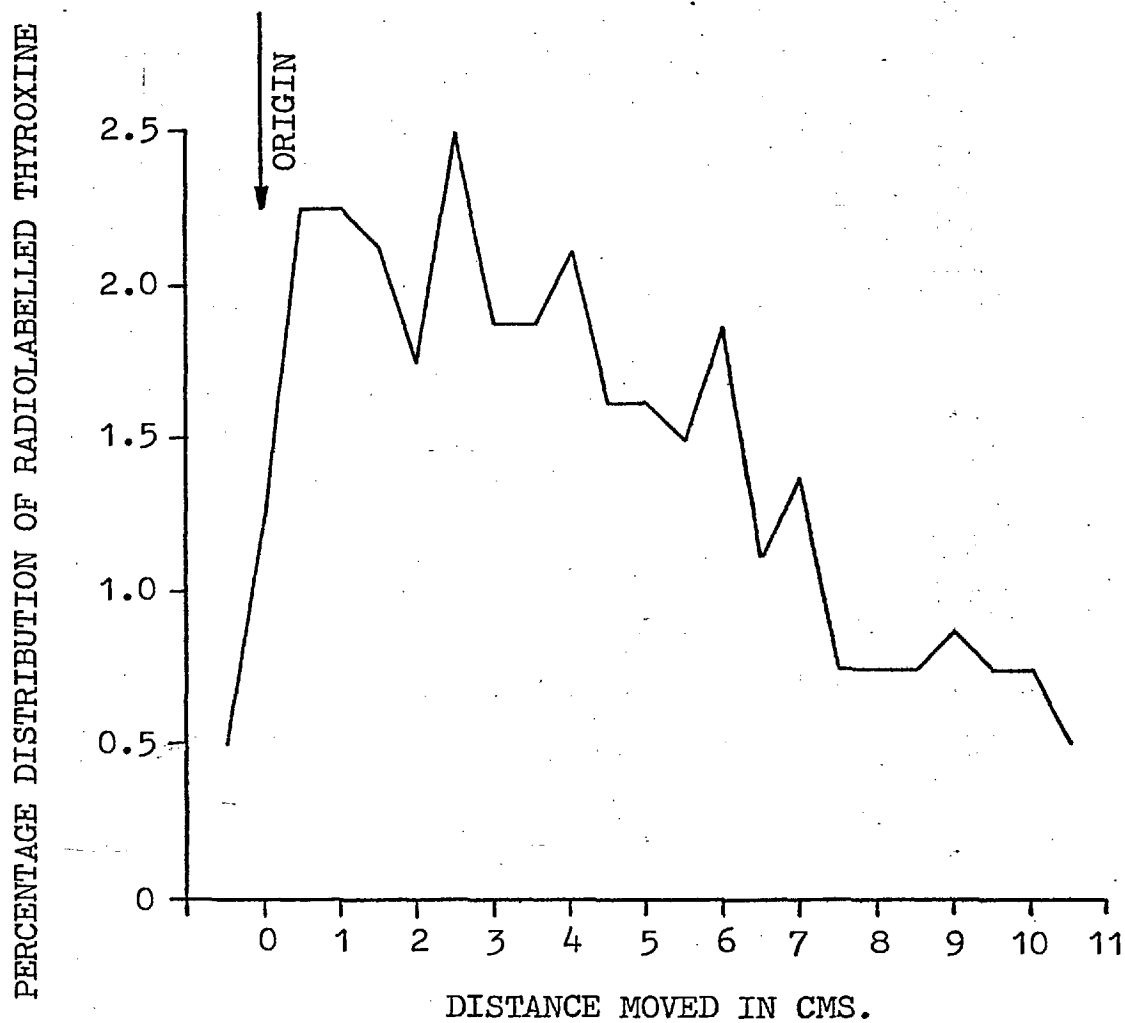


FIGURE 36

DISTRIBUTION OF RADIOACTIVITY ON A STANDARD LINE
PLATE AFTER A 15 HOUR IMMUNOELECTROPHORESIS.

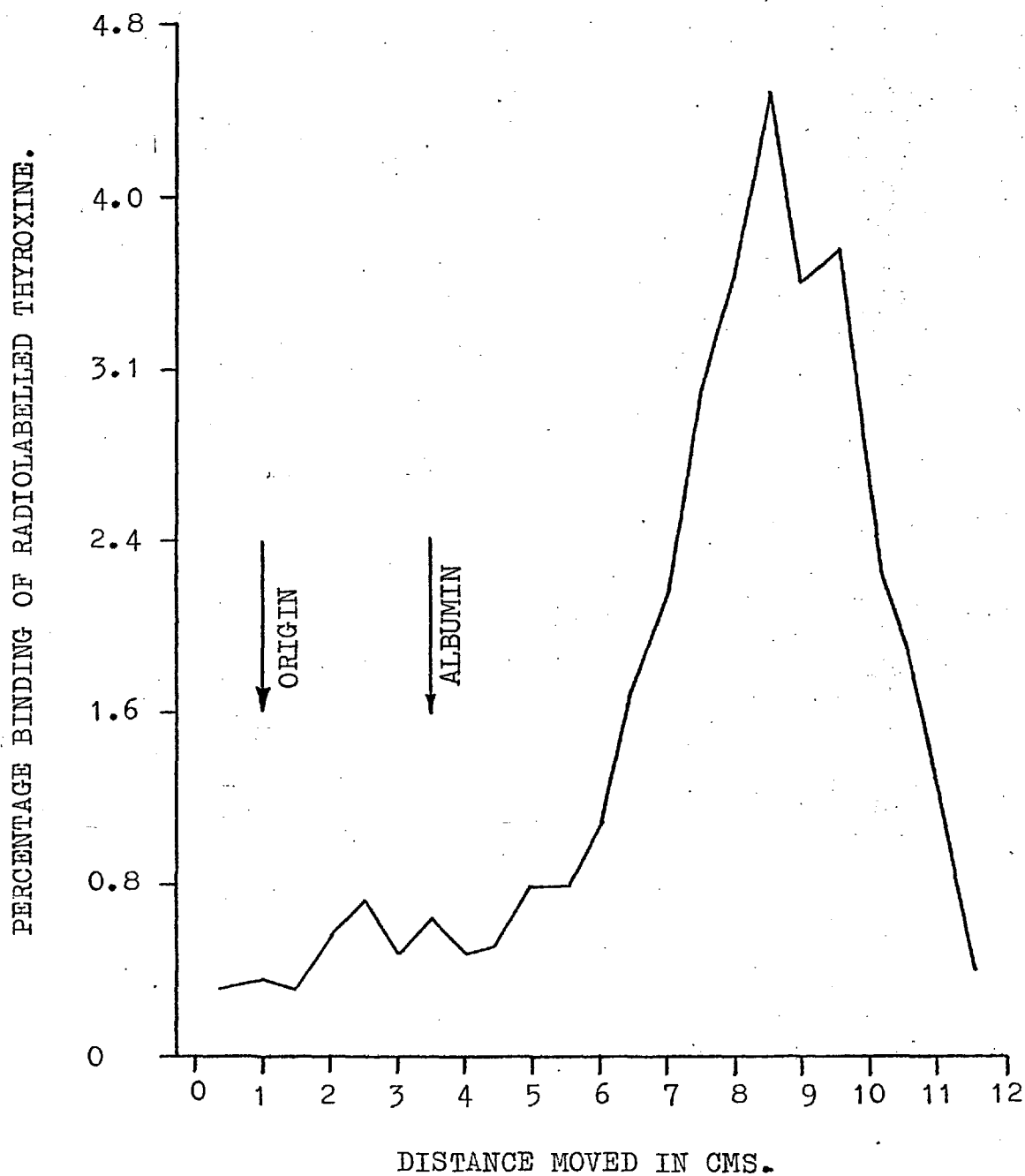


TABLE 8MOVEMENT OF RADIOACTIVITY ($T_{1/2}^{125}$) ONON STANDARD LINE PLATE

RUNNING TIME (Hr.)	DISTANCE RUN BY RADIOACTIVITY		RATE OF MOVEMENT (cm./hr.)	
	PEAK 1 (cms.)	PEAK 2 (cms.)	PEAK 1	PEAK 2
1	1.5	0	1.5	-
2	3.0	0.75	1.5	0.375
4.66	7.0	1.25	1.5	0.27
12.5	OFF	3.00	OFF	0.24
16		3.5		0.22
20		4.0		0.20
24.		5.0		0.21
25.5		5.5		0.22
48		8.75		0.18
64		OFF		OFF

4.2.5.2. IDENTIFICATION OF LINE PLATE

PROTEINS

The individual proteins on the Line plate were identified using composite Line/Laurell plates. Using the same sample, a first dimension Laurell separation was performed and placed on a second dimension glass plate. A line sample was then laid anodal to the first dimension Laurell gel and the antibody gel was poured over the remaining plate area anodal to the first dimension Laurell gel. The plate was then run on the modified Line system.

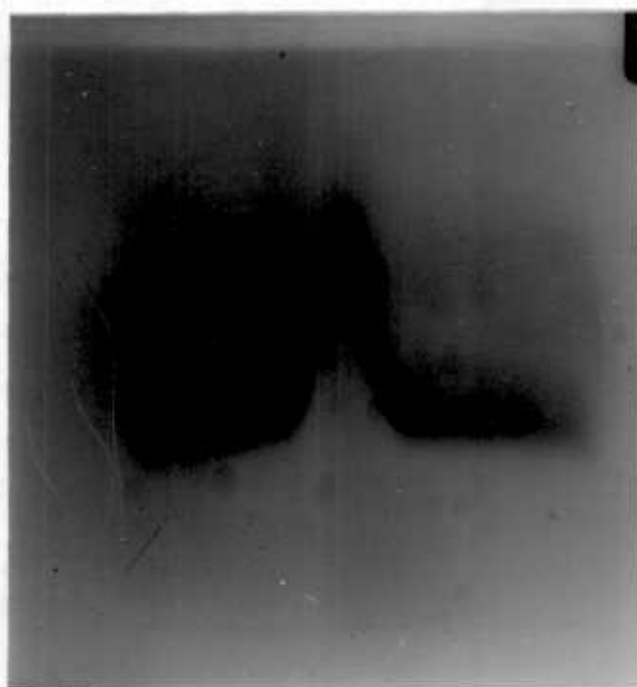
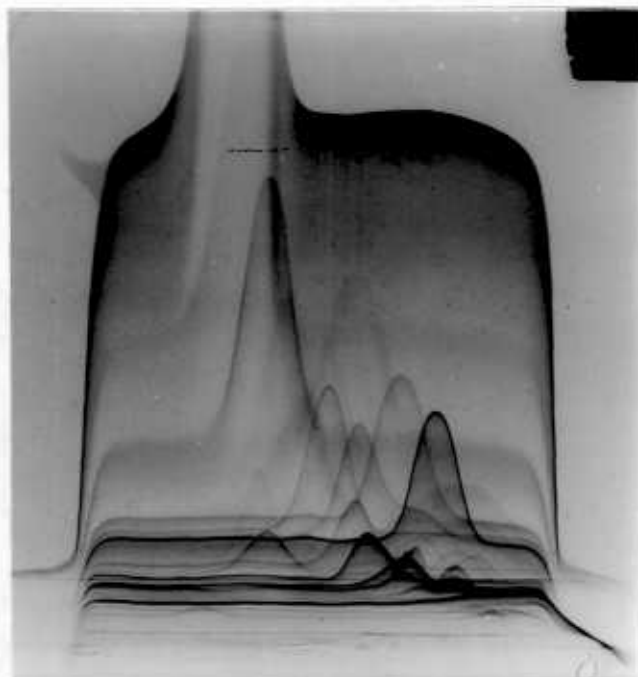
The autoradiograph of the electrophoresed plate showed antigen/antibody precipitin lines (Fig. 37) with the characteristic Laurell peaks sitting on their corresponding Line. Identification of the precipitin lines therefore followed from the associated known Laurell peaks.

The main thyroxine binding proteins showed sufficiently characteristic precipitation that with a little experience each protein could be identified from the Line plate alone without recourse to the composite Line/Laurell plate.

The Line/Laurell technique also confirmed protein binding of hormone during the Line method by indicating an association between the serum proteins and radioactivity.

FIGURE 37

PHOTOGRAPH OF A STAINED PLATE AND THE CORRESPONDING
AUTORADIOGRAPH OF A COMPOUND LINE/LAURELL PLATE



4.2.5.3. DISCUSSION OF LINE PLATE
 PRECIPITIN IDENTIFICATION

The results in this Section demonstrate the mobility and precipitation of radiolabelled thyroxine with and without the "buffer" gel. The results suggest that the hormone is "bound" by some other factor than normal serum transporting proteins, but what the factor is remains unclear. The agarose itself is possibly a non-specific binding agent, as filter paper has been shown to be, but the evidence does not support this. Reports from other studies tend to confirm that agarose does not bind thyroxine unless specifically induced to do so (Pensky & Marshall, 1969). Another and more interesting possibility is that an antibody is present in the antiwhole human serum against thyroxine. The presence of an antibody would fit the results showing thyroxine to migrate ever slower as it runs through the antibody. As more antibody attached to the hormone it would slow down until it stopped in the same way as the serum proteins precipitate.

4.2.6. REPRODUCIBILITY OF THE
 MODIFIED METHODS

Before using the modified methods for examination of test sera, their reproducibility and standardisation were examined.

Reproducibility of TBG concentration and the Standard Curves of TBG concentration against serum volume were investigated using the Modified Rocket Technique and different standard sera. Using the Modified Line Technique, reproducibility of both TBG concentration and percentage binding of radio-labelled hormone were examined.

4.2.6. REPRODUCIBILITY OF THE MODIFIED

METHODS

4.2.6.1. MODIFIED ROCKET TECHNIQUE

REPRODUCIBILITY

i) Reproducibility

Both the intra-assay and interassay reproducibility were studied so that the accuracy on an individual plate and between separate plates could be assessed.

Intra-assay Reproducibility

A 17 x 11 cm. twenty sample plate was produced with alternate samples of the Standard serum (TDP) and a normal serum. After electrophoresis against antiwhole human antiserum (B.W.), the plate was autoradiographed and the TBG heights were measured from the top of the sample hole to the tip of the TBG peak. The Standard serum TBG was expressed as the peak height in centimetres while the normal serum sample TBG was calculated as a percentage of the Standard serum TBG. The results in Table 9 show the mean, standard deviation and standard error of the mean of the samples used.

The intra-assay reproducibility was repeated on five other plates run at different times, but with only the Standard serum, and the results were consistent with those in Table 9 and are shown in Table 10.

Interassay Reproducibility

The interassay reproducibility was determined by running the Standard serum (TDP) and a normal serum on fifty plates. The plates were run on the Modified System and the TBG peaks were measured from the autoradiographs. The mean, standard deviation and standard error of the mean were calculated and are presented in Table 11.

ii) Standard Curves

A standard curve of TBG concentration against Rocket

TABLE 9

MODIFIED ROCKET TECHNIQUE : INTRA-ASSAY REPRODUCIBILITY

	<u>NUMBER OF</u> <u>OBSERVATIONS</u>	<u>MEAN</u>	<u>S.D.</u>	<u>COEFFICIENT</u> <u>OF VARIATION</u>	<u>S.E.M.</u>
Standard Serum	10	3.83 (cms.)	0.026 (cms.)	0.68	0.01
Normal Serum	10	121 %	0.8 %	0.66	0.25

Units of Standard Serum = Peak height in cms.

Units of Normal Serum = $\frac{\text{Peak height of Normal}}{\text{Peak height of Standard}} \times 100$

TABLE 10

MODIFIED ROCKET TECHNIQUE : INTRA-ASSAY REPRODUCIBILITY

USING FIVE SMALL PLATES

	<u>NUMBER OF</u> <u>OBSERVATIONS</u>	<u>MEAN</u> <u>(cms.)</u>	<u>S.D.</u> <u>(cms.)</u>	<u>COEFFICIENT</u> <u>OF VARIATION</u>	<u>S.E.M.</u>
Serum 1	11	3.97	0.079	1.99	0.024
Serum 2	11	3.92	0.060	1.53	0.018
Serum 3	11	4.11	0.119	2.89	0.036
Serum 4	11	4.21	0.050	1.19	0.015
Serum 5	11	4.15	0.027	0.65	0.008

TABLE 11

MODIFIED ROCKET TECHNIQUE : INTER-ASSAY REPRODUCIBILITY

	<u>NUMBER OF OBSERVATIONS</u>	<u>MEAN</u>	<u>S.D.</u>	<u>COEFFICIENT OF VARIATION</u>	<u>S.E.M.</u>
¹ Standard Serum	50	4.06 (cms.)	0.12 (cms.)	2.96	0.02
² Normal Serum	50	113.6 (%)	2.19 (%)	1.93	0.31

1 Units of Standard Serum = Peak height in cms.

2 Units of Normal Serum = $\frac{\text{Peak height of Normal}}{\text{Peak height of Standard}} \times 100$

height was necessary to quantitate TBG in a given test sample. However, due to the difficulty of isolating pure TBG from serum, a pure TBG standard was not available. The Standard serum (TDP) was therefore used in place of pure TBG to construct a standard curve. Seven aliquots of the radiolabelled standard serum between 0.6 μ l and 4.2 μ l were pipetted, in increasing volumes, into the sample holes punched in the "buffer" gel (Table 12). After immunoelectrophoresis and autoradiography the TBG rocket heights were measured and plotted against sample volume. The TBG standard curve constructed did not form a straight line, but the normal working region was acceptably linear (Fig. 38). Linearity of the whole curve was more closely approached when the log volume was plotted against peak heights (Fig. 39).

Standard curves of TBG rocket heights against serum volume were also constructed using a normal serum and serum from a young female (28 years) taking oestrogen (Minovlar) to extend the TBG standard curve above the normal range. All curves followed the same pattern, and became more linear when the log volume was plotted (Figs. 40 & 41).

Protein-Protein interactions Related to the Standard Curves

It has been postulated that TBG undergoes a protein-protein interaction with albumin, (Inada & Sterling, 1970) which could affect electrophoretic mobility. However, although the modified Rocket technique is relatively independent of mobility, this interaction was considered and investigated to eliminate the possibility of interference with the antigen/antibody interaction on which the modified Rocket method relies. To investigate this the standard (TDP) was progressively diluted with serum from an individual (N.F.) with congenitally very low TBG (Table 13) (see also Section

TABLE 12

TABLE OF ALIQUOTS OF A STANDARD SERUM

	T U B E N U M B E R						
	1	2	3	4	5	6	7
Volume of Standard Serum (TDP) μ l	0.6	1.2	1.8	2.4	3.0	3.6	4.2
Log Volume	1.78	0.08	0.26	0.38	0.48	0.56	0.62
TBG Rocket Height (Cms.)	2.2	3.5	4.3	4.8	5.4	5.7	5.9

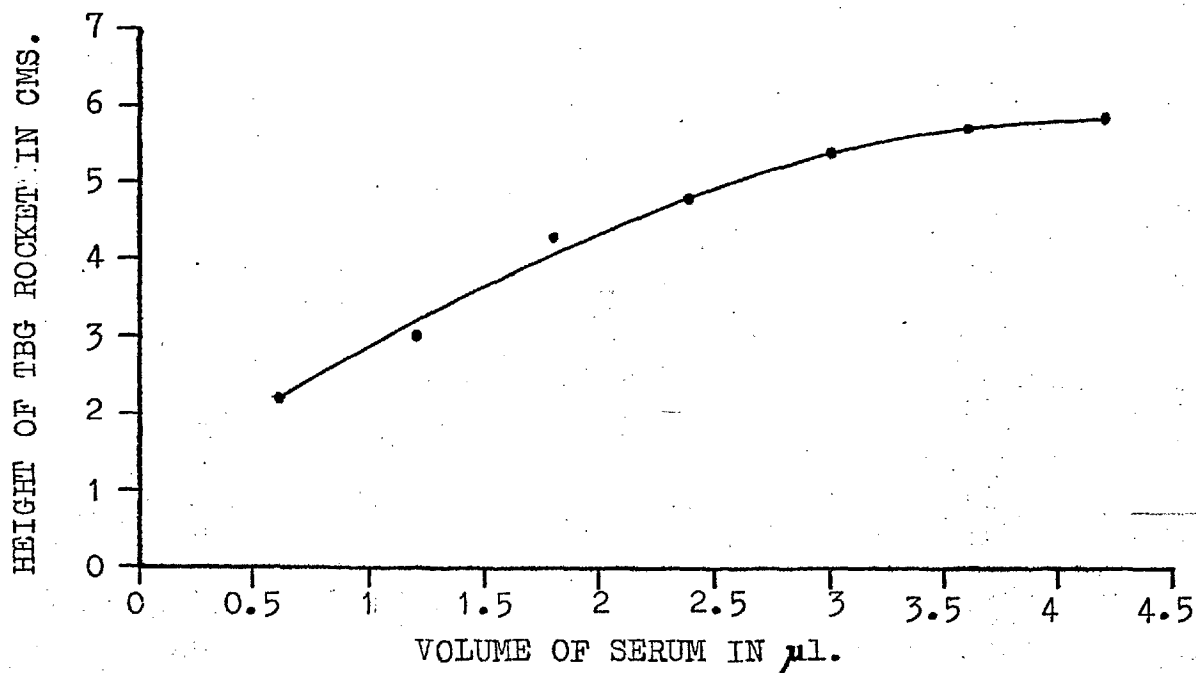
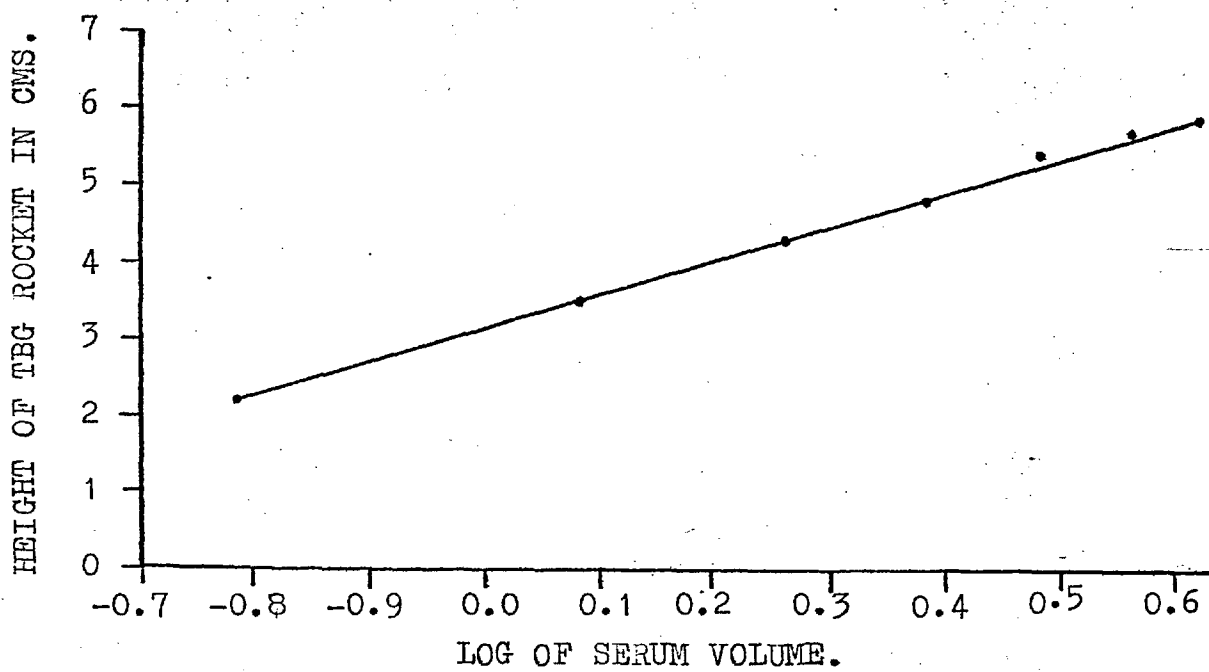
FIGURE 38TBG STANDARD CURVE USING TDP SERUM.FIGURE 39LOG TBG STANDARD CURVE USING TDP SERUM.

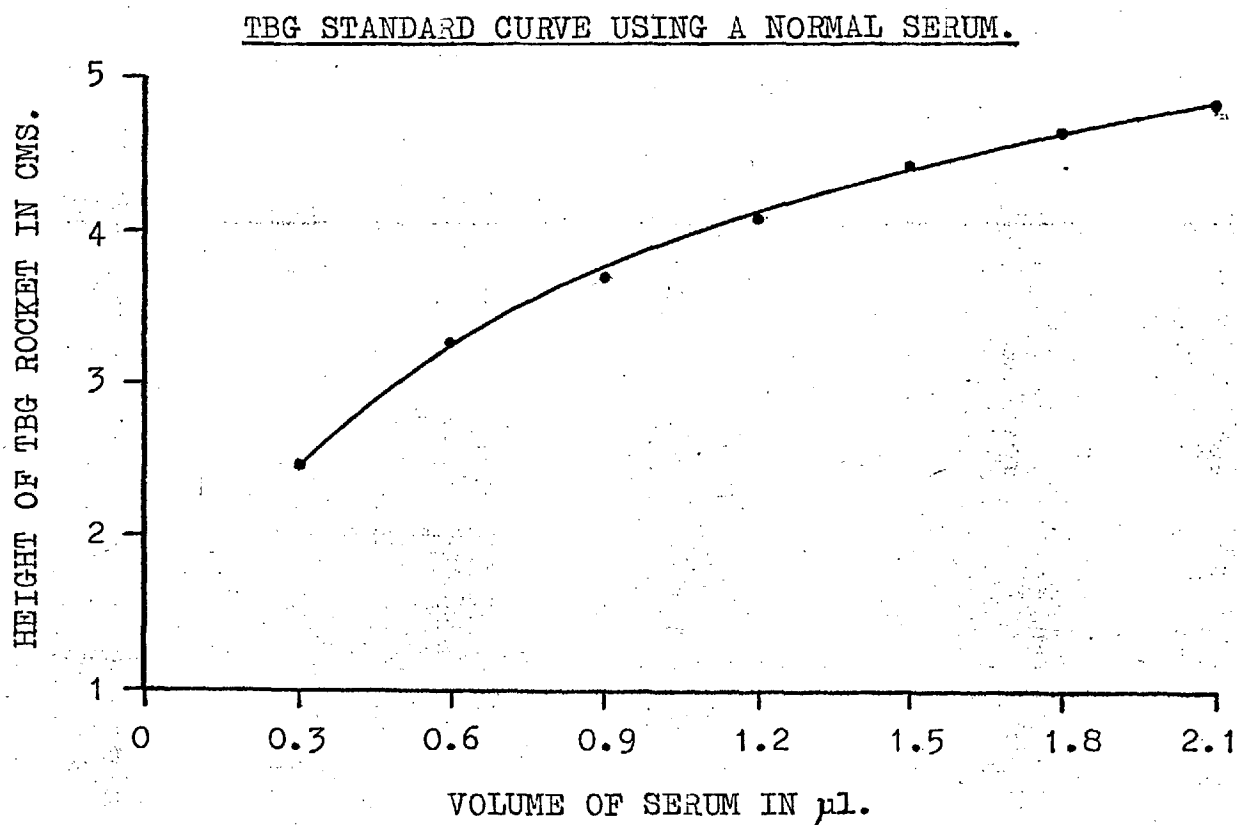
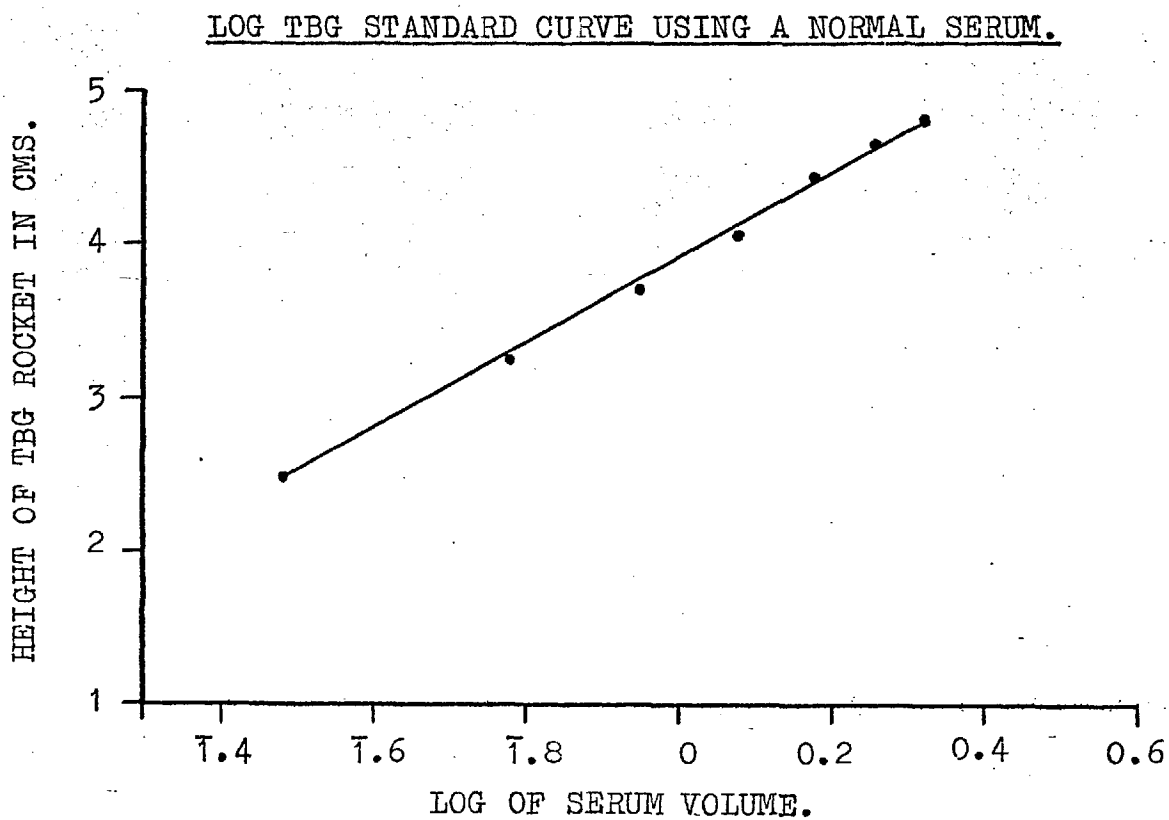
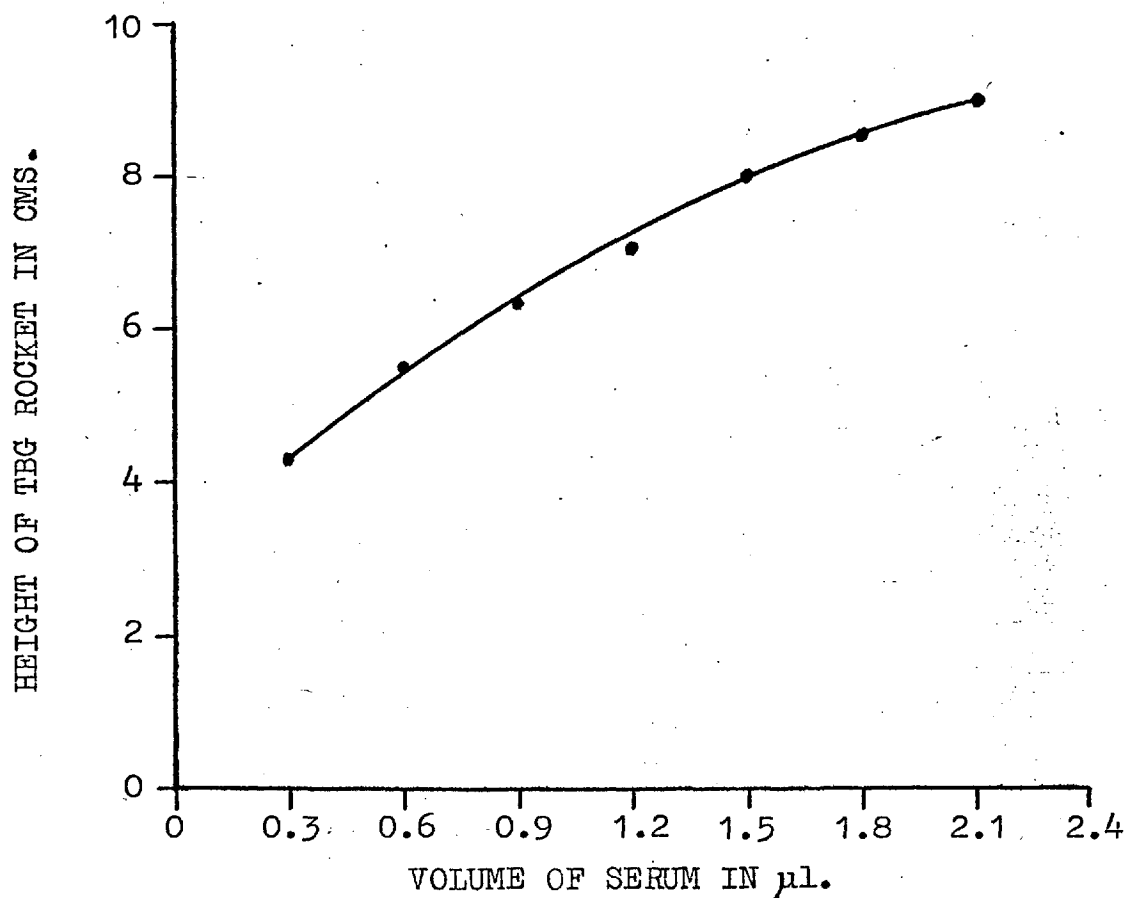
FIGURE 40aFIGURE 40b

FIGURE 41

(i) TBG STANDARD CURVE USING A SERUM CONTAINING
ELEVATED TBG CONCENTRATION.



(ii) LOG TBG STANDARD CURVE USING AN ELEVATED TBG CONCENTRATION.

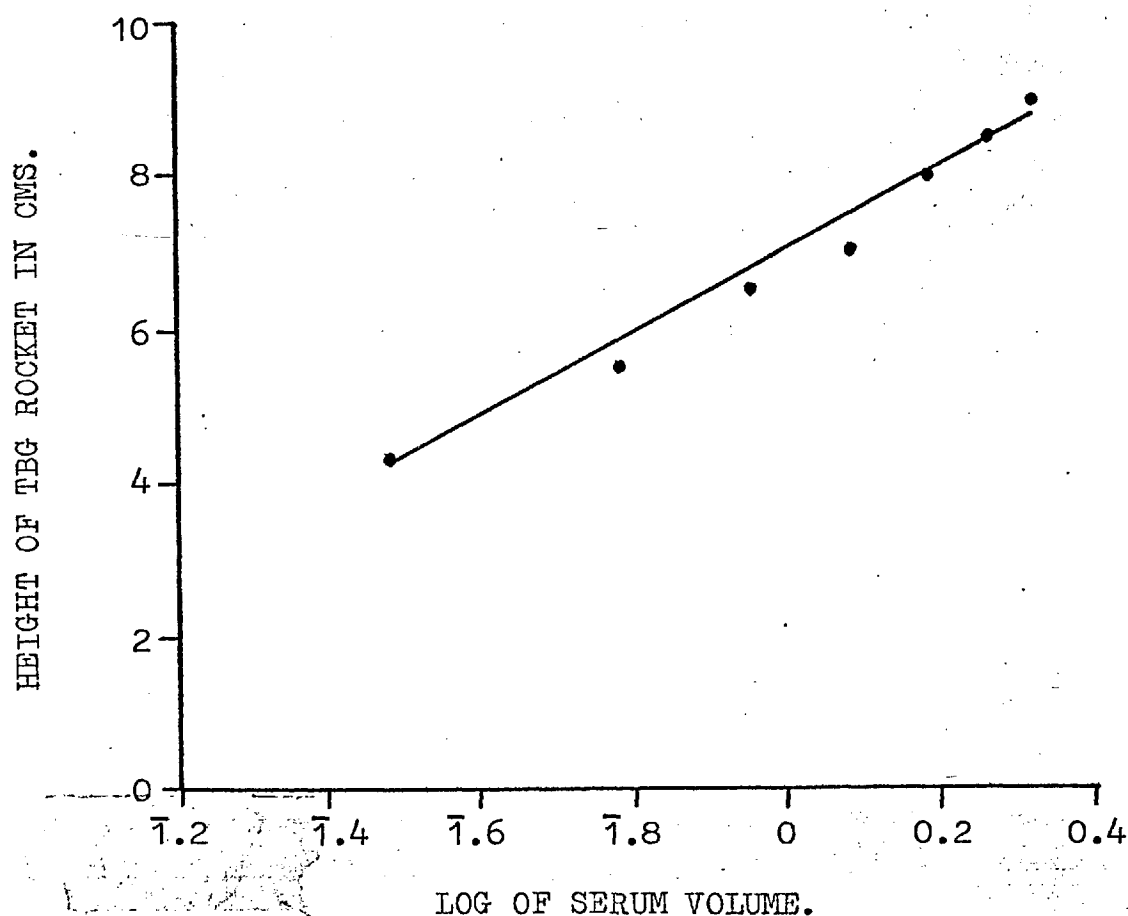


TABLE 13

TABLE OF DILUTION OF STANDARD SERUM WITH AN A-TBG SERUM

	T U B E N U M B E R							
	1	2	3	4	5	6	7	8
Volume of Standard Serum (TDP) μ l	0	10	20	30	40	50	50	70
Volume of Dilutant (N.F. serum or Saline)	35	30	25	20	15	10	5	0
Total Volume	35	40	45	50	55	60	65	70
Dilution Ratio	-	1 in 4	4 in 9	3 in 5	8 in 11	5 in 6	12 in 13	NEAT
Effective Volume of Standard Serum	0	1.05	1.9	2.5	3.05	3.5	3.9	4.2
Log Volume	-	0.02	0.28	0.40	0.48	0.54	0.59	0.62
TBG Rocket Height (Cms.)								
N.F. Serum	0	3.1	4.3	4.8	5.2	5.5	5.7	5.8
Saline	0	2.75	4.2	4.7	5.2	5.45	5.7	5.8

4.3.1.8.)). This dilution resulted in the constant concentration of all the serum proteins except TBG.

The TBG peaks on the autoradiograph increased from undetectable, in the congenitally low TBG serum, to normal level in the undiluted standard. These results were compared with those of a similar experiment using the standard serum (TDP) diluted in exactly the same way with saline (Figs. 42 & 43). The two resulting graphs of TBG rocket height against volume of standard serum are exactly parallel indicating that any protein-protein interactions that occurred did not affect this assay system.

The investigation of protein-protein interaction was repeated using the same normal serum and serum from a young female taking oestrogen. Each group of standard curves were found to be parallel.

FIGURE 42

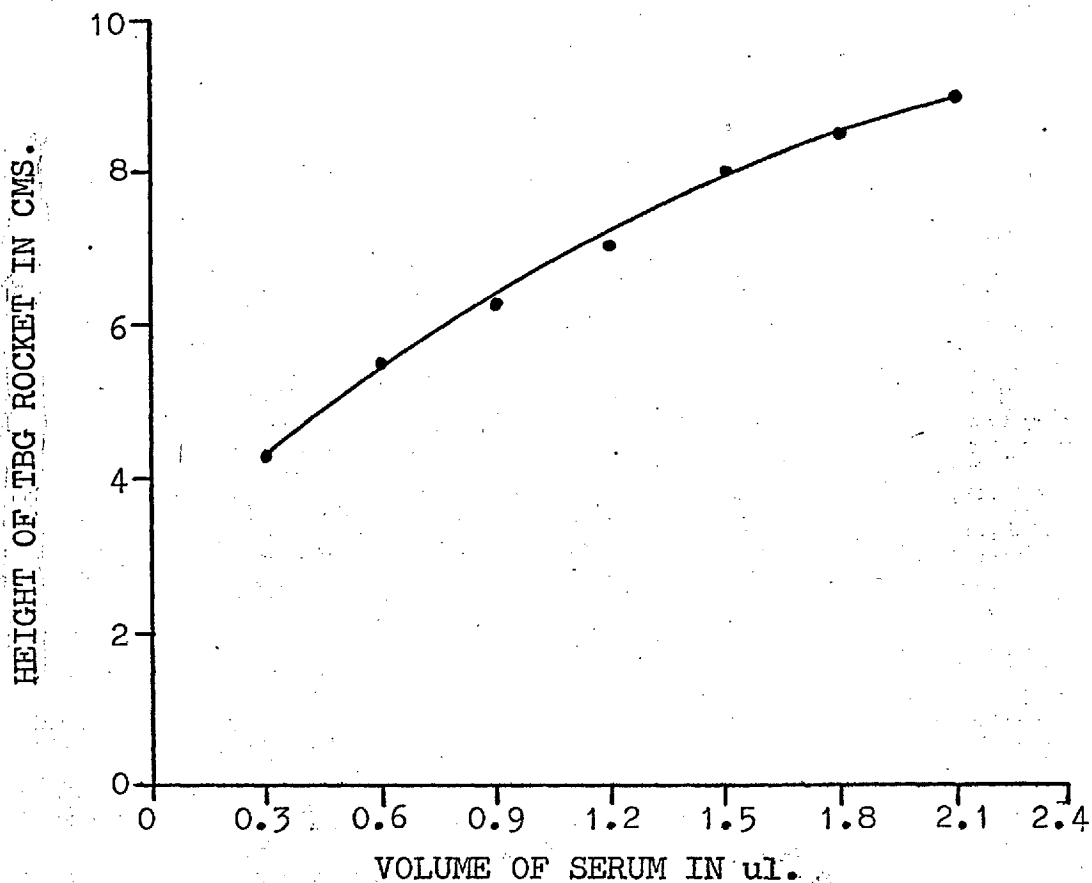
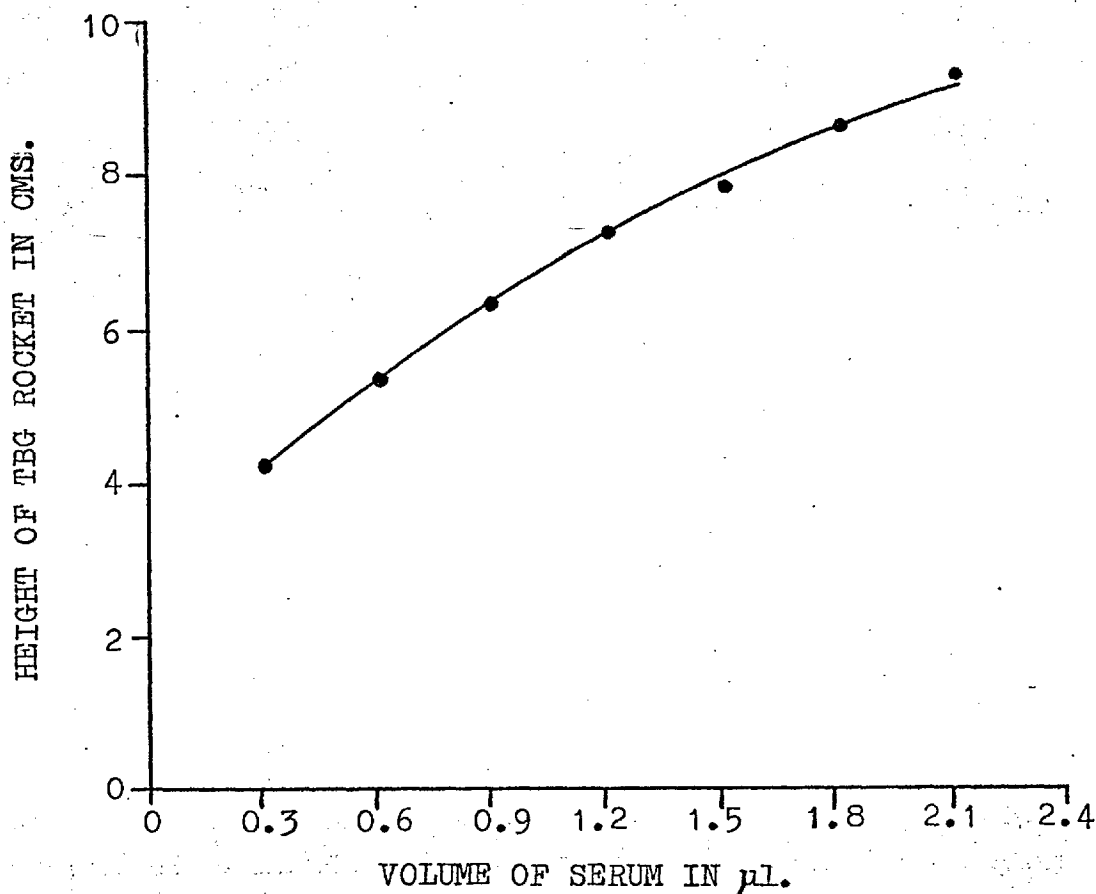
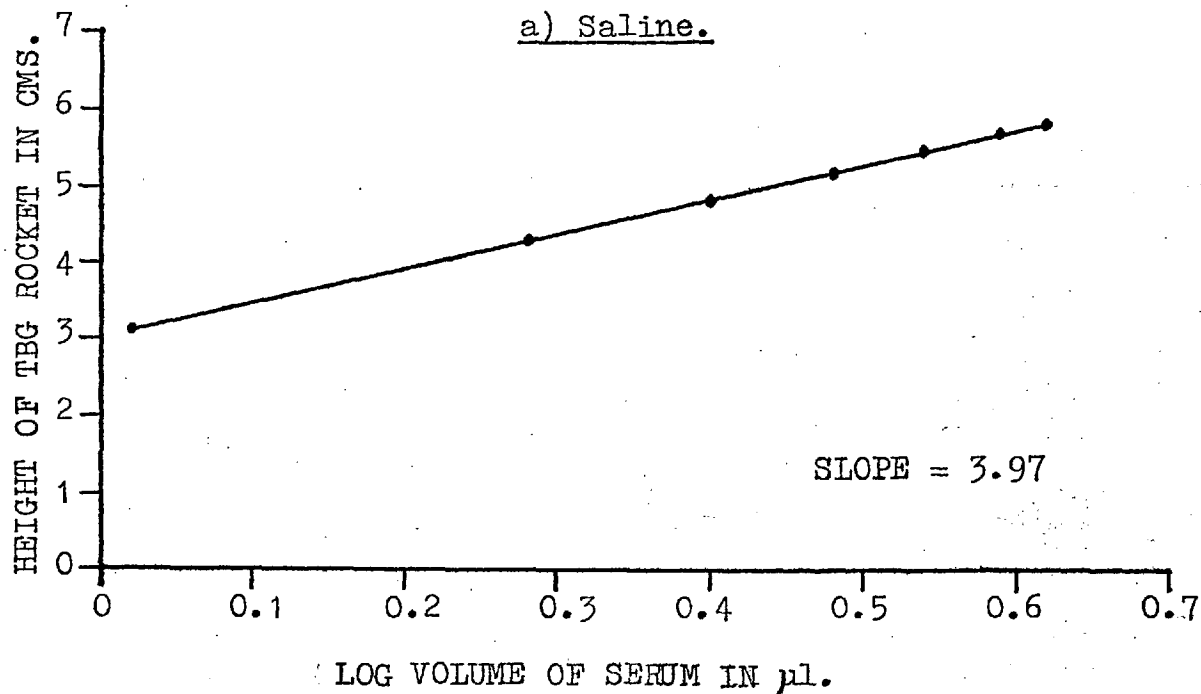
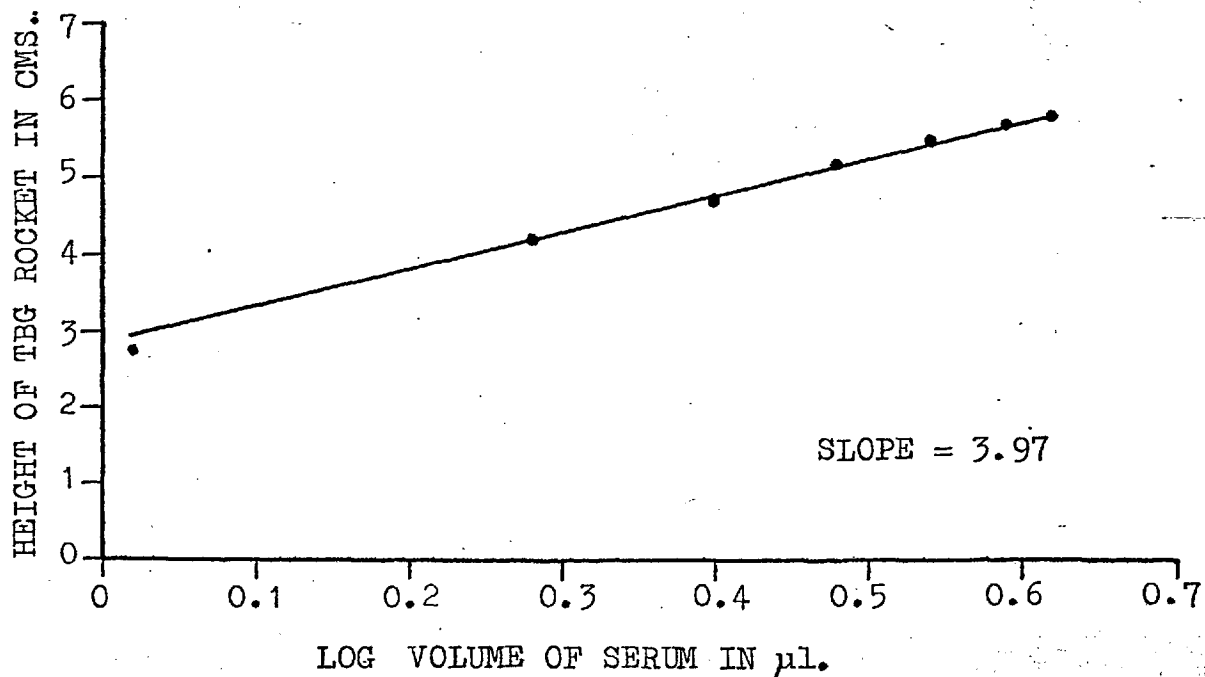
STANDARD CURVES OF TBG CONCENTRATION.a) Increasing Volume of Undiluted Serum.b) Serum Progressively Diluted with Saline.

FIGURE 43
STANDARD CURVES OF TBG CONCENTRATION USING TDP
DILUTED WITH:
a) Saline.



b) A Serum Containing Immeasurable
TBG Concentration.



4.2.6.2. MODIFIED LINE TECHNIQUE

REPRODUCIBILITY

i) TBG Estimation Reproducibility

Only interassay reproducibility is applicable using the Line Technique as only one sample may be applied to a plate. The interassay reproducibility was investigated using ten plates of the same serum sample with a 9 step range of added radiolabelled hormone between 0.2 and 20 times physiological level (Table 14). A "buffer" gel and constant concentration of antiserum were used on each plate. After electrophoresis the gel was cut up and counted and the distance moved by TBG was measured from the origins (Tables 14 & 15). The gel sections were cut 0.25 cm. wide so that if the peak was at 4.125 cm. the cutting accuracy would result in the peak being found at either 4.0 or 4.25 cms. Considering this inherent latitude of measurement the TBG was found to remain extremely constant provided the same serum sample and antibody concentration were used. Also, the Line values for TBG were found to closely correlate (Table 15) with the Rocket values, and to be independent of the amount of radiolabelled hormone equilibrated with the serum within the range of hormone concentrations used during these experiments.

ii) Reproducibility of the Percentage Binding of Thyroxine by TBG

The modified Line Technique was reserved for estimation of the percentage binding of thyroxine by TBG because of its advantage over the modified Rocket Technique in quantitating thyroxine binding.

The percentage binding of thyroxine by TBG changes according to the total hormone concentration in the serum,

TABLE 14

MODIFIED LINE TECHNIQUE REPRODUCIBILITY

SERUM	TOTAL T4 CONCENTRATION (nmol/l)	DISTANCE RUN BY TBG (cms.)	% BINDING BY TBG	ANTISERUM TYPE AMOUNT (ml.)
1	120	4.0	60	BW 1.5
		3.75	46	
		3.75	47	
		3.75	51	
		3.75	66	
	125	3.75	66	
		4.0	41	
		3.5	49	
		3.75	46	
		3.5	53	
	150	4.0	-	
		4.75	36	
		4.0	36	
	200	3.75	38	
		3.75	37	
		4.0	40	
		3.75	40	
		4.0	41	
	300	4.25	42	
		3.75	46	
		3.75	45	
		4.0	32	
		4.25	16	
	4,300	4.75	8	
2	120	3.75	63	BW 1.5
	150	4.0	57	
	200	4.0	58	
	300	4.0	48	
	600	4.0	36	
	600	4.25	30	
	800	4.25	21	
	1,100	4.25	23	
	1,600	4.0	20	
	2,100	4.25	14	
3	50	4.25	81.5	BW 1.5
	50	4.5	81	
	100	4.5	63	
	100	4.5	68	
	100	4.0	63	
	500	4.5	36.5	
	500	4.5	34.5	
	500	4.75	35.5	
4	20	4.75	84	BW 1.5
	100	4.75	61	
	500	4.75	46	

TABLE 15

MODIFIED LINE TECHNIQUE REPRODUCIBILITY
OF TBG ESTIMATION

SERUM	NUMBER	MEAN	S.D.	COEFFICIENT OF VARIATION	S.E.M.	TBG BY ROCKET
1	24	3.93	0.316	8.04	0.065	116%
2	10	4.08	0.168	4.12	0.053	120%
3	8	4.44	0.222	5.00	0.078	130%
4	3	4.75	0.0	0.0	0.00	151%

and presumably also with changing concentrations of TBG and other major thyroxine binding proteins. However, under experimental conditions when serum has been extracted, the protein concentrations are constant. The reproducibility of percentage binding by TBG was examined at several total hormone concentrations and estimations of binding at these levels were performed over a period of about six months. The results are shown in Table 16 and can be seen to be largely constant for a given total thyroxine concentration.

TABLE 16

MODIFIED LINE TECHNIQUE REPRODUCIBILITY
OF PERCENTAGE BINDING OF THYROXINE BY TBG

SERUM	TOTAL T ₄ nmol/l CONCENTRATION	NUMBER	MEAN	S.D.	COEFFICIENT OF VARIATION	S.E.M.
1	120	8	53.25	9.53	17.9	3.37
1	125	2	49.5	4.95	10.0	3.50
1	150	3	36.7	1.16	2.79	0.67
1	200	7	41.6	3.10	7.45	1.17
1	300	1	32	-	-	-
1	600	1	16	-	-	-
1	4,300	3	8	1.00	12.5	0.35
3	50	2	81.25	0.35	0.43	0.25
3	100	3	64.67	2.89	4.47	1.67
3	500	3	35.50	1.00	2.81	0.58

4.2.6.3. DISCUSSION OF MODIFIED METHODS

REPRODUCIBILITY

The most crucial test of a new quantitative technique is its reproducibility. Extensive experiments were therefore performed using the modified techniques with standard sera to investigate this factor.

Standard curves for TBG were produced using the modified Rocket Technique, because only this method was used for TBG quantitation whereas the modified Line Technique was reserved for quantitation of percentage binding of thyroxine.

i) Reproducibility and Standard curves for TBG estimation using the Modified Rocket Technique

a) Reproducibility

The Modified Rocket Technique reproducibility is shown in Tables 9 to 11. The actual values of the means are dependent on the units of measurement, that is in centimetres for the Standard Serum (TDP) and as a percentage of this serum for the normal serum (I.D.). The standard deviations of the two means are 0.026 cm. and 0.12 cm. for the Standard serum intra- and inter-assay results and 0.8% and 2.19% for the normal serum results. In both instances the intra-assay standard deviation is significantly less than the interassay standard deviation which is to be expected because the inherent errors are magnified when samples are run at different times and on different plates.

Laurell has suggested (Laurell 1966) that the optimum rocket height is between 2 and 5 cms. when the relative error is roughly constant. Using the modified techniques as described the peaks were usually in the range of 3.5 to 5 cms. except during pregnancy or oestrogen therapy when the peak could be as high as 7.0 cm. Examination of the standard

curves of TBG shows the practice of dilution of sera with high TBG concentration to be of little necessity as the relationship between TBG concentration and rocket height is very constant at high concentration and only falls significantly from linearity in the low concentration sera (Figs. 38, 39, 40, 41, 42 & 43).

The standard deviations of the interassay mean compares very favourably with both the Nielsen et al. (1972) and Kranz et al. (1974) studies which report means of 95 ± 3.7 (S.D.) Arbitrary Units and 96 ± 4 (S.D.) Units respectively, for autoradiographic determinations, compared with the present study of $113.6 \pm 2.19\%$ (S.D.). The percentage sign in the present study is analogous to the arbitrary units in both the other studies which also refer to standard sera. The Kranz et al. (1974) study reports some interesting reproducibilities which implies that the more samples run using the autoradiographic method, the better is the reproducibility, that is $\pm 8\%$ in five plates on one day and $\pm 4.4\%$ on nineteen different days, while using the staining technique the reproducibility declines from $\pm 3\%$ in five plates on one day to $\pm 4.9\%$ on nineteen different days. Thus the reproducibility values of the two techniques reverse over a longer period of study and autoradiography becomes progressively better. During routine examination of different sera the time period of study becomes extremely long and these results seem to indicate that if long studies are performed the autoradiographic method is preferred. This would tend to support the better reproducibility of the present study which collected reproducibility data for more than three months. The superior reproducibility of the current study over the Kranz et al. (1974) report could also be explained by the absolute heights of the rockets which were

almost twice as high in the present investigation compared with the other study. No information regarding the height of rockets was mentioned by Nielsen et al. (1972) who also showed inferior reproducibility to the present work.

b) Standard Curves

No pure preparation of human TBG was available during the time of study so a pool serum was chosen as a standard. This standard serum was part of a thousand donor pool (TDP), but since it was diluted with acid: citrate : Dextrose at collection, and normal sera were related to it, the normal sera in this study show TBG values slightly greater than 100%. All serum samples in this study are quoted as percentages of the standard, but a Reference serum (Nat. Institute for Biol. Standard Serum No. 68/449) was included to enable inter-laboratory comparison. This standard Reference serum (68/449) had a TBG concentration 1.21 (121%) times greater than the working standard (TDP).

The standard or calibration curves are shown in the Results (Figs. 38 to 43) and indicate that the relationship between rocket height of TBG and amount of TBG is close to linear. The plot of TBG rocket height against concentration or volume of serum is probably not precisely linear as the concentration of protein is proportional to the area under the peak not to the peak height. However, the error is extremely small once the peak height is more than about 2.5 cms. because the base dimension of the rocket being constant, relatively decreases as the peak height increases. The curves may be made more linear by the mathematical manipulation of plotting rocket height against the log volume of serum used (Figs. 39, 40b, 41b & 43).

A secondary result from the calibration curve investigation indicated that during these modified techniques, any protein-protein interaction that occurs in vivo, as suggested by Inada & Sterling (1970), does not affect the system. This would tend to be confirmed theoretically from the preceding discussion (Section 4.2.6.1.) when it was postulated that the techniques only worked because any protein-protein interactions that did occur in vivo were disrupted during the initial electrophoretic separation in the "buffer" gel.

Also resulting from the calibration curves of the mixed normal and a-TBG serum is the finding that in the supposed a-TBG serum there is in fact no protein immunologically recognised as TBG as opposed to a mutant form of the protein which simply cannot bind thyroxine (Section 4.3.1.8.).

ii) Reproducibility of the Modified Line Technique

a) Reproducibility of TBG Estimation

The interassay reproducibility of TBG estimation involving the modified Line Technique was investigated using four serum samples and the results have been shown in Tables 14 & 15. Although the study is relatively small compared with the Modified Rocket analysis, the reproducibility was found to be extremely good provided the serum sample and antibody type and concentration were constant. The amount of radiolabelled thyroxine added to the samples varied between 20 and 2000 nmol/L, but the position of the precipitated TBG was found to be independent of hormone concentrations.

This method, in common with the modified Rocket Technique, employs a unidimensional measurement for estimating the TBG position, but in this case the area under the line may be easily calculated as the sample may be made a standard width.

The results from modified Rocket and Line Techniques are

consistent, but the range between the highest and lowest concentration of TBG is far wider using the modified Rocket technique implying greater sensitivity.

b) Reproducibility of Percentage Binding of Thyroxine by TBG

The modified Line Technique was employed mainly for the estimation of thyroxine binding by TBG and data was collected from a number of sera to investigate the reproducibility. One serum sample was equilibrated with known levels of radiolabelled hormone and using constant conditions the percentage binding of hormone to TBG was estimated over a period of six months. Although the size of the study was small the time period between estimations was sufficiently large to allow valid conclusions.

The percentage binding of thyroxine to TBG using the serum (I.D.) is remarkably constant using 150 and 200 nmol/l of thyroxine giving TBG $37\% \pm 0.5\%$ and $42\% \pm 4\%$ respectively. Using a total thyroxine concentration of 120 nmol/l the percentage binding is far less constant with a mean of $53\% \pm 13\%$. When referring to the modified Line Results (Section 4.3.2.1.), it is found that the total hormone concentrations of 150 to 300 nmol/l result in a relatively constant percentage binding by TBG (Figs. 65 & 66), not only when using the I.D. serum, but using P.P. serum also. In contrast to these results, are those found for total thyroxine levels between 100 and 150 nmol/l when the percentage binding by TBG changes rapidly for very small differences in hormone content. Therefore, small pipetting errors when aliquots of radiolabelled hormone were being equilibrated with the serum sample could affect the total hormone content and therefore the amount of thyroxine bound to TBG. In this range of total hormone, i.e. 100-150 nmol/l, the percentage binding to TBG could theoreti-

cally be found in the range 37 - 76%. The actual range found during these experiments was 41 - 66%.

Reproducibility of duplicate experiments was always very close throughout the entire study.

4.2.7. QUANTITATIVE ESTIMATION OF HORMONE

BINDING TO TBG

The Modified Line Technique was designed to quantitate the percentage recovery of hormone after electrophoresis and the percentage binding of hormone to TBG.

To demonstrate quantitative binding, the percentage recovery of radiolabelled hormone was investigated on sixteen plates using five sera with seven exogenous concentrations of radiolabelled thyroxine between 20 and 4200 nmol/l. Two different antisera were used, Burroughs Wellcome and Meath, to observe any differences of percentage recovery. All plates were run in the phosphate pH 7.4 buffer. Eight sets of two plates were run, with the same sample on each pair of plates. One set was run using the modified Line Technique including the "buffer" gel whilst the second set was run using the standard form of the Line Technique. Each pair of plates was treated identically. The percentage recovery of hormone was calculated and individual plates from each set were compared.

On each plate using the modified Line Technique the percentage recovery of radiolabelled hormone was 100% irrespective of the amount of exogenous hormone added (Table 17).

On the plates using the standard form of the technique, however, only one plate resulted in 100% recovery and this was on the plate with the lowest exogenous addition of hormone, i.e. 20 nmol/l. On each of the remaining plates where the standard method was used, the percentage recovery of hormone was far below 100% and was found to be between 85% and 12% (Table 17). There appeared to be no relationship between hormone concentration and percentage recovery on the standard plates.

Distribution of hormone was investigated and is discussed in Section 4.3.2.1.

TABLE 17

COMPARISON OF THE PERCENTAGE RECOVERY OF
RADIOLABELLED HORMONE USING THE MODIFIED
AND STANDARD LINE TECHNIQUES AND A RANGE
OF HORMONE LEVELS.

<u>SERUM</u>	<u>ANTISERUM</u>	<u>EXOGENOUS</u> <u>RADIOLABELLED</u> <u>$^{125}\text{I T}_4$</u> <u>(nmol/l)</u>	<u>PERCENTAGE</u> <u>RECOVERY</u>	
			<u>MODIFIED</u>	<u>STANDARD</u>
I.D. 1671	¹ B.W.	20	100	100
I.D. 1786	B.W.	25	100	85
J.M. 1327	² M.	40	100	20
W.B. 1333	M.	40	100	15
J.M. 1576	B.W.	60	100	12
M.G. 1389	M.	70	100	57
R.V. 1571	B.W.	1100	100	75
I.D. 1553	B.W.	4200	100	48

1 Burroughs Wellcome Antiwhole Human Antiserum.

2 Meath (Paines and Byrne) Antiwhole Human Antiserum.

4.2.7.1.

DISCUSSION OF HORMONEBINDING TO TBG

To be able to draw reliable conclusions from hormone binding studies, 100% of the initially added radiolabelled hormone must be accounted for on the electrophoresis plate. To investigate this percentage recovery, the standard and modified Line Techniques were studied.

On each of the modified method plates, 100% recovery was achieved, but using the standard method, only one plate resulted in complete recovery, whilst the remaining seven plates lost between 15% and 88% of the hormone.

The hormone remaining on the standard plates was non-uniformly distributed, and whether it was 100% or 12% remaining, it was always located at the anodal end of the plate and not associated with any known binding protein (Section 4.2.5.).

These results confirm the validity of using the Modified Line method in quantitative hormone binding analyses.

4.2.8. CONCLUSION TO METHODOLOGICALSECTION

New highly reproducible techniques have been developed to perform specific quantitative investigations. These techniques allow direct estimation of serum TBG concentrations and of the percentage binding of thyroxine by this major thyroxine carrier.

The two methods of Modified Rocket and Modified Line have been used in the subsequent section of Quantitative Results to investigate TBG concentration in different sera and to determine the relationship between total thyroxine concentration and percentage binding of thyroxine by TBG. The methods used in the Quantitative Results Section are those described in Section 4.2.3.4. with the modification of omission of the washing procedure to maintain maximum radiolabelled hormone recovery.

4.3.

QUANTITATIVE RESULTS

The Quantitative Results Section is divided into two distinct parts:-

- i) Direct estimation of TBG concentration in serum using the Modified Rocket Technique,
- ii) Direct estimation of thyroid hormone binding by TBG using the Modified Line Technique.

4.3.1. DIRECT ESTIMATION OF TBG INVARIOUS INDIVIDUALS

The results of TBG estimation in this Section were determined using the Modified Rocket Technique described in Section 4.2.3.4. All values are expressed as a percentage of the standard serum (TDP).

To enable expression of the TBG results in terms of absolute values (i.e. mg/l) the two standard sera used in this study i.e. TDP and I.D. were kindly analysed by Dr. D.B. Ramsden. The results were:-

TDP 6.92 mg/l $\pm$ 0.33 S.D. n = 47

I.D. 9.6 mg/l $\pm$ 0.3 S.D. n = 60

whilst the Modified Rocket values in this study were:-

TDP 100%

I.D. 116%

By extrapolating the graph formed by these two values the normal range of TBG from this study was 7.8 - 15.6 mg/l (105 - 149%) for males and 8.5 - 16.3 mg/l (109 - 153%) for females with means of 12.1 mg/l (130.2%) for males and 12.7 mg/l (133.4%) for females.

4.3.1.1.

NORMAL RANGE OF TBGCONCENTRATION IN SERUM

i) Three groups of normal healthy volunteers were selected and 2-5 mls. of blood were collected by venepuncture from each individual. Those individuals who were pregnant or taking oestrogen or other drugs were not included in these groups of subjects.

The three groups were:-

- I. Volunteers from the Clinical Investigation Laboratory at Northwick Park Hospital (N.P.H.).
- II. Employees of a local factory who were taking part in an epidemiological investigation (samples kindly supplied by Dr. T.W. Meade).
- III. Elderly people from the N.P.H. area who were being screened for occult disease (samples kindly supplied by Dr. M.J. Denham).

The samples were allowed to clot and the serum was separated and then stored at -20°C until assayed. All serum samples were treated in an identical manner and run in the modified phosphate pH 7.4 system together with two working standards and one internal standard as control. The TBG peaks were measured on the final autoradiographs using a cm./mm. clear plastic ruler. The results of the subject groups are presented (Table 18) subdivided according to sex and age (Figs. 44 & 45).

The tables show that plasma TBG increased significantly ($P < 0.0005$) in men between the third and fourth decades, but not significantly thereafter. A similar trend was evident in women ($P < 0.0005$). On pooling the male and female age-group results there was a significant difference ($P < 0.0025$) between the two groups with the values in women being higher.

TABLE 18

SERUM TBG CONCENTRATION OF EUTHYROID
MALES AND FEMALES AS GROUPS AND AS TEN

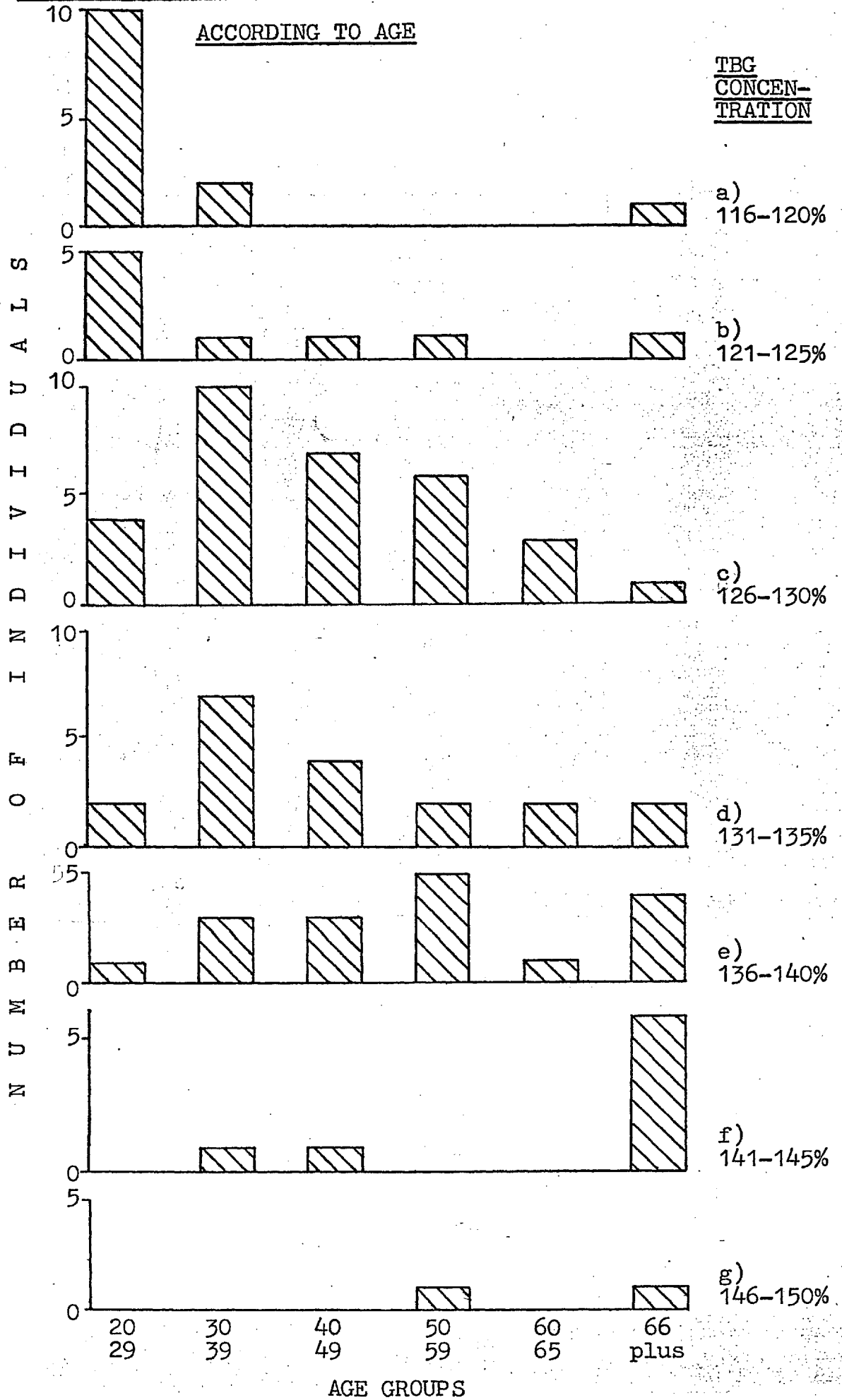
YEAR AGE GROUPS

AGE GROUP	MALE			FEMALE		
	NUMBER	MEAN	S.D.	NUMBER	MEAN	S.D.
20-29	22	123	6.7	11	128	5.3
30-39	24	131	6.2	8	130	6.8
40-49	16	132	5.3	14	134	5.7
50-59	15	133	5.9	9	136	4.1
60-65	6	131	3.7	-	-	-
66 yrs. plus	17	135	11.1	13	138	11.7
TOTAL	100	130	8.1	55	133	8.0

Units = Percentage TBG Concentration of Working Standard Serum.

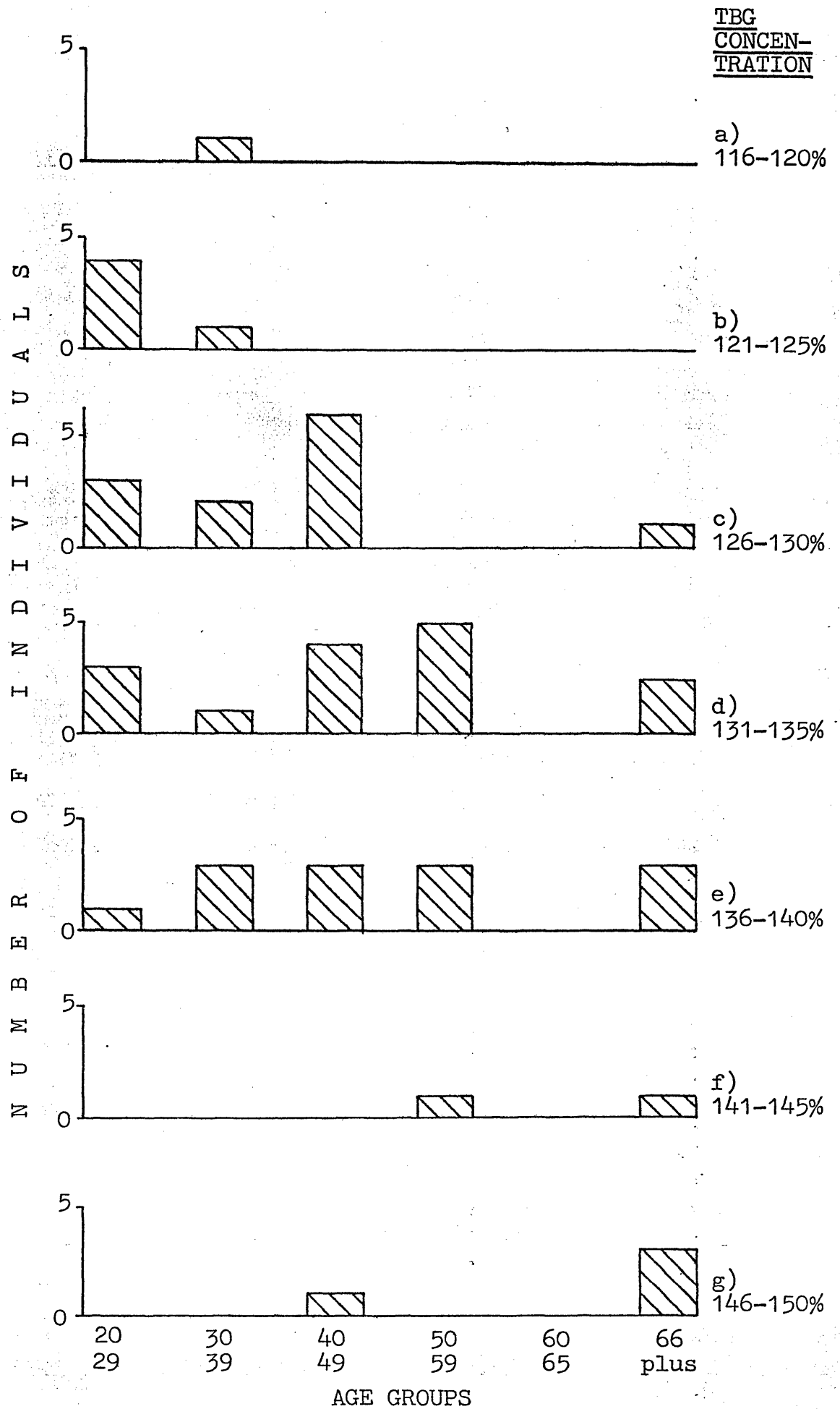
FIGURE 44

DISTRIBUTION OF TBG CONCENTRATIONS IN MALES



DISTRIBUTION OF TBG CONCENTRATIONS IN FEMALES

ACCORDING TO AGE



ii) Blood samples were collected from hospital in-patients with normal thyroid functions and the separated sera were treated in an exactly similar manner to the normal samples. The results of this group of patients are shown in Table 19 and are compared with normals of both sexes.

There was no significant difference between the TBG concentration in normal male subjects and male patients and the standard deviations were similar. In the group of female patients the TBG concentration was found to be greater than the normal female subjects, though with no significance, but the standard deviation of the hospitalized group was wider.

TABLE 19

SERUM TBG CONCENTRATIONS OF
HOSPITALISED EUTHYROIDS

AGE GROUP	MALE			FEMALE		
	NUMBER	MEAN	S.D.	NUMBER	MEAN	S.D.
20-29	-	-	-	3	132	6.0
30-39	2	129	9.2	6	139	21.0
40-49	-	-	-	9	141	9.3
50-59	5	128	3.4	17	134	9.4
60-69	5	134	7.0	4	125	11.5
66 yrs. plus	9	131	8.6	10	128	8.2
TOTAL	21	131	7.2	49	134	11.8
'Normal' } Total }	100	130	8.1	55	133	8.0

Units = Percentage TBG Concentration of Working
Standard Serum.

4.3.1.1.1. DISCUSSION OF NORMAL RANGE OF TBG CONCENTRATION

a) In Healthy Volunteers

The normal TBG range for males and females as groups and for 10 year age categories were calculated and are shown in Table 18. The male group TBG range is slightly wider, and the mean lower, than the female values which is in accord with results from other laboratories using direct methods of estimation. In contrast to the present work which demonstrated a significant difference between TBG levels in men and women ($P < 0.0025$), these studies (Bradwell et al. 1976; Levy et al. 1971; Chopra et al., 1972) reported no significant difference. Each of these earlier studies included far fewer individuals in the two groups. One laboratory compared 45 males with only 6 females (Levy et al., 1971). Another study while using more evenly matched groups (20 males; 39 females) also found no significant difference (Chopra et al., 1972). The third group, while not stating a significant difference, provided results with a 10% difference between the means (Bradwell et al., 1976). However, using capacity measurements, Harvey (1971) and Braverman, Foster & Ingbar (1967), do confirm a significantly higher female TBG concentration over males ($P < 0.05$ & $P < 0.01$ respectively).

The ten year age categories show an interesting distribution of TBG values. In the male category the 20-29 year age group has 41% of the individuals in the TBG concentration group 116% and 120%, whilst the subsequent TBG groups contained decreasing numbers of this age group (Fig. 44). The lowest, 110% - 115%, TBG group contained

only two individuals, both in the 20-29 year age bracket. The distribution of TBG values in the 20-29 year group therefore shows a marked deflection towards the lower end of the TBG range and is significantly ($P < 0.0005$) different from the spread of TBG values in the other four age groups where most individuals of a given age group lie in either the 126% - 130% or 136% - 140% groups (Fig. 44). This biphasic TBG pattern with age has also been demonstrated by Hesch, Gatz, Jüppner & Stubbe (1977) and by Braverman, Dawber & Ingbar (1966). Both Hesch et al. (1977) and the present study found the lowest TBG concentration in the 19-29 year age range, but Braverman et al. (1966), who only studied individuals in the first, fourth and seventh/eighth decades, found the lowest concentration in the 31 - 40 year group.

The distribution of TBG values in the different age groups has two important implications. Firstly, a mixed sample of euthyroid individuals will statistically show a bias of TBG values which follow the bias in age groups.

Secondly, a 20-29 year old subjects' TBG should be examined within the limits of the normal range for this age group (Fig. 44) and not simply with the normal range and mean.

The reasons for the change of TBG levels in the different age groups are probably many and involved, but one important factor is almost certainly the androgen/oestrogen fluctuation with age. In males the androgen content of urine, which reflects the serum concentrations, increases from less than 1 mg./24 hour in the newborn to a mean, peak concentration of about 15 mg./24 hour at about 25 years. The 17-ketosteroids are measured in the urine as they are the

AP principal metabolites of biological androgens. After the peak at about the age of 25 years, the 17-ketosteroid excretion falls slowly with increasing age to become almost asymptotic after 80-85 years at a mean concentration of about 3.5 mg./24 hour (Ferrazzini, 1956). These results are supported by Nathanson, Towne & Aub (1941), who recorded total urinary oestrogens in children and adolescents. The urinary oestrogens of males were found to increase very slightly from birth to puberty (14 - 15 years), but to remain fairly constant thereafter.

The relevance of these changes lies in the finding that androgens decrease TBG serum concentration whilst oestrogens cause an increase in their concentration (Ingbar, & Woeber, 1974). The relative changes in these two hormones could therefore influence the liver to increase or decrease the production and/or clearance of TBG thereby influencing circulating concentration.

In the female the system is more complex since the much higher mean oestrogen concentrations exhibit marked changes during life, and the androgen concentrations, though only two thirds the male level, follow a less regular pattern. Superimposed on these basic changes is the menstrual cycle when the concentration of circulating oestrogen is changing according to the time through the 28 day cycle (Speroff & Van de Wiele, 1971).

The urinary excretion of 17-ketosteroids follows the same pattern as in males up to about 25 years though the mean maximum is only two thirds that of males, i.e. about 10 mg./24 hours. The concentration then falls to about half the maximum by 35 years when it remains constant until 55 years, when the fall continues and eventually becomes

asymptotic at about 75-80 years at one fifth of its mean maximum concentration, i.e. about 2 mg./24 hours (Ferrazzini, 1956).

The urinary oestrogen concentration also follows the male trend but only until 10-11 years when it starts to substantially rise to mature levels at about 16-18 years. The onset of oestrogen rise corresponds with the time of menarche. The adult oestrogen concentration is maintained with 28 day period fluctuations until menopause at about 47 years when it abruptly falls (Kirk, 1951).

These hormonal changes reflect in the TBG content of women's serum so that the concentration of this protein follows a general age related pattern. In the youngest age group, 20-29 years, the TBG concentration is relatively evenly distributed between 121% and 135% as would be expected from the oestrogen/androgen ratio. As age increases, however, the hormone balance is displaced towards an oestrogen bias and the TBG distribution between 30 and 49 years is in the higher TBG category of 126% - 135%. The number of individual sera in the 126% - 130% and 131% - 135% TBG groups is slightly different however, and the older age group (40-49 years) has its peak number of sera in the lower TBG group whilst the reverse is true in the younger age group (30-39 years) and this difference, though small may be a reflection of the hormonal changes discussed above. In the higher age group (50-66+ years) after menopause and the secondary fall in androgens, the TBG concentration significantly increases to contain the greatest number of subjects in the 131% - 135% and 141% - 150% TBG groups (Fig. 45). Again these changes perhaps reflect the hormonal

changes and the shift of balance in the oestrogen/androgen relationship.

Previous investigations of TBG concentration in serum have usually ignored the age factor, but if the sample size is sufficiently large and of even distribution with respect to age, then the study would be valid. However, with a balanced sample the distribution of male TBG concentration is not Gaussian and the curve is displaced toward the higher values (Fig. 44). Similarly, with TBG in female serum the mean is displaced toward the higher values because of the high value "tail" with increasing age (Fig. 45).

The more recent direct assays have reported various results. Kranz et al. (1974) have shown a mean TBG value related to a standard serum of $89 \text{ A.U.} \pm 14 \text{ S.D.}$, but there was no mention of age or of sex differentiation. Nielsen et al. (1972) who also used the Laurell Rocket technique have given no values for a normal range. Bradwell et al. (1976) obtained a pure TBG sample and recorded its concentration in mg/l , but their results show the rather wide range of $6.3 - 16.1 \text{ mg/l}$ for males (mean = $11 \text{ mg/l} \pm 2.8 \text{ S.D.}$; $n = 24$) whilst their female range is $6.7 - 15.0 \text{ mg/l}$ (mean = $12.1 \text{ mg/l} \pm 2.3 \text{ S.D.}$; $n = 20$). The study makes no reference to age distribution in the same sex, but even with this, the range is wider than those from other studies, including the present study (Chopra et al., 1972; Levy et al., 1971; Drysdale et al. 1975). However, assuming the age ranges in the male and female groups to be similar, the female mean value is slightly higher than the male mean which is in accord with the present study.

b) In hospitalized Euthyroid Patients

All the studies involving direct measurement of TBG that have quoted normal ranges for TBG concentration have all used normal, healthy volunteers (Levy et al., 1971; Chopra et al., 1972; Castro & Urgarte, 1974; Kranz et al., 1974; Bradwell et al., 1976), but when investigating TBG concentrations, quite frequently the individuals are hospitalized and not healthy. A second set of "normals" was therefore selected which included a hospitalized group of patients who were suffering from disease known not to be associated with changes in TBG concentration to determine whether the generally "unwell" had altered TBG concentrations.

The hospitalized patients had a higher mean age than the normal euthyroids so that the slightly higher TBG mean for male and female groups is not significant. This similarity between normal and hospitalized groups bears out the work of Oppenheimer et al. (1963). The much wider standard deviation of the hospitalized group is presumably due to non-specific factors influencing TBG or to "illness" affecting the liver which both synthesises and catabolises TBG. Another factor involved in TBG changes is that found in aged sick patients where TBG levels have been shown to be reduced in about one fifth of patients even in the absence of thyroid disease (Jeffrys, Farran, Hoffenberg, Fraser & Hodgkinson, 1972). A decreased albumin was also found under these conditions which were frequently associated with critical conditions or with drug therapy.

The similarity of the normal and hospitalized euthyroid group means implies that any change from this common mean

during specific disease must be real and indicate a significant alteration in TBG metabolism provided blood volume or plasma dilution remains constant.

4.3.1.2. TBG CONCENTRATION IN HYPER-
AND HYPOTHYROID PATIENTS

Blood samples were collected from patients diagnosed on clinical and biochemical grounds as either hyperthyroid or hypothyroid. All samples were collected before treatment to the patients had begun, and the sera were separated and stored at -70°C until they were assayed. The TBG rocket heights were measured from the autoradiographs and recorded as percentages of the TDP serum TBG. The results are shown in Table 20 and compared with the normal individuals and hospitalized patients. The mean TBG value of the 28 female hypothyroid patients is significantly ($P < 0.005$) lower than the normal female group, and the scatter of values is greater. The 21 female hyperthyroid patients also have a significantly ($P < 0.005$) lower mean than the normal, with greater scatter.

Men with thyroid disease are less common than women with the disease and only four hyperthyroids and nine hypothyroids were available. However, with the nine subjects in the hypothyroid group, the mean TBG level was substantially higher than both the normal and hospitalized groups, and with greater scatter. Conversely, the male hyperthyroid mean shows a substantially lower mean compared to the normal and hospitalized groups (Tables 19&20). The male hyper- and hypothyroid means are not significantly different from normal, because the numbers in the two groups are too small to represent a significant sample.

TABLE 20

SERUM TBG CONCENTRATION IN HYPO- AND HYPERTHYROIDISM.a) HYPOTHYROID SERA

AGE GROUP	MALE			FEMALE		
	NUMBER	MEAN	S.D.	NUMBER	MEAN	S.D.
20-29	-	-	-	-	-	-
30-39	1	136	-	3	123	10.4
40-49	-	-	-	3	127	6.4
50-59	1	135	-	7	130	4.7
60-65	4	150	19.3	7	139	12.0
66 yrs. plus	3	132	19.8	8	127	14.5
TOTAL	9	141	17.8	28	131	11.6
'Normal'	100	130	8.1	55	133	8.0
TOTAL						

b) HYPERTHYROID SERA

AGE GROUP	MALE			FEMALE		
	NUMBER	MEAN	S.D.	NUMBER	MEAN	S.D.
20-29	1	112	-	3	124	7.6
30-39	-	-	-	3	131	6.0
40-49	1	140	-	4	120	13.5
50-59	1	124	-	5	134	12.8
60-65	1	116	-	2	142	8.5
66 yrs. plus	-	-	-	4	131	12.7
TOTAL	4	123	12.4	21	130	11.8
'Normal'	100	130	8.1	55	133	8.0
TOTAL						

Units = % TBG Concentration of Working Standard Serum

CONCENTRATION IN HYPER- AND HYPOTHYROIDISM

The TBG concentration in sera from both hyper- and hypothyroid females is significantly ($P < 0.005$) lower than normal females found in this study and the standard deviation of both are much wider. The age range of the hyperthyroids is very similar to the normal group, but that of the hypothyroids is displaced towards the older group of 60-69 years.

The TBG in sera from males suffering from thyroid disorders is substantially different from the control group results. The male hyperthyroids, even though only four individuals' sera were examined, show a lower mean than the normal male and hospitalized groups. These results also show a lower TBG concentration than any individual age group TBG result. Three of the four sera show TBG values below 125%, yet only one of the patients was younger than 45 years. This mean of 123% is 7% lower than the normal male group mean and indicates a marked deviation from the TBG distribution related to age.

The nine male hypothyroid patients also show a deviation from the normal and hospitalized control groups, with a mean value 11% higher than the normal male mean. The mean age of this group, however, is 61.5 years which is much higher than the control group mean age, but even in the 60-69 year normal group, the TBG mean is only 131% which is 10% lower than the hypothyroid mean.

Most workers using the indirect binding capacity method (Oppenheimer et al., 1963; Robbins & Rall, 1957; Robbins & Rall, 1960; Shimizu et al., 1972; Sutherland & Simpson-Morgan, 1975) have found no significant difference between hyperthyroid and normal sera TBG levels. However, one group using the

M.B.C. method (Nusynovitz & Strader, 1972) has found a small elevation of TBG content in hyperthyroid sera which is in direct contrast to most of the studies using direct methods of measurement (Levy et al., 1971; Chopra et al., 1972) which have shown hyperthyroid serum to contain a significantly smaller amount of TBG in common with the present study. One direct method using a solid phase assay (Castro & Ugarte, 1974) however, has shown a definite elevation of TBG content in hyperthyroid serum which contradicts the results from both the male and female sera in the present study and of the subjects in the two other direct method studies (Levy et al., 1971; Chopra et al., 1972). In the Castro and Ugarte (1974) results, however, no precise mention is made of the number of patients in the study or of their age and sex.

Although three studies, including the present work, indicate a fall of TBG concentration in hyperthyroid serum using direct methods of measurement, the results should only be taken as an indication since only thirty one hyperthyroids in all were included in these results. More prolonged studies using the direct measurement methods should therefore be conducted so that not only hyperthyroid sera may be compared with normal, but age and sex differences may also be included.

The male hypothyroids (141%) in this study had an 11% higher TBG mean than normal males (130%), but this result is not significant because only nine individuals were studied. The female hypothyroid mean (131%), however, is significantly different ($P < 0.005$) from normals (133%), though it is only 2% lower, because the number (28) in the study was large.

The hypothyroid TBG results reported in the literature indicate no significant difference between these patients

and normal sera TBG using either direct or indirect methods of study (Castro & Ugarte, 1974; Sutherland & Simpson-Morgan, 1975; Wellby et al., 1974; Chopra et al., 1972; Levy et al., 1971). However, none of these studies mention examination of age and sex of the hypothyroid patients and it could be that with a large predominance of female patients, who have about eight times the incidence of thyroid disease, a virtually normal TBG concentration would be found. Thus, if the female hypothyroid mean (131%) is multiplied by eight and added to the male hypothyroid mean (141%) and the result divided by nine, the result is 132% which is almost identical to the mean of the normal male (130%) and female (133%) TBG concentrations, i.e. 131.5%. If the above studies do have the usual 8 : 1 female bias, then their results may not indicate the difference found in this study, because of the compensating difference in male and female TBG concentrations discussed.

The different results from hypothyroid and hyperthyroid sera within the studies prompts the need of a more comprehensive study so that changes in TBG levels at different ages and between the sexes may be adequately determined. The results of the present investigation while indicating that female TBG concentrations significantly decrease in both hyper- and hypothyroidism, do not show a similar change in male subjects.

4.3.1.3. TBG CONCENTRATION IN SERUM FROM PREGNANT WOMEN, FOETAL CORDS & IN AMNIOTIC FLUID

Blood was collected from women at various stages of pregnancy and from the umbilical cords of neonates at birth. Sera were separated from the samples and stored at -20°C until assayed in exactly the same way as the "normal" samples.

Amniotic fluid samples were obtained between 38 and 40 weeks. These amniotic fluid samples were treated in the same way as the other samples except that twice the volume i.e. $4\ \mu\text{l}$ was used on the electrophoresis plate because the protein content was lower than in plasma. The results from these three groups are shown in Tables 21 to 24 and are compared with the normal female group.

The sera from pregnant women were found to have a significantly higher ($P < 0.0005$) mean value (19.8%) than normal females and with a standard deviation about twice as (1.91) wide. The cord serum TBG levels were found to be almost exactly between the normal and pregnant serum values, that is 10% (10.5%) higher than the normal female values, but the standard deviation was again nearly twice (1.86) that of normal.

The amniotic fluid TBG concentration was very low in comparison with normal serum, but by using $4\ \mu\text{l}$ of the fluid the TBG peaks on the autoradiographs were readily measurable (Fig. 46). It was interesting that the highest TBG concentration was found in a woman bearing twins.

TABLE 21SERUM TBG CONCENTRATION IN NEONATES (CORD)PREGNANT FEMALES AMNIOTIC FLUIDS ANDOESTROGEN ADMINISTERED FEMALES.

	PREGNANT FEMALES	CORD SERUM	AMNIOTIC FLUIDS	NORMAL FEMALE	OESTROGEN ADMINISTERED FEMALES
NUMBER	34	58	12	55	21
MEAN	158	147	74	133	158
S.D.	15.3	15.0	11.2	8.0	12.0

UNITS = PERCENTAGE TBG CONCENTRATION OF WORKING
STANDARD SERUM.

TABLE 22SERUM TBG CONCENTRATION IN NEONATES (CORD)GROUPED BY MATERNAL AGE

MATERNAL AGE GROUP	NUMBER	MEAN	S.D.
20-29	38	149	14.6
30-39	20	144	15.7
TOTAL	58	147	15.0

SERUM TBG CONCENTRATION IN NEONATES (CORD)GROUPED BY FOETAL SEX

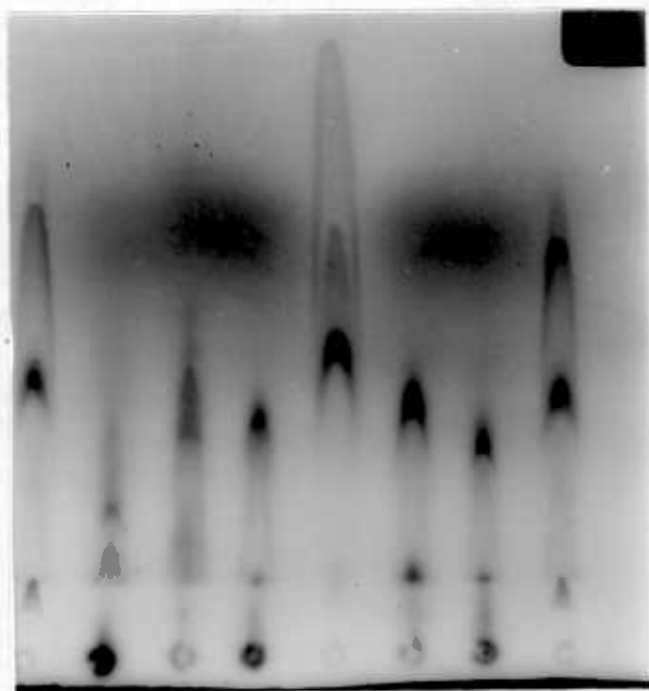
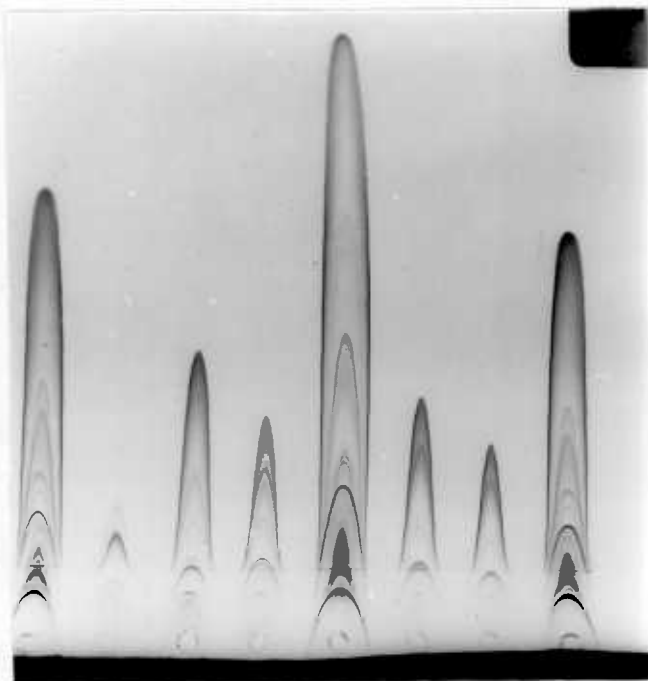
MATERNAL AGE GROUP	MALE NEONATES			FEMALE NEONATES		
	NUMBER	MEAN	S.D.	NUMBER	MEAN	S.D.
20-29	20	153	13.8	18	144	14.4
30-39	10	144	18.9	10	145	12.8
TOTAL	30	150	15.9	28	144	13.6

TABLE 24SERUM TBG CONCENTRATION IN PREGNANTFEMALES GROUPED BY AGE

AGE GROUP	NUMBER	MEAN	S.D.
20-29	27	159	15.4
30-39	7	157	16.2
TOTAL	34	158	15.3
'NORMAL' FEMALES	55	133	8.0

FIGURE 46

PHOTOGRAPH OF A STAINED PLATE AND THE CORRESPONDING
AUTORADIOGRAPH OF AMNIOTIC FLUID SAMPLES.



4.3.1.3.1.

DISCUSSION OF TBGCONCENTRATION IN PREGNANT & FOETAL SERUM, &AMNIOTIC FLUIDa) Pregnant & Foetal Blood TBG

The results from estimation of TBG in sera from pregnant females and from neonates (Umbilical cord blood) are shown in Table 21 and have been substantiated by many studies using either indirect (Sutherland & Simpson-Morgan, 1975; Nusynowitz & Strader, 1972; De Nayer et al., 1965; Dowling, Freinkel & Ingbar, 1956 (a); Robbins & Nelson, 1958) or direct (Bradwell et al., 1976; Levy et al., 1971; Chopra et al., 1972) methods.

The TBG in sera from pregnant women was about 20% above the normal mean level while the foetal cord sera TBG was almost exactly midway between the pregnant and normal women's mean values. The scatter of both pregnant and cord results were wider than those of normal sera which may reflect either different responses to the oestrogen increase of pregnancy, to the relatively different amounts of oestrogen secreted by the expectant mothers, or to a difference in plasma dilution.

During examination of the results from foetal cords the maternal age was noted and the effect of maternal age on foetal TBG concentration at the time of birth was investigated. Of the 58 mothers of known age, 38 were between 20-29 years old and the remaining 20 were between 30 and 39 years. The mean values and standard deviations are shown in Table 22. The 20-29 year old mothers showed a significantly ($P < 0.01$) higher mean TBG concentration in their foetuses than did those from mothers of 30-39 years. The foetal sex of the 58 samples was then sought to carry this "maternal age/foetal TBG" investigation further. The re-

sults of male and female neonates TBG concentration related to maternal age are shown in Table 23 and a remarkable result emerges. Whilst the mean TBG concentration of all female neonates, and male neonates born of mothers between 30 and 39 years, are virtually identical, the TBG concentration of male neonates born of mothers between 20-29 years shows a mean TBG concentration significantly higher than the other groups ($P < 0.01$). The significance of a higher TBG concentration in male neonates of younger mothers is obscure and it is hard to imagine a different sensitivity to maternal oestrogen only in those male fetuses with younger mothers.

A similar analysis was performed using the sera from pregnant females and the mean from the 20-29 year group was significantly higher ($P < 0.01$) than from the 30-39 year group (Table 24). The foetal sex from these women was unfortunately unavailable.

b) TBG concentration of Amniotic Fluid

The TBG content of amniotic fluid has not been as extensively studied as foetal blood TBG. The thyroxine content has been examined, but no relationship was made with actual protein levels (Hollingsworth & Austin, 1971; Chopra & Crandall, 1975).

In this study, twelve different amniotic samples were studied giving a TBG range of 54-93% of the Standard serum with a mean of 74%. This mean value is far lower than the normal serum TBG level even though twice the volume of fluid, in comparison to serum, was added to the sample holes. This low TBG level reflects the generally low protein content of amniotic fluid which contains only 0.5 gm/100 ml. of protein compared with serum which contains about 7 gm./100 ml.

However, the TBG concentration appears to be disproportionately high in comparison to the amniotic fluid/serum total protein relationship.

The TBG presumably leaks into the amniotic fluid from the mother's serum with T_4 and T_3 bound to it. The function of the complex in this fluid is not obvious, however, as there is no direct route into the foetal circulation. Since fluid is swallowed by the foetus the hormones could be released after digestion of the protein and absorbed, though the amount would be small in comparison to the production of thyroxine by the foetus itself after about two months.

4.3.1.4.

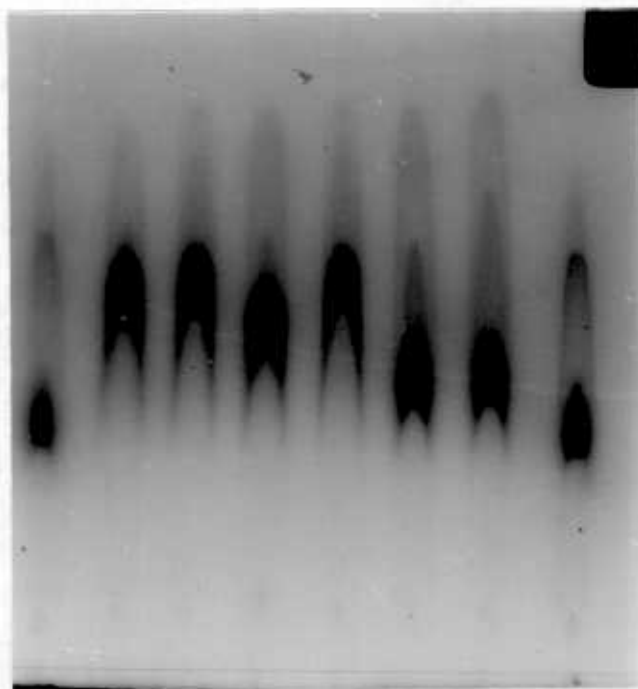
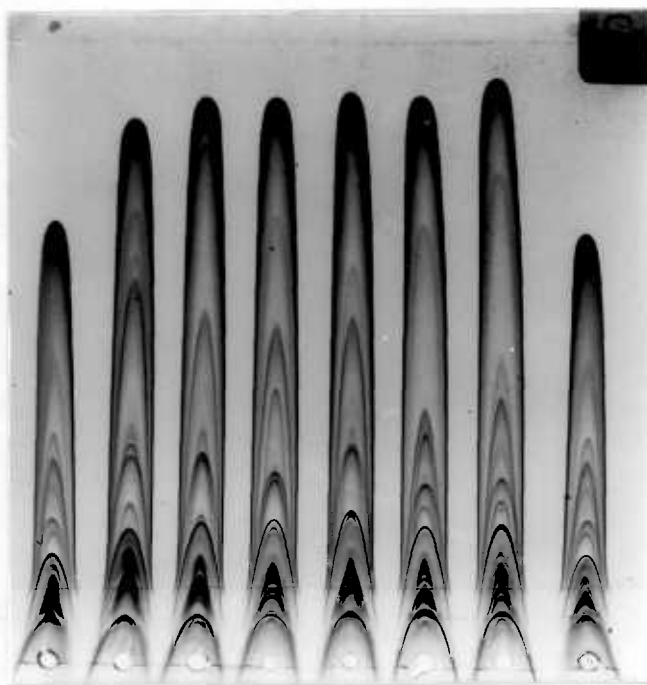
EFFECT OF OESTROGENADMINISTRATION ON TBG CONCENTRATION IN WOMEN

Blood was collected from twenty one women (aged between 22 and 44 years) who were taking oestrogen preparations. The sera were separated and stored at -20°C until being run on the Modified Rocket System.

After immunoelectrophoresis and autoradiography the TBG peaks were measured and related to the Standard Sera on each plate (Fig. 47). The results gave a mean TBG value of 158 (Table 21), which was significantly higher ($P < 0.0005$) than the normal female concentration, but no different from pregnant females.

FIGURE 47

PHOTOGRAPH OF A STAINED PLATE AND THE CORRESPONDING
AUTORADIOGRAPH OF FEMALE SERUM AFTER OESTROGEN
ADMINISTRATION.



4.3.1.5. SERIAL EFFECT OF OESTROGEN ON
THE TBG CONCENTRATION IN WOMEN

The effect of oestrogen on TBG concentration over a period of one menstrual cycle was studied in two married women. Two healthy women not taking oestrogen and two healthy males were included in the study as controls. The sera were assayed using long (24 hole) plates so that all samples from each subject could be run on the same plate (Figs. 53 & 54).

The results are presented graphically (Figs. 48-50) as percentage TBG rocket heights against time in days.

The two male controls were first examined to determine the constancy of the base line. Since the male is presumed to have no sex hormone cycle the TBG concentration should remain relatively constant. Any observed changes are deductively due to bleeding procedure differences and/or experimental variation. The results from the two males included in this study showed extreme constancy of TBG concentration except when one subject (P.B.) contracted a mild virus infection and was treated with antibiotics. During this treatment period a slight rise in TBG concentration occurred (Fig. 49).

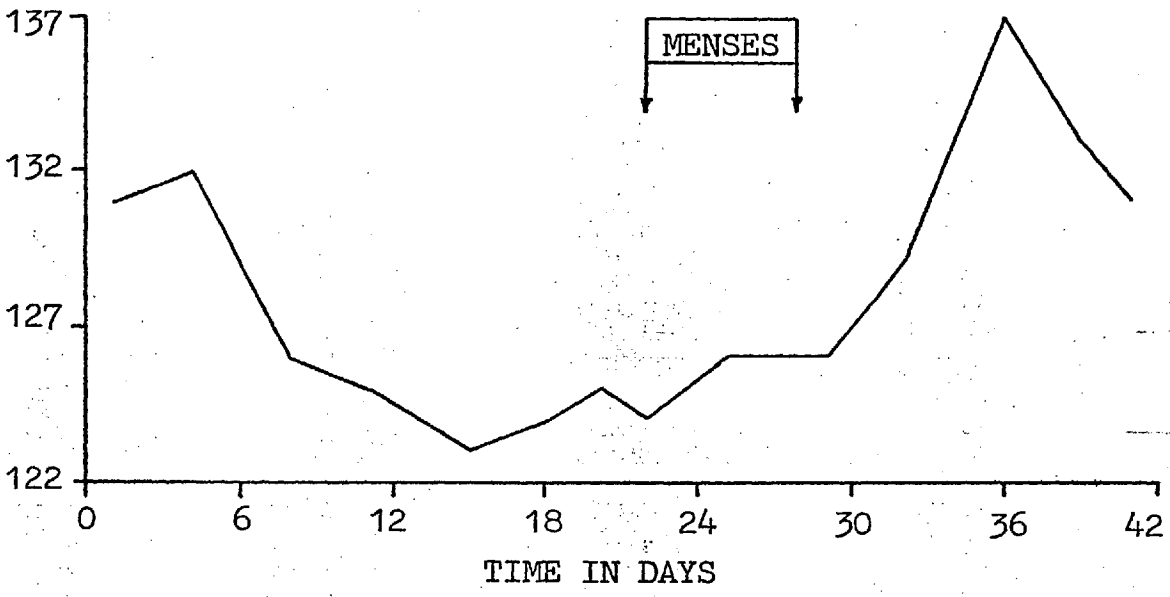
The two control females with physiologically determined menses displayed obvious cyclic TBG variations. The absolute TBG values were well within the normal female TBG range with peak to trough variations of 10 and 14%. The times of peak and trough values of TBG concentration in these control women basically followed the oestrogen cycle with the TBG concentration at or near its low point during menstruation, while the peak value was approximately half way between consecutive menses (Fig. 48).

FIGURE 48

CYCLIC CHANGES OF TBG CONCENTRATION IN
TWO WOMEN

TBG ROCKET HEIGHT AS PERCENTAGE OF STANDARD.

i) Subject I.Y.



TBG ROCKET HEIGHT AS PERCENTAGE OF STANDARD.

ii) Subject H.C.

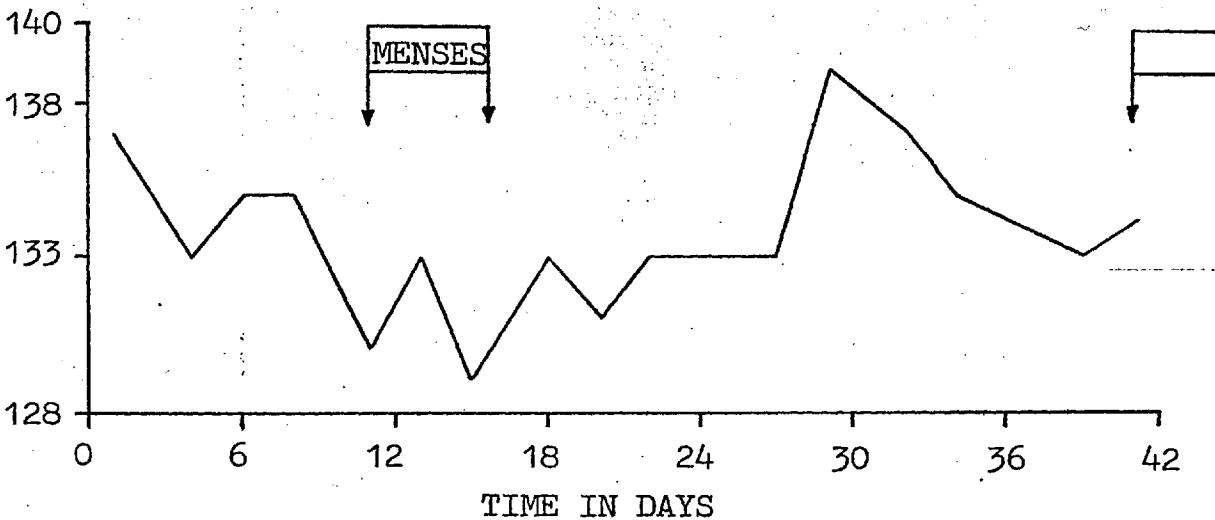


FIGURE 49

CYCLIC CHANGES OF TBG CONCENTRATION
IN TWO MALES

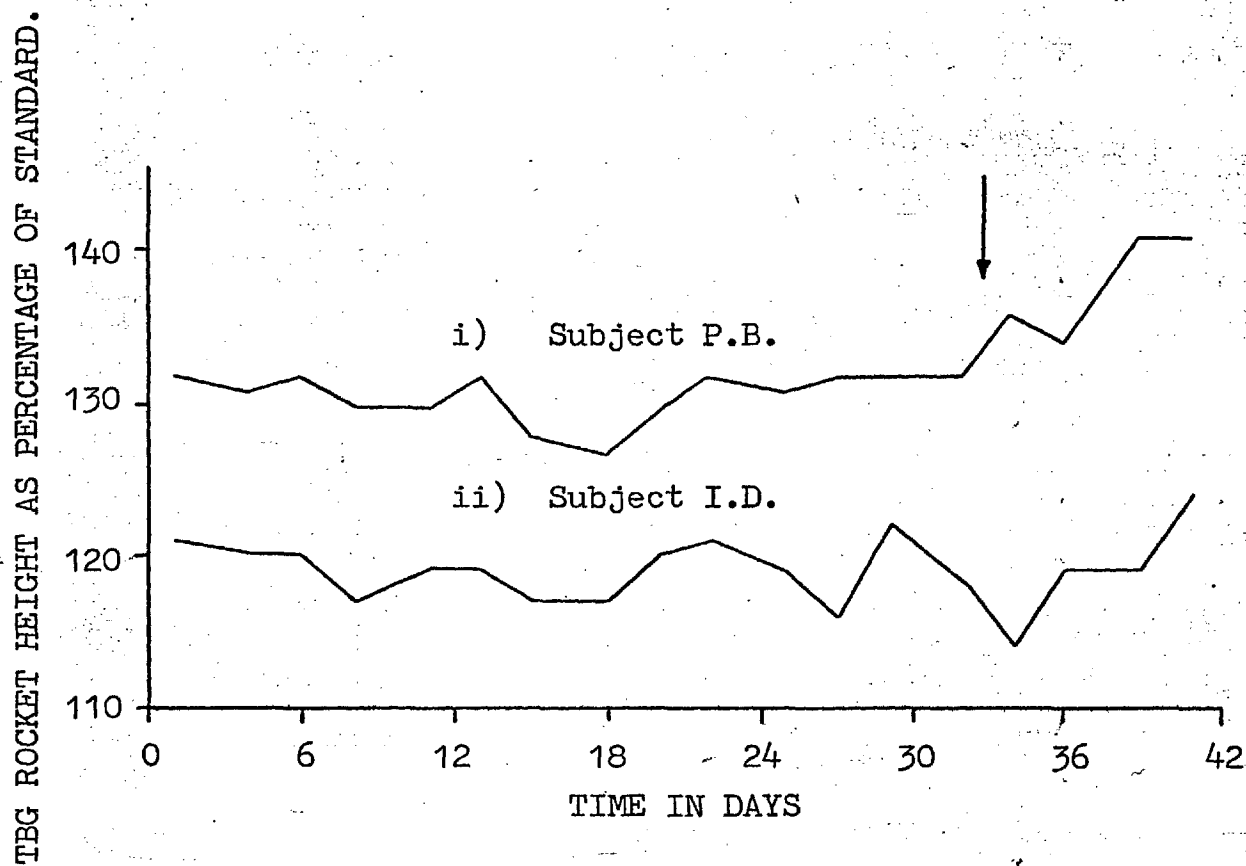
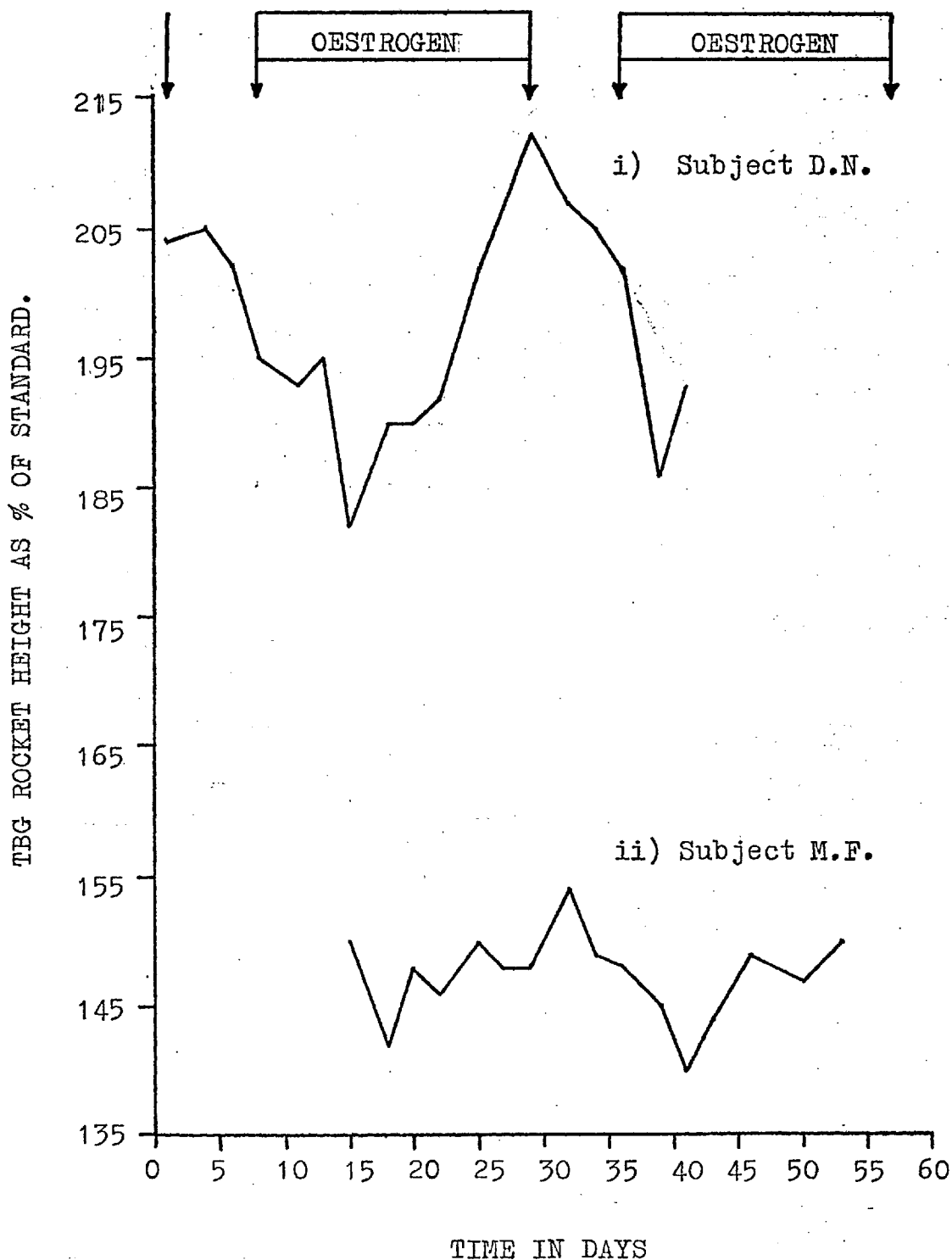


FIGURE 50

CHANGES IN TBG CONCENTRATION IN DIFFERENT
INDIVIDUALS DURING A ONE MONTH PERIOD.

Changes in TBG concentration in two females
during Oestrogen administration.



The TBG of the two women taking oestrogen, both of whom were 28 years of age, was considered in terms of the constancy of the male TBG concentrations and cyclicity of the control females. The amounts of oestrogen taken by these women were equal, but the formulations were different (Minovlar: 0.05 mg. ethinyl oestradiol + 1 mg norethisterone acetate; Ovulen "50": 0.05 mg. ethinyl oestradiol + 1 mg. ethynodiol diacetate). One of the subjects (D.N.) had been pregnant four years before the study and had an absolute TBG concentration far higher than the other female, who had not been pregnant. Both women taking oestrogen displayed absolute TBG levels well above the normal female range (Fig. 50). They also demonstrated cyclic TBG variations, but in marked contrast to the control women's cyclicity. A peak in TBG concentration occurred at 1 - 3 days after the final oestrogen pill of a series, and a nadir 5 - 9 days after the first pill of the next series. The peak to trough TBG differences in the two women were 16 and 32% compared with the maximum male differences of 5 and 8%, and control women's 10 and 14% (Figs. 48, 49 & 50).

4.3.1.6. SERIAL EFFECT OF STARTING AND STOPPING OESTROGEN ON THE TBG CONCENTRATION IN WOMEN

The effect of starting or stopping administration of the oestrogen pill on TBG concentration was studied in two women. One woman (G.J.) who had not previously been taking the contraceptive pill started, whilst the second woman (C.P.) had been regularly taking oestrogen for three years, but decided to stop. Blood samples were obtained at 5 to 10 day intervals over a ten week period, and the sera were separated, stored and then run in the same way as all previous samples. The results are shown graphically (Figs. 51 & 52).

The woman (G.J.) who started taking a high oestrogen preparation showed a marked, sustained rise in TBG concentration out of the normal female range and up into the elevated range of the oestrogen pill users. A maximum TBG concentration was reached by the time of the final pill of the first month and then fell during menstruation (Figs. 51 & 53). The TBG again rose to the same maximum concentration after starting the second series of oestrogen pills.

The subject (C.P.) who had decided to stop taking her low oestrogen preparation again showed a cyclic pattern of TBG concentration, with a peak 1 - 3 days after the final pill and a nadir midway between the menses. After taking the final pill, the TBG concentration continued to rise to a peak at 3 days, then decreased over the following 40 days until it entered the normal "non-pill" range. However, even after 46 days, TBG concentration was not consistently within the normal range and there was no menstruation for the two months immediately following the final pill (Figs. 52 & 54).

FIGURE 51

CHANGES IN TBG CONCENTRATION OVER A TWO MONTH PERIOD IN A FEMALE SUBJECT WHO
STARTED TAKING AN OESTROGEN PREPARATION.

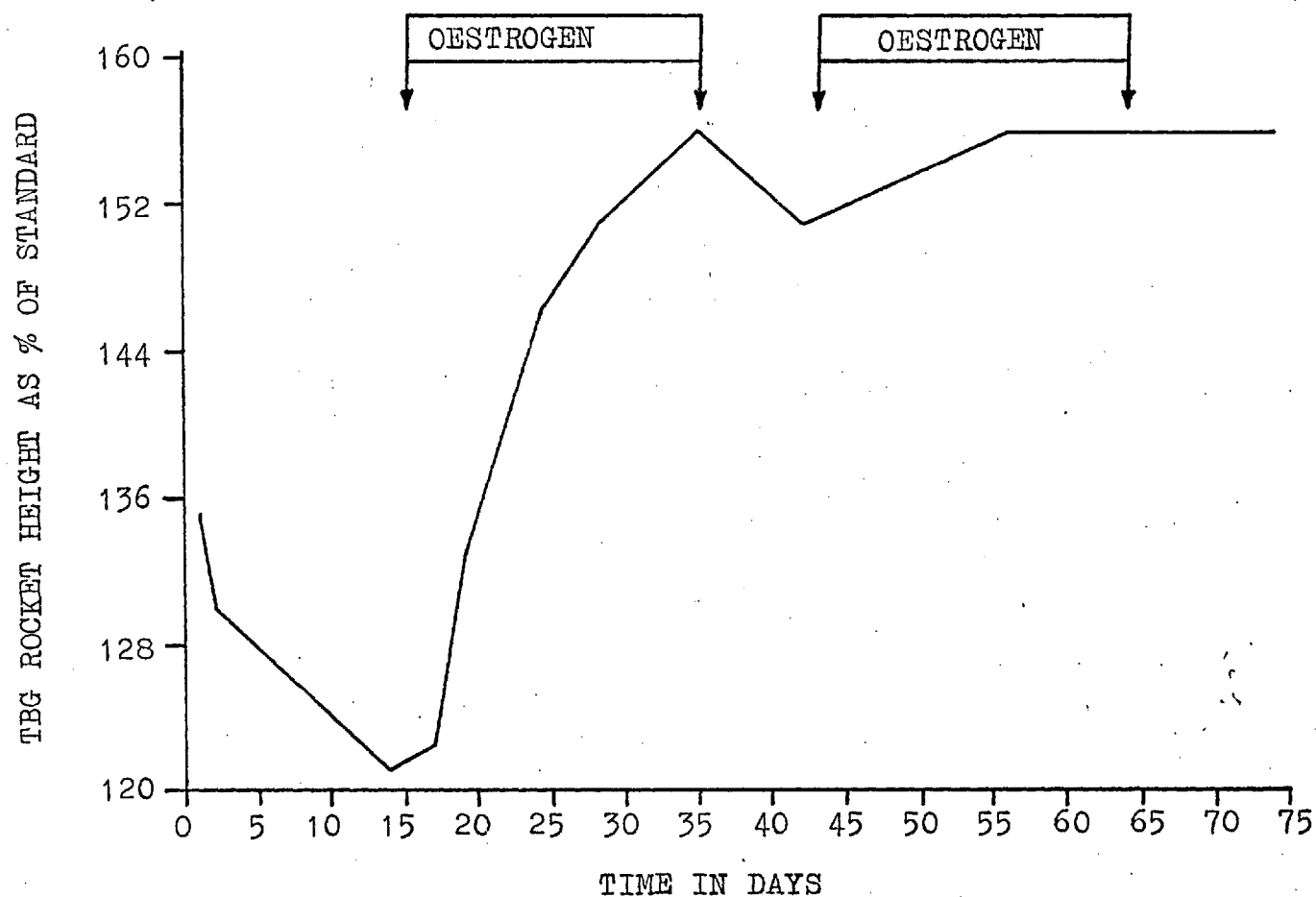


FIGURE 52

CHANGES IN TBG CONCENTRATION OVER A TWO MONTH PERIOD IN A FEMALE SUBJECT WHO STOPPED

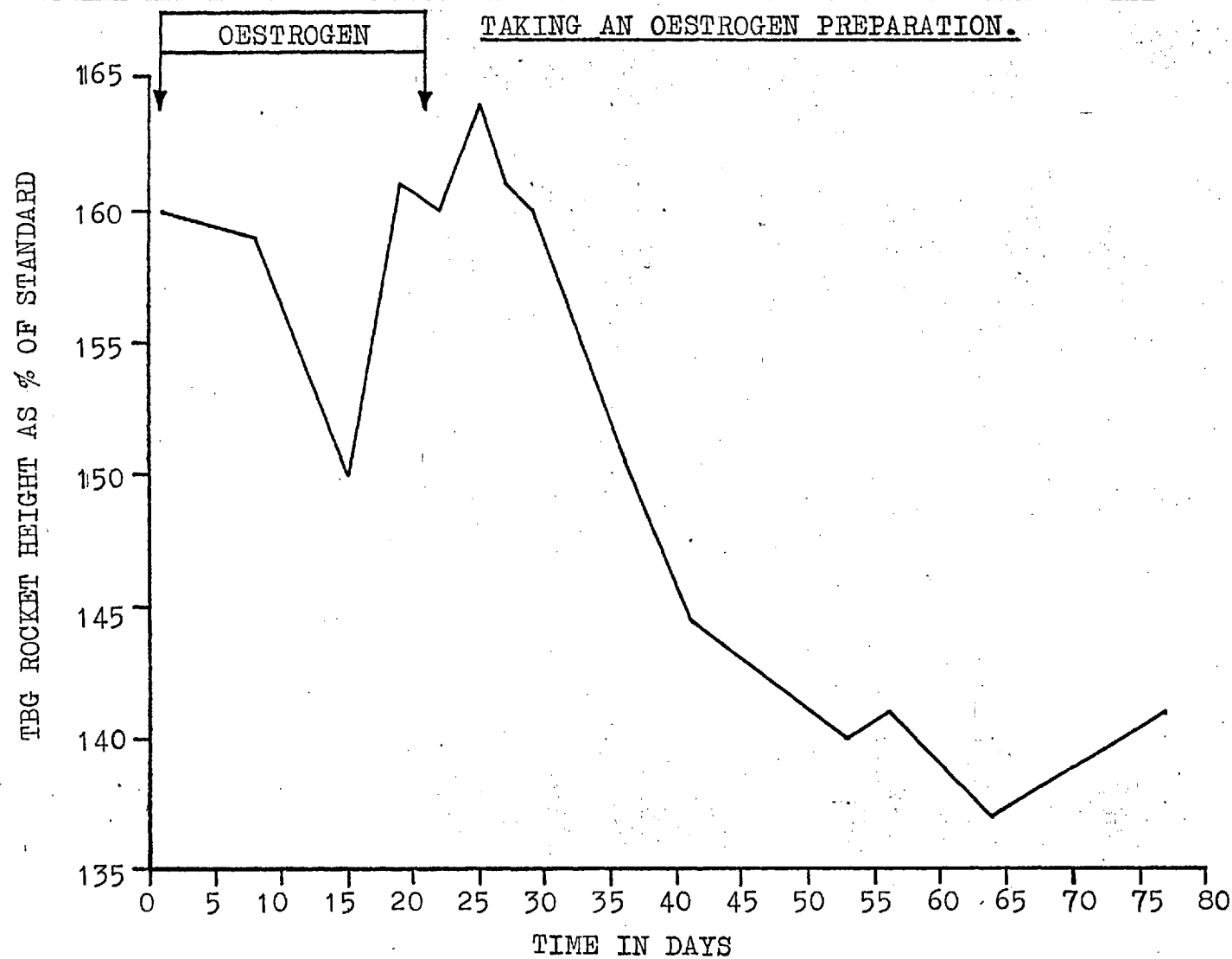


FIGURE 53

PHOTOGRAPH OF A STAINED PLATE AND THE CORRESPONDING
AUTORADIOGRAPH OF THE TBG CHANGES OF A FEMALE
STARTING OESTROGEN

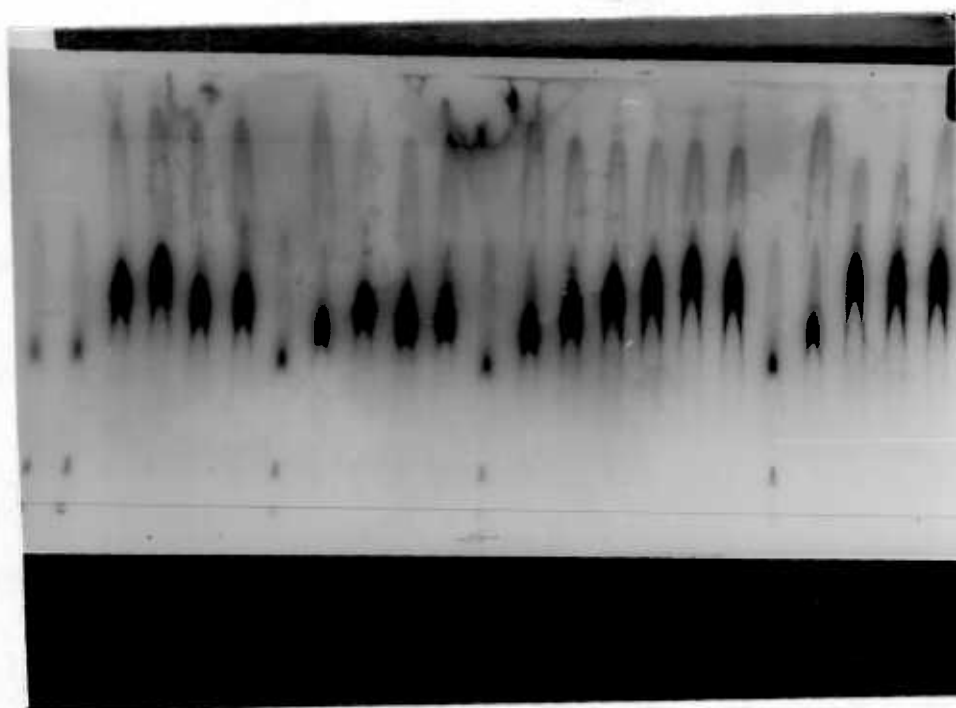
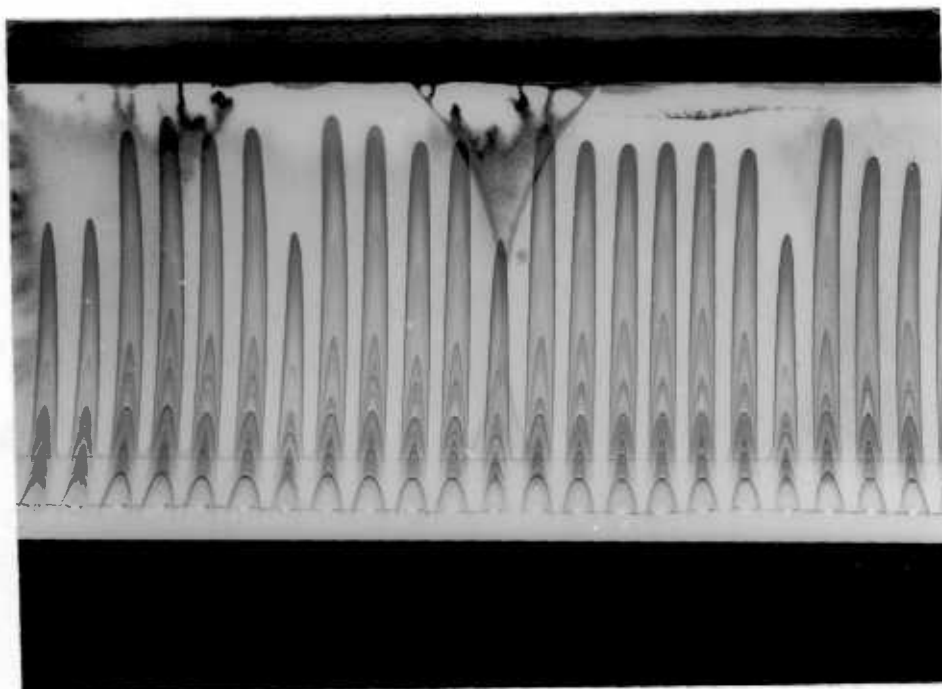
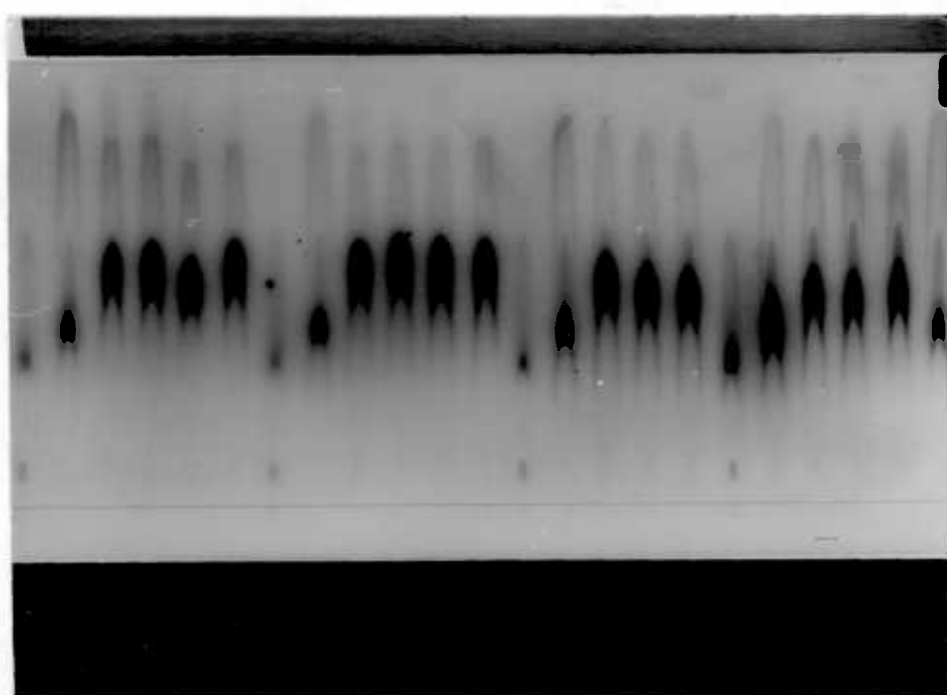
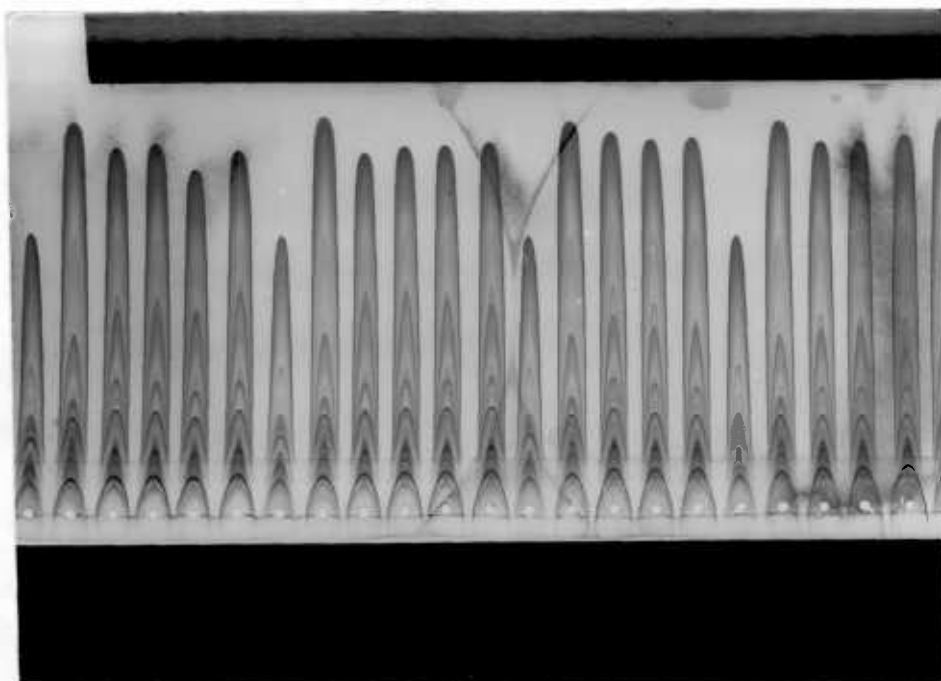


FIGURE 54

PHOTOGRAPH OF A STAINED PLATE AND THE CORRESPONDING
AUTORADIOGRAPH OF THE TBG CHANGES OF A FEMALE
STOPPING OESTROGEN



4.3.1.6.1. DISCUSSION OF OESTROGEN

EFFECTS ON TBG CONCENTRATION IN WOMEN

The effect of oestrogen in causing an increase in serum TBG concentration is well documented (Laurell, Kullander & Thorell, 1968; Jeppsson, Laurell & Rannevik, 1973; Dowling, Freinkel & Ingbar, 1956(b); Engbring & Engstrom, 1959) and the present study is in agreement with those results. The twenty-one women included in this study gave a mean TBG concentration of the same value (158%) and significantly ($P < 0.0005$) elevated level, over normal, as that found in pregnant (158% and $P < 0.0005$) women (Table 21).

The effects of abrupt changes in circulating oestrogen levels on serial TBG concentrations were also studied and compared with TBG concentration during the normal 28 day ovulating cycle. Eight subjects were studied. Two normal women (H.C., I.Y.) and two men (P.B., I.D.) were included to determine physiological TBG range. Those taking oestrogen were one woman who started taking oestrogen (G.J.), two who had been taking oestrogen for a number of years (D.N., M.F.), and one who had been taking oestrogen for three years and stopped during the investigation (C.P.). Two major differences between the control and oestrogen taking groups were apparent:

- i) the TBG concentration of those taking oestrogen was higher than the controls.
- ii) the TBG cyclicity was reversed in the oestrogen group compared with the controls.

In the women with physiologically controlled menstruation the TBG concentration (120% - 140%) was within the normal female range and showed marked cyclicity, with the peak appearing approximately at the mid-point of the cycle and

with the nadir at the beginning or during menstruation.

The male subjects' TBG concentration was extremely constant (116% to 121% and 130% to 138%) and well within the normal male range for their age group. A slight rise in TBG concentration occurred in P.B. serum after he had contracted a mild infection and was treated with antibiotics. (Fig. 49).

Before starting to take oestrogen, the first woman (G.J.) also displayed marked cyclicity of TBG with the concentration range well within the normal group. Day one of the cycle was on the first day of menstrual bleeding and the first oestrogen was taken on day 5. Between days 5 and 26 when the oestrogen was being taken, the TBG showed a consistent and significant elevation out of the "normal" range and up into the oestrogen users range (Fig. 51). On day 26 when the final "Pill" was taken the TBG level had risen to a maximum 156%. The serum was taken less frequently after this first cycle. However, a further three samples were taken over the next cycle of "pills", when the elevated TBG level common to oestrogen takers was maintained. The final "pill" was taken on day 28 of the second series, but a full ten days after this when the final blood sample was taken, the TBG level was still at the maximum recorded concentration of 156%.

In the two women already taking oestrogen, pronounced cyclicity of TBG concentration was observed, but with a different phase. A peak of TBG concentration occurred at about 1-3 days after the final pill and a nadir 5-9 days after the start of the next series of oestrogen pills. Thus the oestrogen and physiologically determined patterns of the cycles, while being basically the same shape, showed a reversal of the TBG cyclicity. The oestrogen pill users were

found to be out of phase with women following the physiological cycle by 14-18 days.

The control women showed a peak TBG value at about the time of ovulation, i.e. day 14-18, when oestrogen levels are also reaching maximum levels, whilst a minimum level was reached at or just before menstruation, again following the oestrogen concentration, which reaches a low at this time. However the women taking oestrogen appeared to have their TBG levels controlled by the exogenous hormone. The peak TBG concentration in the oestrogen taking women occurred at day 1-3 of the cycle, which was 1-3 days after the final pill, whilst the minimum TBG level occurred at day 10-14 which was 5-9 days after commencement of the next series of pills. This indicates a time lapse for stimulation of synthesis, or inhibition of degradation, of TBG which is longer than the latent period occurring before inhibition of synthesis, or stimulation of degradation, since the TBG level starts to fall after the final pill much quicker than it rises after commencing the next series of oestrogen pills.

The physiologically determined oestrogen cycle follows a diphasic change (Speroff & Vande Wiele, 1971), but the dip of oestrogen at the time of ovulation is relatively short and the concentration of oestrogen does not fall as low as the menstrual level. The secondary peak of oestrogen is 9-10 days before onset of menstrual bleeding and by 2-4 days before, this has fallen to its lowest level and in the control females in this study, corresponded to the lowest TBG concentration.

The subject (C.P.) who had been taking a low oestrogen preparation (Minilyn) for three years was studied for one cycle prior to stopping the oestrogen. It indicated a

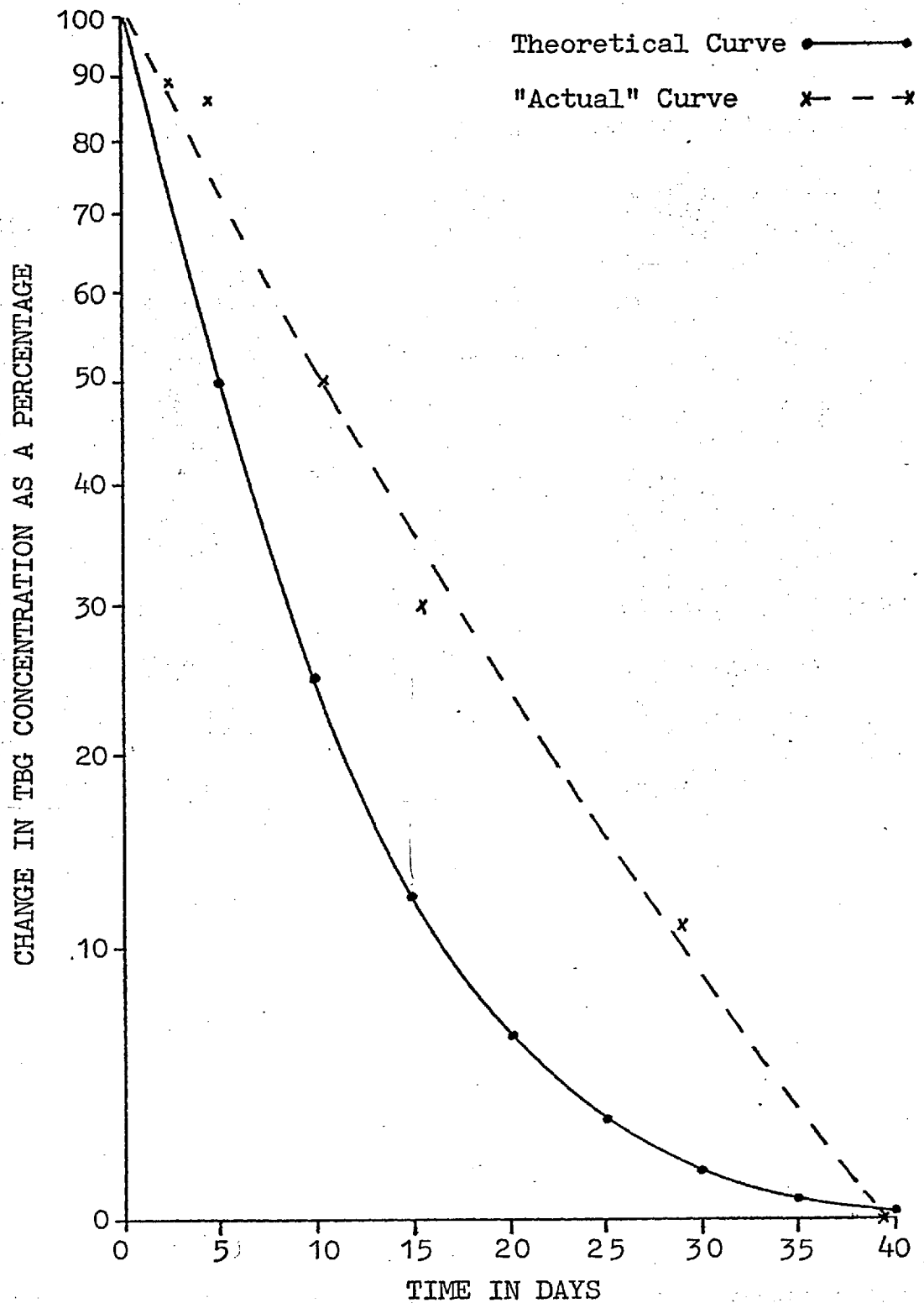
pattern of cyclicity close to the previous oestrogen users with a minimum TBG just over half way through the cycle and a maximum level about 3 days after the final "pill" of the series. After the final "pill" the TBG rose to a maximum, in conformity with the previous subjects. At about day 3 and thereafter, the TBG concentration fell consistently for 32 days when the normal range was entered for the first time. The level then rose again slightly, but then continued to fall for a further 8 days. The final 12 days indicated a rise, but the taking of only one sample during this time made accurate monitoring impossible. The general trend during the final 24 days of study indicated a return to a normal cycle with the TBG only just higher than the usual control levels. This final cyclicity of TBG on the upper level of normal TBG concentrations may have been the re-adjustment before commencing physiological control of the cycle, since the first menstrual bleeding after finishing the oestrogen pills followed this more normal TBG cycle (Fig. 52). The period of time (36 days) taken to return to normal TBG concentration range is in agreement with that found (6 weeks) by Robbins & Nelson, (1958) who studied thyronine binding protein levels after parturition.

The time taken (36 days) for the TBG to fall back to normal concentration after cessation of the oestrogen was far longer than that taken for the TBG to rise to maximum levels (20 days) in the woman starting to take the oestrogen. The reason for the longer degradation compared with the synthesis is probably due to the half-life time of TBG, which is about 5 days (Cavalieri et al. 1975), resulting in a gradual clearance from serum. When the theoretical half-life ($t_{\frac{1}{2}}$ circa 5 days) curve of TBG is compared with the dis-

appearance curve of TBG from C.P. serum ((where a 100% decrease is equal to a fall from maximum TBG level (i.e. 164% of the TDP) to the non-oestrogen supplemented level (i.e. 134% of the TDP) as arbitrary values in this case)), the curves are found to be extremely close (Fig. 55). The differences between the theoretical and observed curves is probably due to the continued cyclic production of TBG under the control of the physiological oestrogen output, and the persistence of stimulatory exogenous oestrogen.

The effect of oestrogen on the TBG content of serum is thus quick to start and is prolonged even after cessation of the oestrogen stimulus. The cyclicity of TBG concentration is also significantly changed from normal so that the maximum/minimum times are reversed.

FIGURE 55

COMPARISON OF "ACTUAL" AND THEORETICAL($t_{\frac{1}{2}}$ circa 5 DAYS) HALF LIFE OF TBG

The "Actual" Half Life has been calculated from the decrease in TBG Concentration using C.P. serum (Fig. 52)

4.3.1.7. TBG CONCENTRATION DURING
LIVER DISEASE

Sera were collected from patients with various forms of liver disease. The samples were stored and assayed as all previous samples.

The two patients with serum hepatitis (Australia Antigen positive) have TBG concentrations far above the normal TBG means. The two patients with drug induced hepatitis also demonstrated elevated TBG concentrations.

The remaining five sera did not show any conclusive TBG changes (Table 25).

TABLE 25TBG ROCKET VALUES FOR LIVER DISEASE PATIENTS

<u>TBG</u> %	<u>Disease/Condition</u>
172	Australia Antigen Positive
179	Australia Antigen Positive
162 F	Hepatoma
188 F	Methyl-dopa induced Hepatitis
143 F	Post-operative Jaundice (Anaesthetic Induced)
154 M	Extra Hepatic Obstructive Jaundice
148 F	Primary Biliary Cirrhosis
133 F	Histologically Proven Cirrhosis
135 M	Secondary Carcinoma of Liver

(Sex when known: M = Male

F = Female)

4.3.1.7.1. DISCUSSION OF TBG CHANGES
DURING LIVER DISEASE

The concentration of TBG is known to become elevated in many patients with infectious hepatitis (Ingbar & Woeber, 1974) and to change non-specifically during hepatic disease generally (Clark, 1967). The present study confirms this previous work, but a greater number of patients are needed for a really positive analysis of the relationship between liver disease and TBG concentration.

4.3.1.8. CONGENITAL TBG ABNORMALS

Sera were collected from a family with an inherited TBG deficiency by Dr. D.B. Grant and were kindly made available for this study. The history and relationships of the individuals within the family have been reported elsewhere (Grant, Minchin-Clarke & Putman, 1974) and it is considered sufficient in this study merely to demonstrate detection of different congenital levels of TBG. The results shown in Figure 56 and Table 26 may be divided into three groups:-

- i) A normal group
- ii) A low TBG group and
- iii) A TBG deficient group

The results were compared with the Grant et al. (1974) results and the order of increasing TBG values were virtually identical. The method used in the Grant et al. (1974) study was a modified form of the Freeman & Pearson (1969) system, using Laurell crossed immunoelectrophoresis, a longer and more tedious method than the current modified Laurell Rocket Technique.

TABLE 26

TBG CONCENTRATION OF THE SAME SAMPLES USING THE
MODIFIED ROCKET TECHNIQUE AND LAURELL CROSSED
IMMUNOELECTROPHORESIS

SUBJECT NUMBER (From Grant et al. 1974)	SEX	AGE, YEAR OF BIRTH	TBG CONCENTRATION	
			MODIFIED ROCKET TECHNIQUE (as %)	LAURELL ⁺ (From Grant et al. 1974)
I 1	M	1889	ND	ND
I 2	F	1888	137	105
II 1	M	1917	126	134
*II 2	-	-	-	-
II 3	F	1911	100	43
II 4	M	1919	120	99
II 5	M	1920	120	93
II 6	F	1926	76	25
III 1	M	1933	121	116
III 2	F	1935	98	53
III 3	M	1936	ND	ND
III 4	M	1936	121	105
III 5	F	1937	93	51
III 6	F	1939	85	50
III 7	F	1945	79	43
III 8	F	1939	133	107
III 9	M	1956	121	88
III 10	F	1963	121	114
IV 1	M	1958	123	102
IV 2	F	1961	94	44
IV 3	M	1959	123	101
IV 4	M	1963	ND	ND
IV 5	M	1964	ND	ND
IV 6	M	1967	126	110

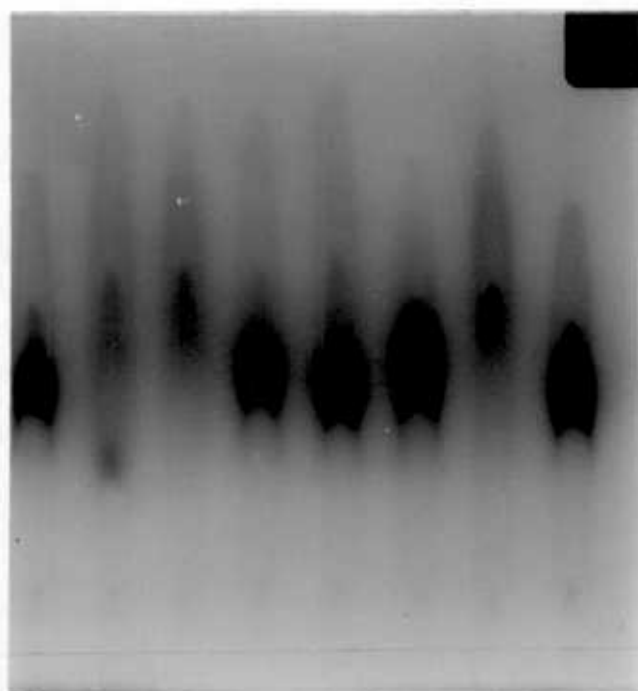
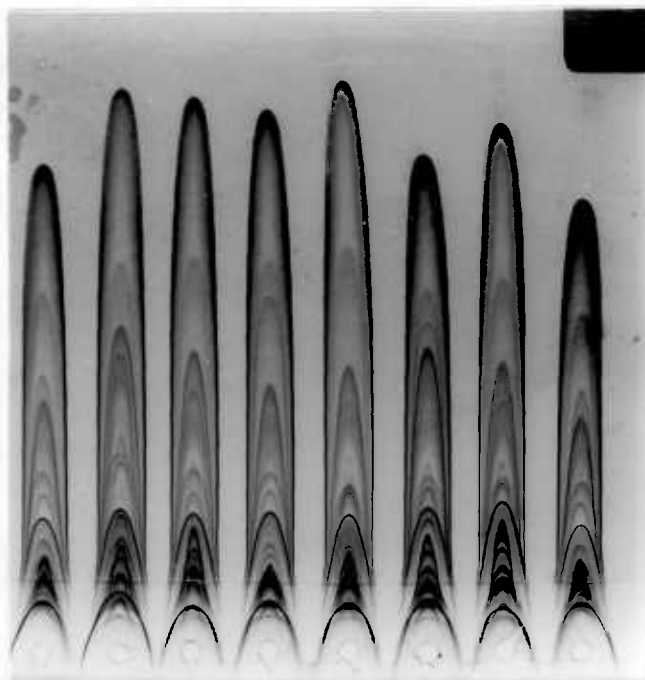
* = Subject Deceased

N.D. = None Detected.

+ = Laurell Crossed Immunoelectrophoresis Technique used by Grant et al. (1974) related the test sera to a standard serum as a percentage.

FIGURE 56

PHOTOGRAPH OF A STAINED PLATE AND THE CORRESPONDING
AUTORADIOGRAPH OF A FAMILY WITH A CONGENITAL
TBG ABNORMALITY



4.3.1.8.1. DISCUSSION OF CONGENITAL

TBG ABNORMALS

The individuals in this study were found to form three distinct groups.

Group one contained twelve normal TBG subjects, eight of whom were consanguineous and four non-consanguineous.

Group two contained seven individuals all of whom were female and consanguineous. The TBG levels were between a half and two thirds the normal female value found in this study (Section 4.3.1.1.).

Group three contained four consanguineous males in whom TBG was not detectable.

This general grouping of subjects is identical with the Grant et al. (1974) study, but the precise order of TBG values is slightly different, presumably due to the accuracy of the methods used. The method used by Grant et al. (1974) was the Laurell (1966) crossed immunoelectrophoresis system modified by Minchin-Clarke & Freeman (1968) and adapted by Freeman and Pearson (1969) for estimating TBG. The Method uses autoradiography, and planimetry of the autoradiograph peaks. However, using this method the accuracy of measurement depends on the operator's judgement since the autoradiograph of the peak's "feet" are difficult to see, and are often not visible at all, and, the edge of the protein peak is hazy. These factors combine to make tracing with the planimeter needle arduous, often inaccurate and time consuming as the process must be repeated until two consecutive values match.

In the present study the modified Rocket technique was used which is an easier, more accurate and conservative method than that used by Grant et al. (1974). A cost comparison of the techniques is interesting. As can be seen

from Table 27 the amount of reagents used in this study is only one sixth of those used, and the time spent obtaining the result is only a quarter of that spent, by Grant et al. (1974).

It has been stated that uncertainty continues regarding the apparent "absence" of TBG (Grant et al. 1974) even after Levy et al. (1971) were unable to detect TBG in the congenital syndrome using a radioimmunoassay. During the present study therefore, an investigation into the nature of the TBG "absence" anomaly was considered. Working on the principle that to be classified as a discrete entity the protein must be immunologically recognisable, an experiment was devised to investigate the apparent "absence" of TBG.

One of the sera from the "TBG absent" group (N.F.) was selected and progressively diluted with a "normal" TBG containing serum (TDP). Neat N.F. serum and dilutants were run on the same plate. If there existed in the N.F. serum an immunologically recognizable "TBG" protein then the TBG peak of the TDP serum would remain the same height, though it would be less black on autoradiography with the higher "a-TBG" serum component. If, however, the TBG peak became smaller in proportion to the TDP serum dilution, i.e. as the N.F. serum component rose, then there would be no immunologically recognizable TBG, and therefore by definition, no TBG, in the serum.

The results of this experiment, discussed in the "Standardization" Section, indicated that the TBG peaks of TDP serum decreased in height in proportion to the same serum diluted with phosphate showing that the TDP "marker" TBG was not being supplemented and therefore no TBG was contained in

TABLE 27

COMPARISON OF MATERIAL AND TIME EXPENDITURE
BETWEEN THE MODIFIED ROCKET TECHNIQUE AND LAURELL CROSSED
IMMUNOELECTROPHORESIS TECHNIQUE WHILST
STUDYING THE SAME SAMPLES.

CONSUMABLE	MODIFIED ROCKET TECHNIQUE	LAURELL CROSSED IMMUNOELECTRO- PHORETIC TECHNIQUE
Number of Plates Used	4	23
Volume of Antiserum Used	10 ml.	57.5 ml.
Volume of Agarose Used	96 ml.	572 ml.
Number of Autoradiographs Used	4	23
Controls Run Per Plate	2	1
Electrophoretic Running Time	15 Hrs.	25 Hrs.
Number of Electro- phoretic Stages	1	2
Time of Autoradiograph Exposure	1 week	4 weeks
Amount of Sample on Plate	2 μ l	4.4 μ l

the N.F. serum (Fig. 43).

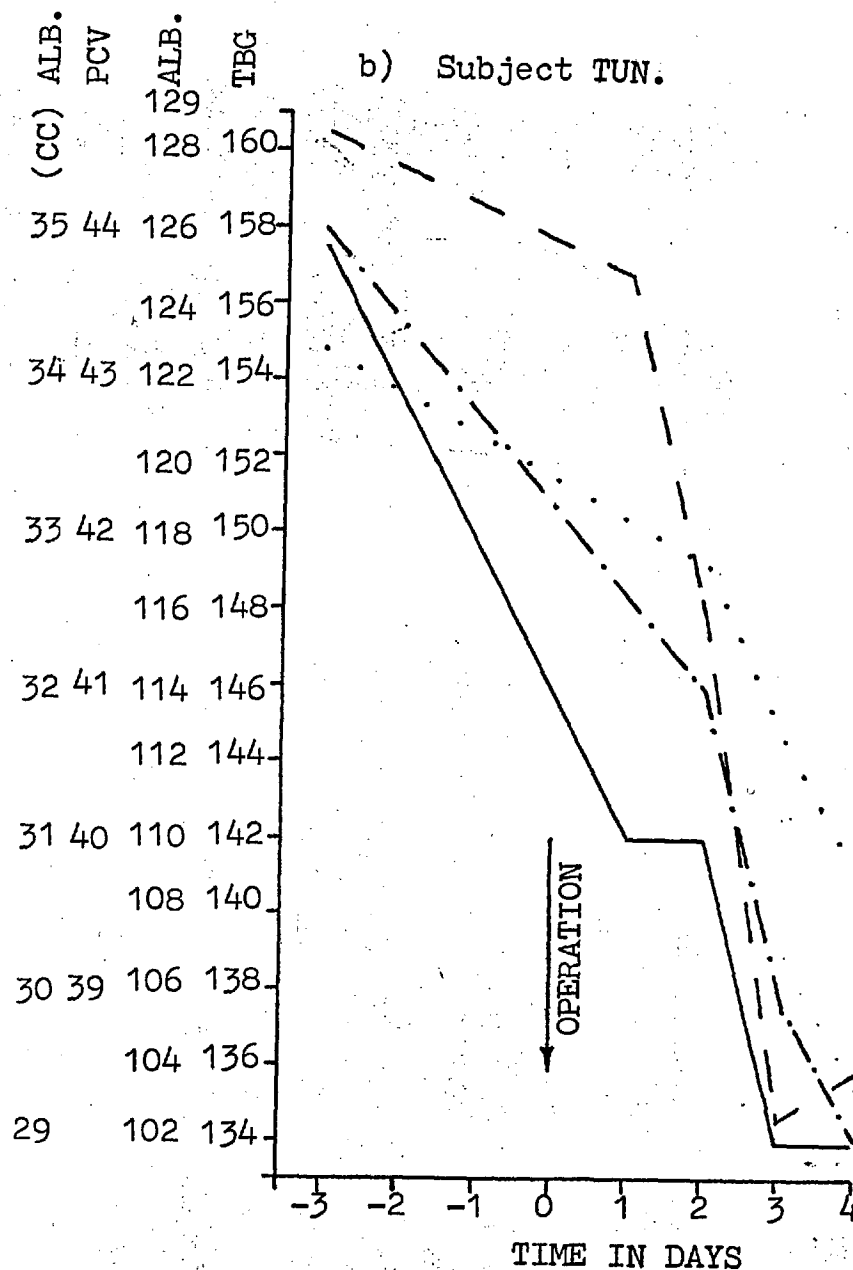
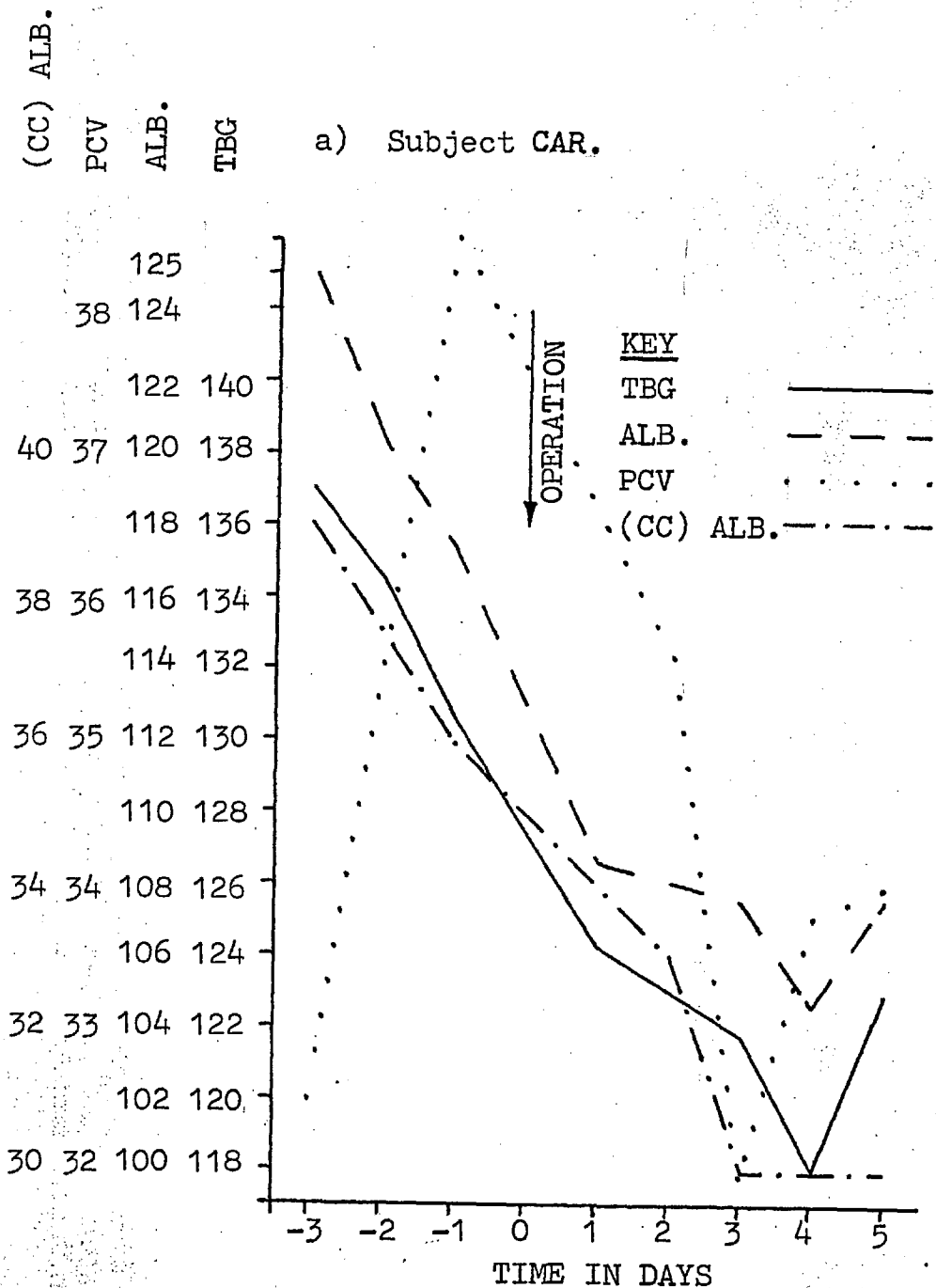
This experiment seems to confirm the results of Levy et al. (1971) and shows that there is no TBG in these individuals, rather than a mutant form of the protein that is unable to bind thyroxine.

4.3.1.9. TBG CHANGES DURING SURGERY

Patients who were to undergo medium grade surgery were bled before operation and if the operation was straightforward, postoperative samples were sent to the laboratory at 1 to 4 day intervals until the patients left hospital. If complications arose either during or immediately after surgery, only one or two samples were sent. Therefore all samples from the individual patients were stored at -20°C until a minimum number of four samples had been collected. The samples from each patient were then run on one Rocket plate. During the five month period of study, 268 samples were collected from 91 patients, but on analysis of the sample data only thirteen patients had four or more samples collected from them. These thirteen individuals form the basis of this study.

The TBG changes during the periods of study are shown in the graphs (Figs. 57a-57m). The Packed Cell Volume (PCV) and chemical albumin estimations are included on the graphs where the information was available.

The general trend of TBG changes was an initial fall of concentration followed by either a slow rise toward pre-operative levels or a continued fall.



CHANGES IN TBG CONCENTRATION
FOLLOWING SURGERY

FIGURE 57

FIGURE 57

CHANGES IN TBG CONCENTRATION
FOLLOWING SURGERY

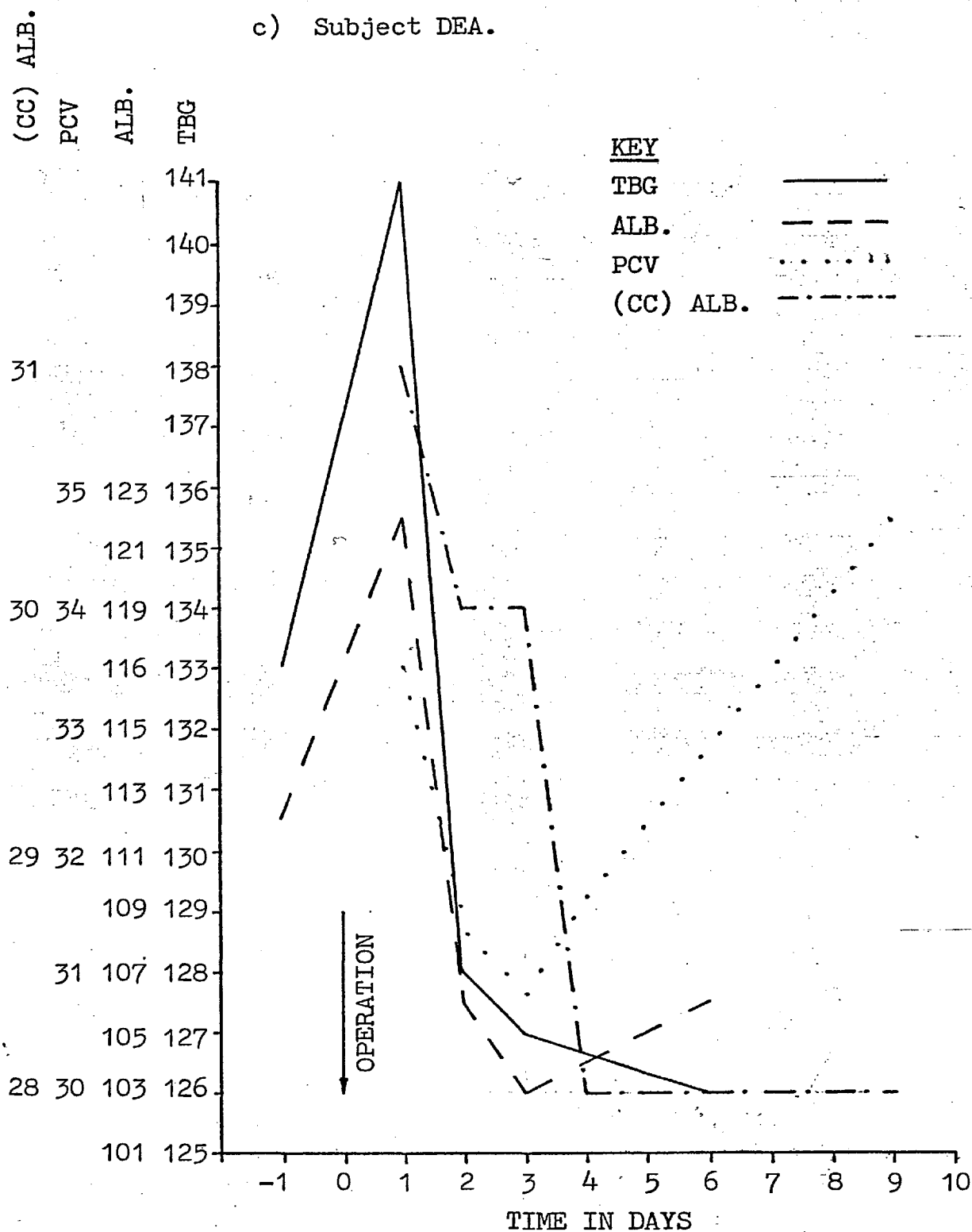


FIGURE 57

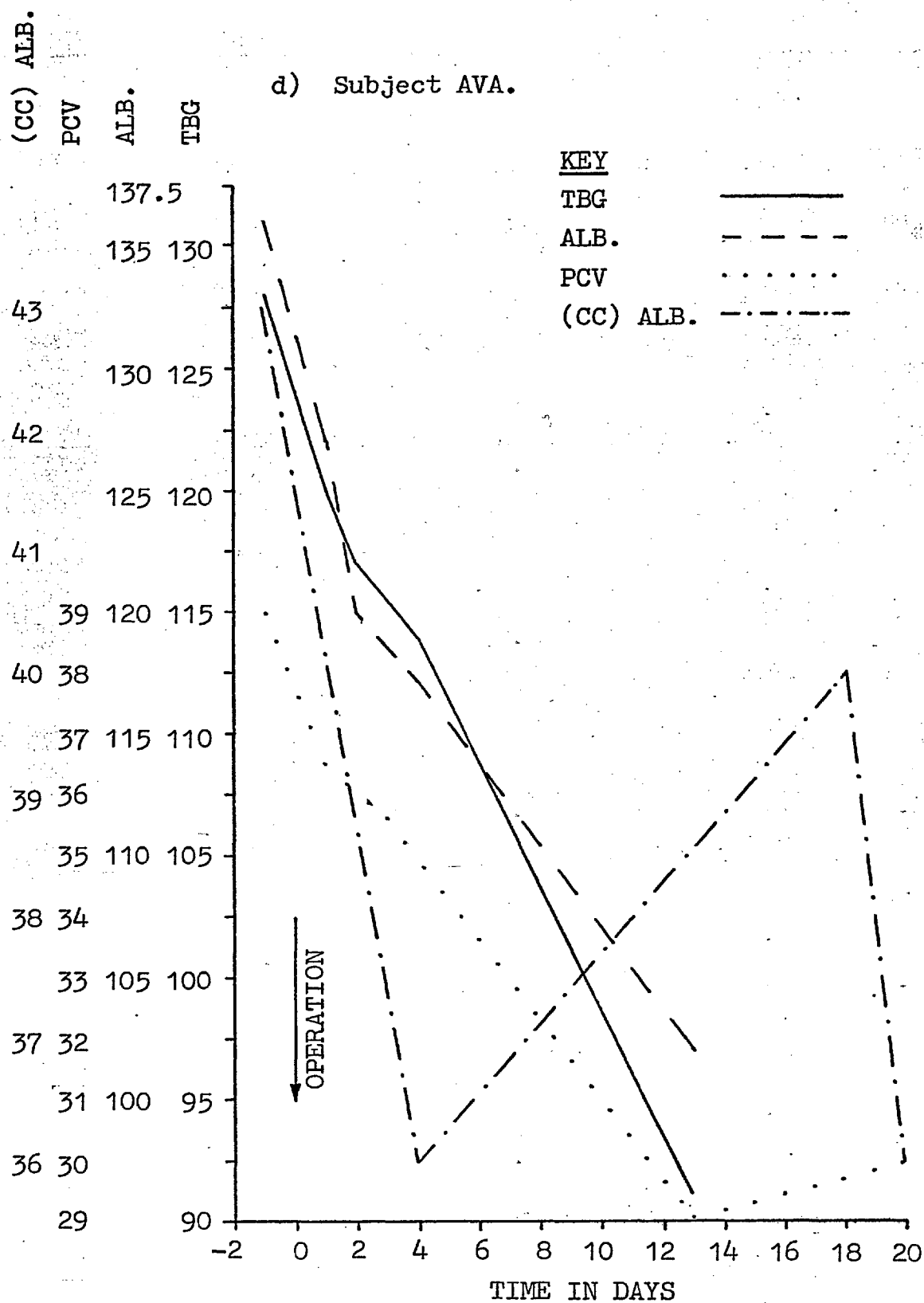
CHANGES IN TBG CONCENTRATIONFOLLOWING SURGERY

FIGURE 57

CHANGES IN TBG CONCENTRATION
FOLLOWING SURGERY

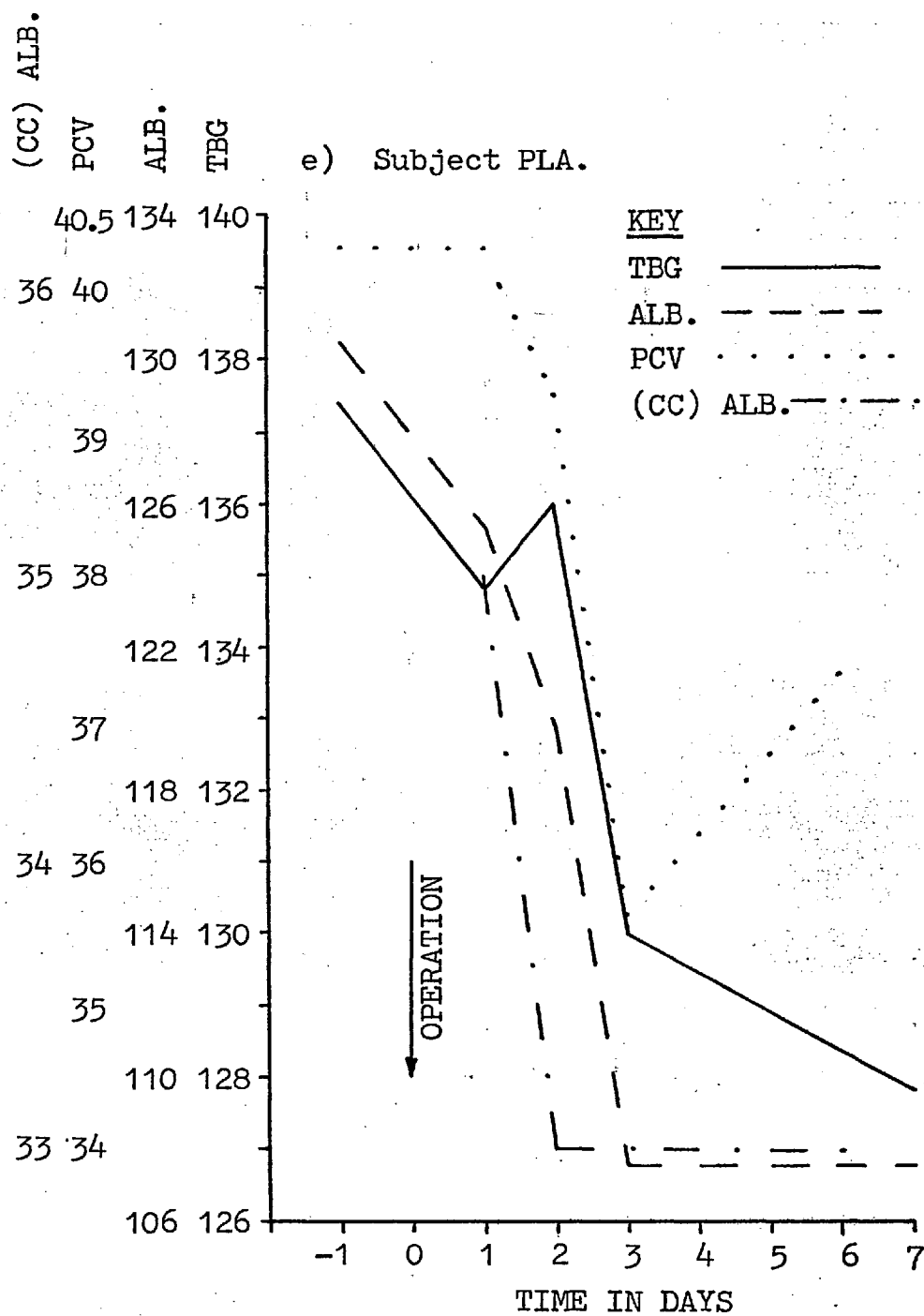


FIGURE 57

CHANGES IN TBG CONCENTRATION
FOLLOWING SURGERY

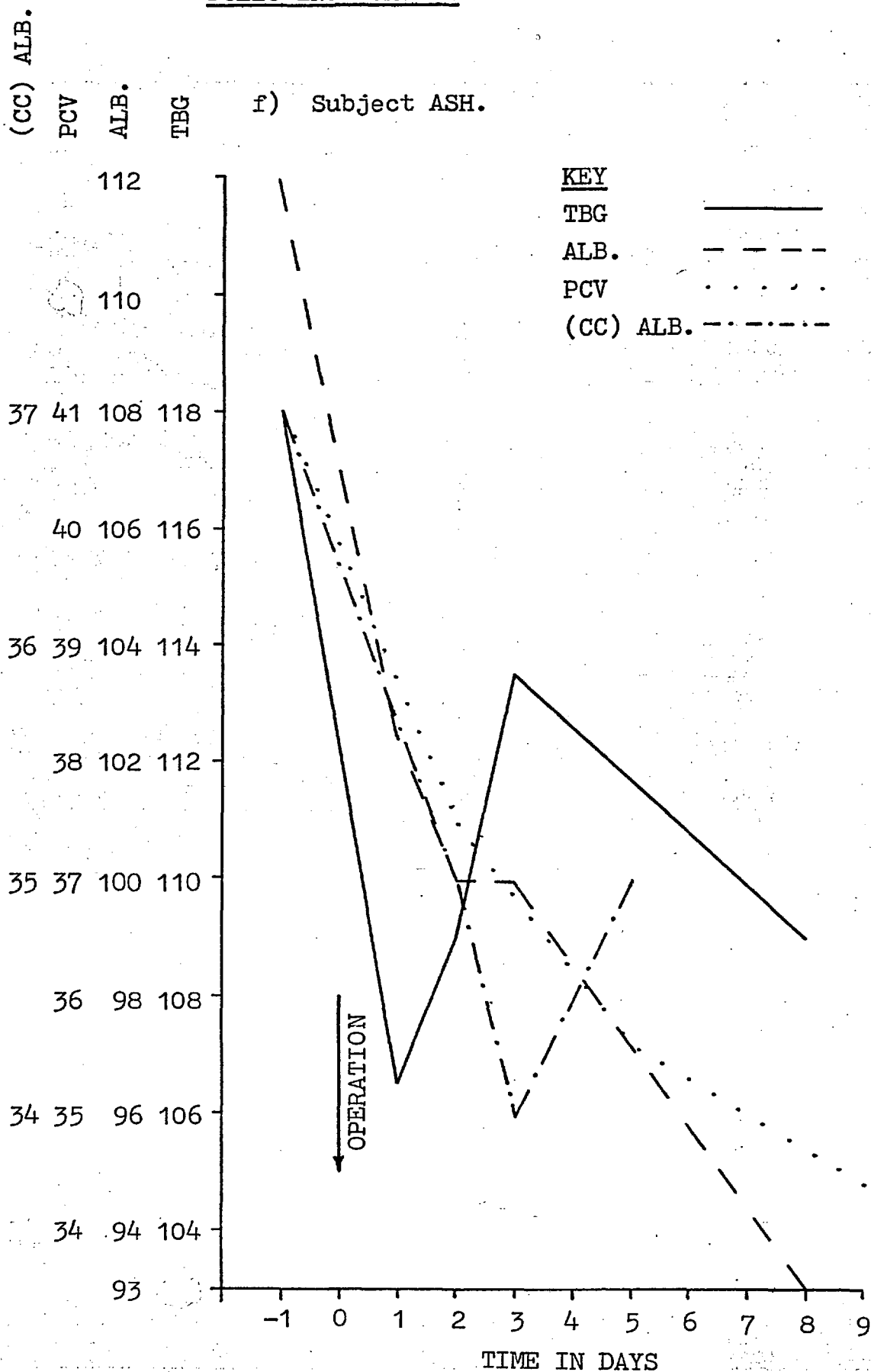


FIGURE 57

CHANGES IN TBG CONCENTRATION
FOLLOWING SURGERY

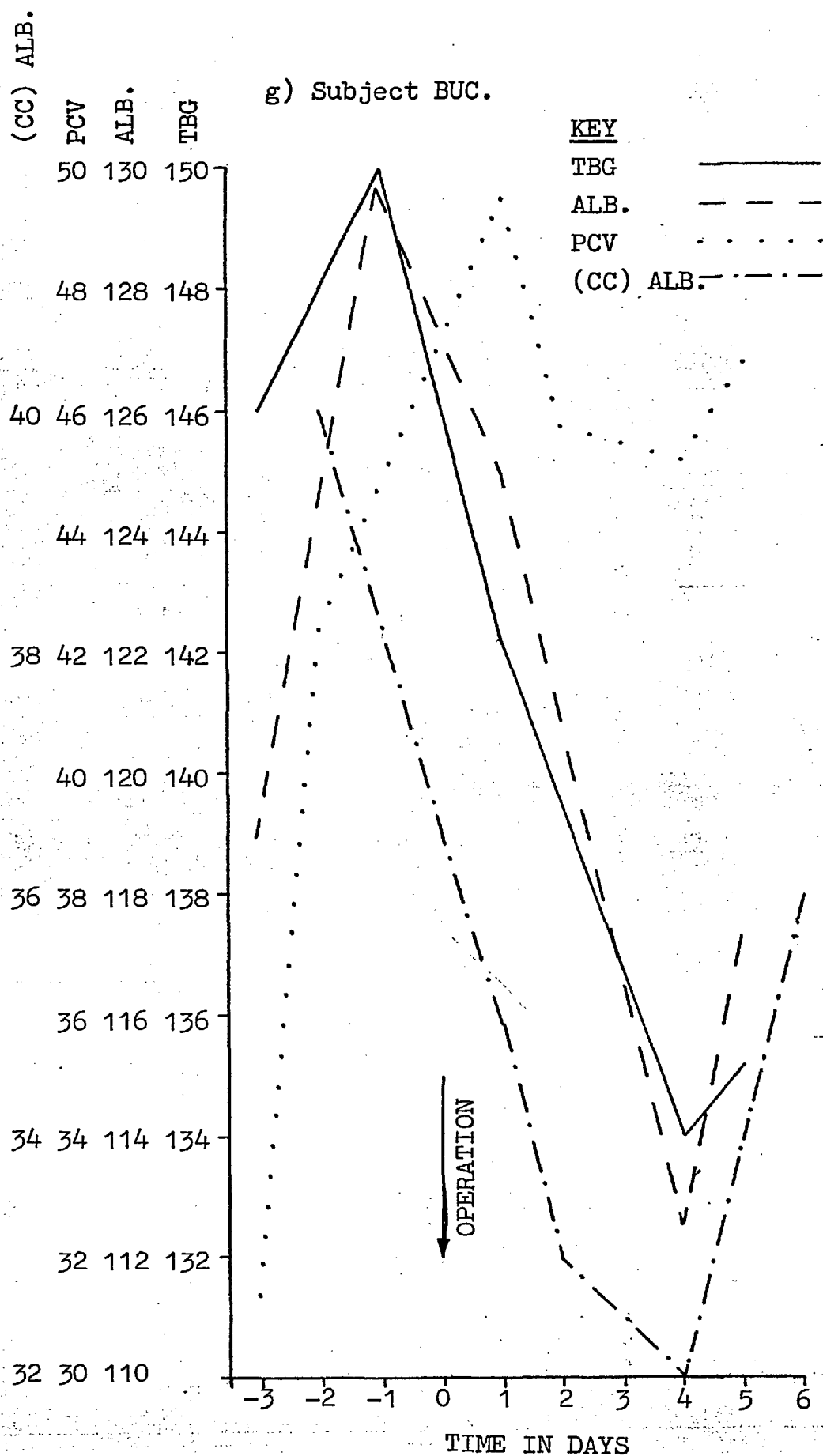


FIGURE 57

CHANGES IN TBG CONCENTRATION
FOLLOWING SURGERY

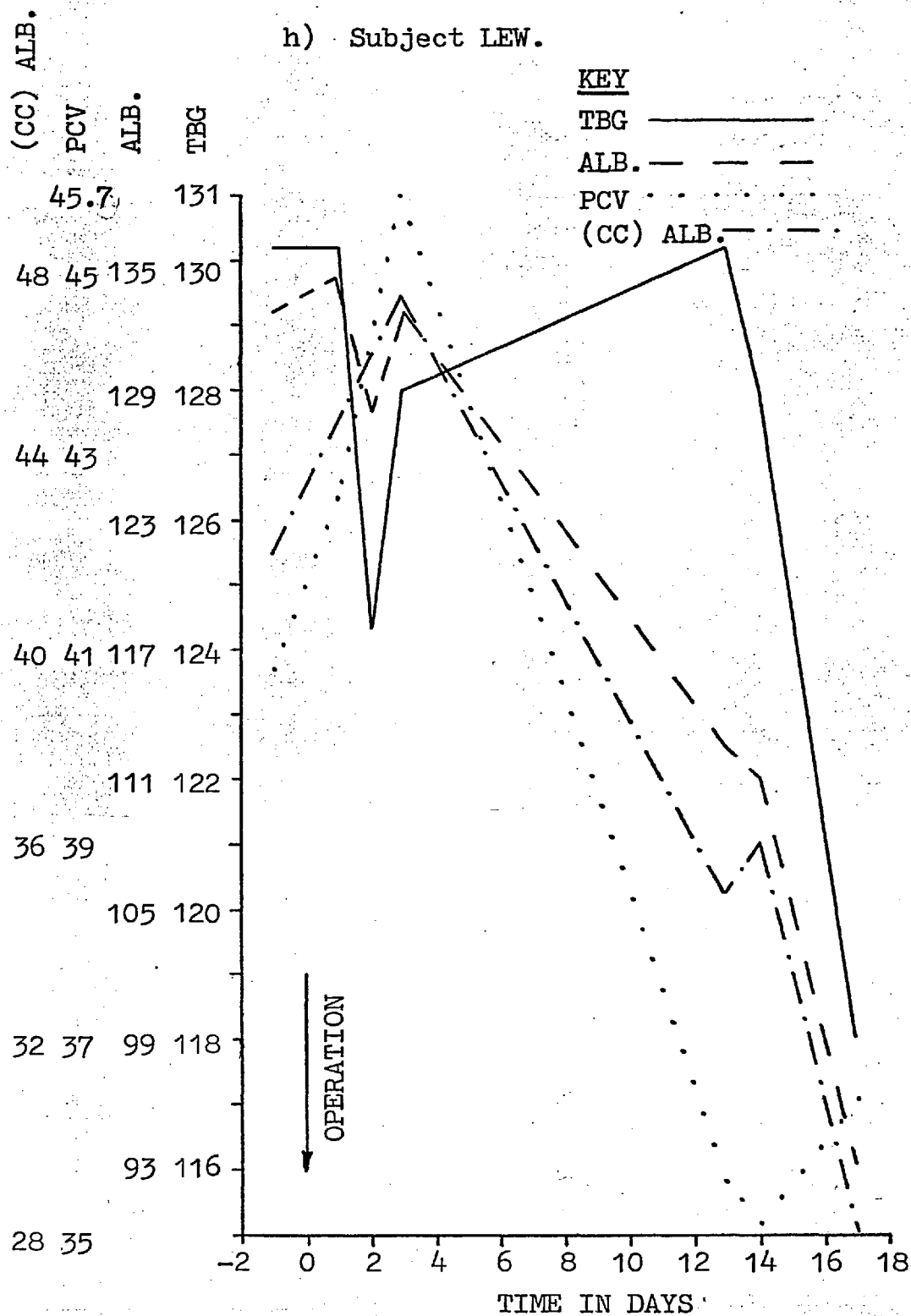


FIGURE 57

CHANGES IN TBG CONCENTRATION
FOLLOWING SURGERY

i) Subject TAY.

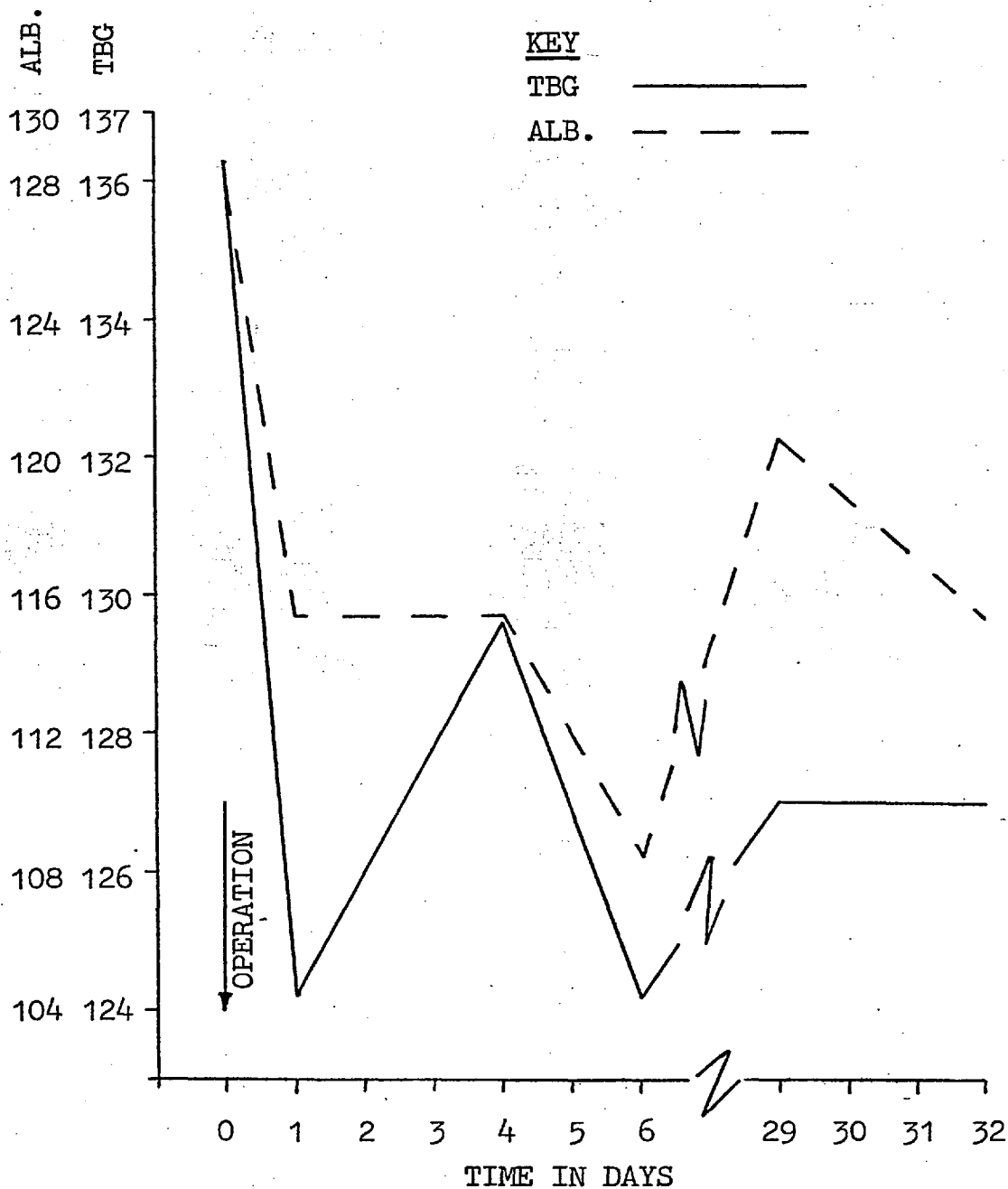


FIGURE 57

CHANGES IN TBG CONCENTRATION

FOLLOWING SURGERY

j) Subject JOH.

KEY

TBG

ALB.

PCV

(CC) ALB.

OPERATION

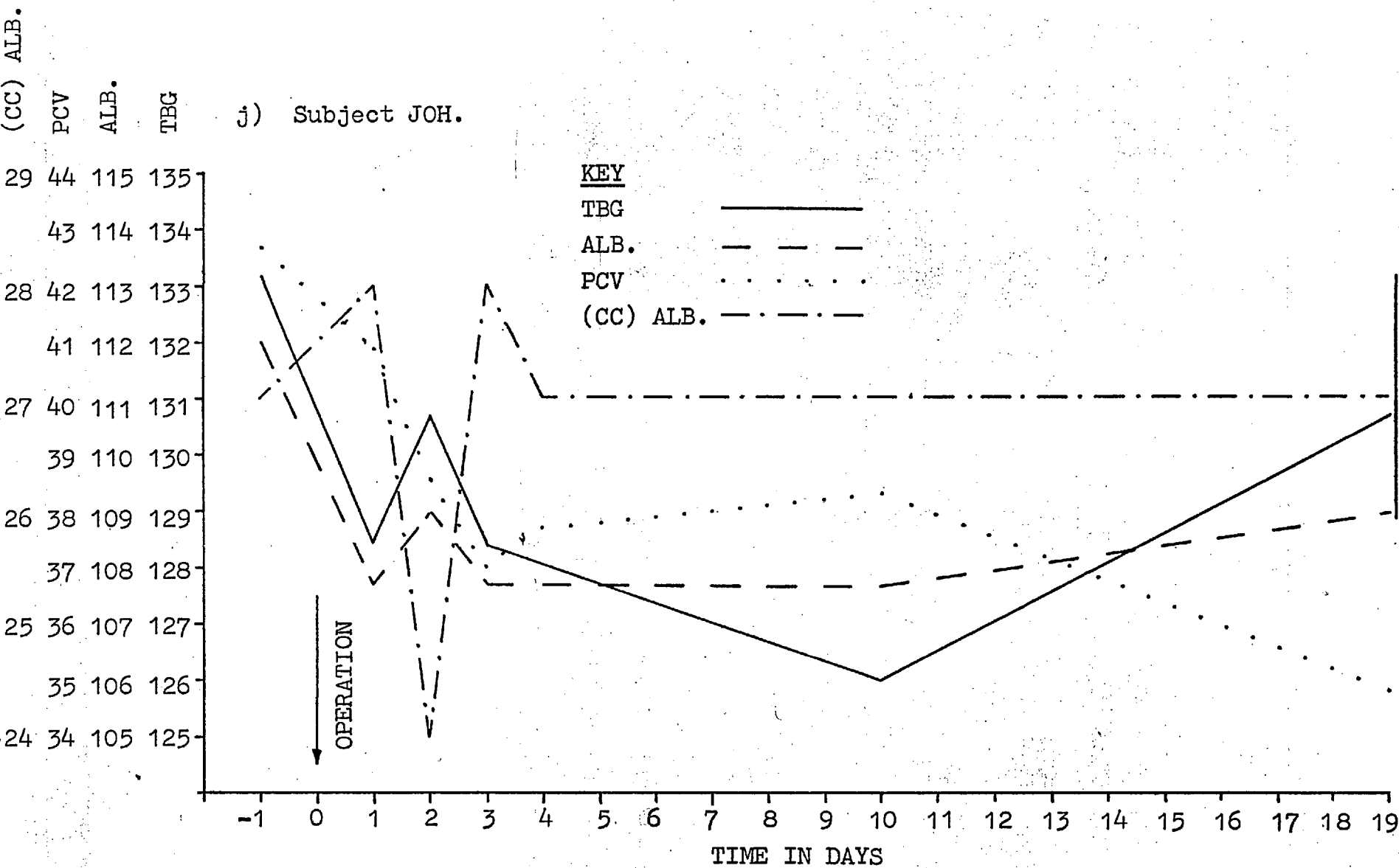


FIGURE 57

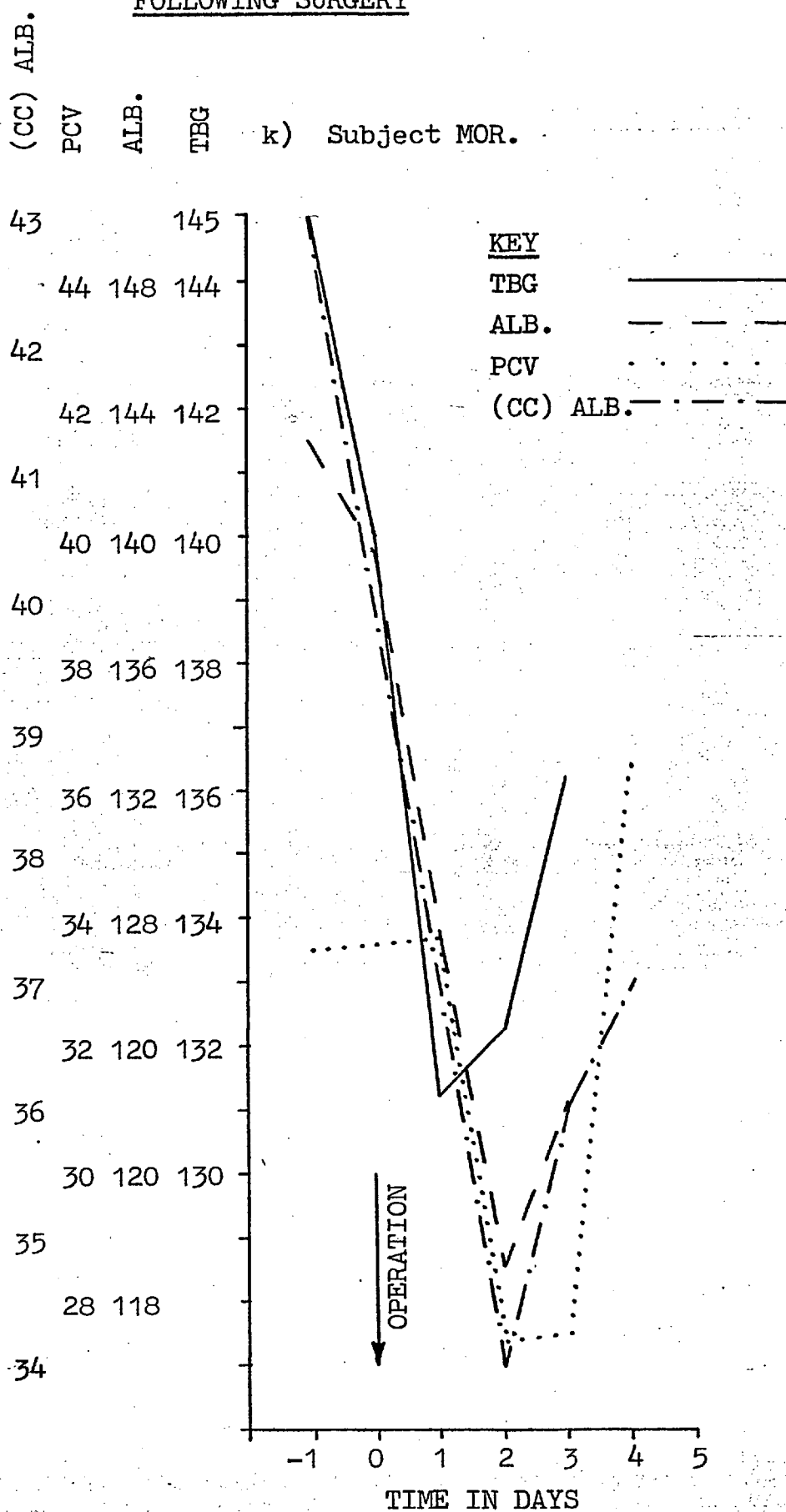
CHANGES IN TBG CONCENTRATIONFOLLOWING SURGERY

FIGURE 57

CHANGES IN TBG CONCENTRATION
FOLLOWING SURGERY

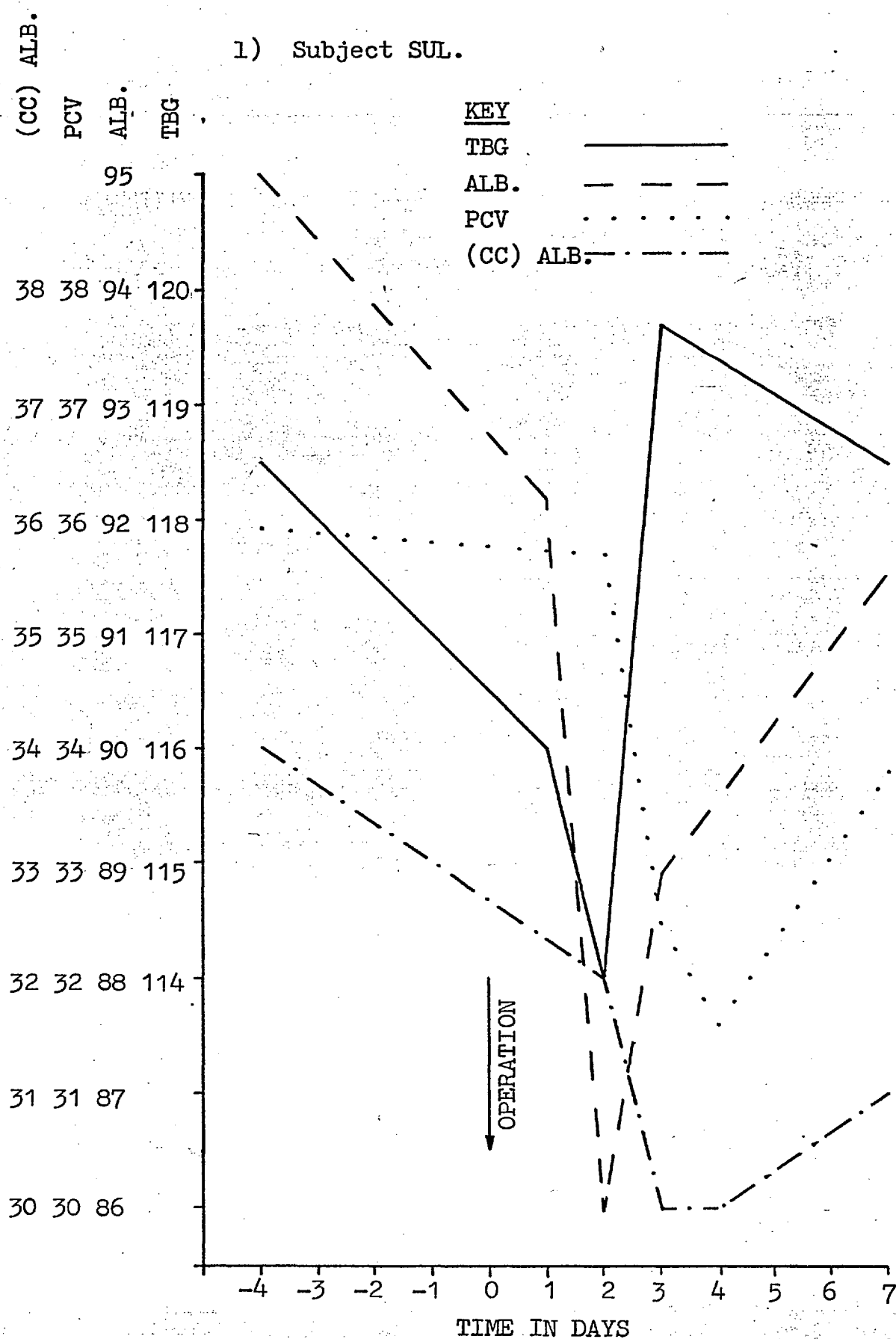
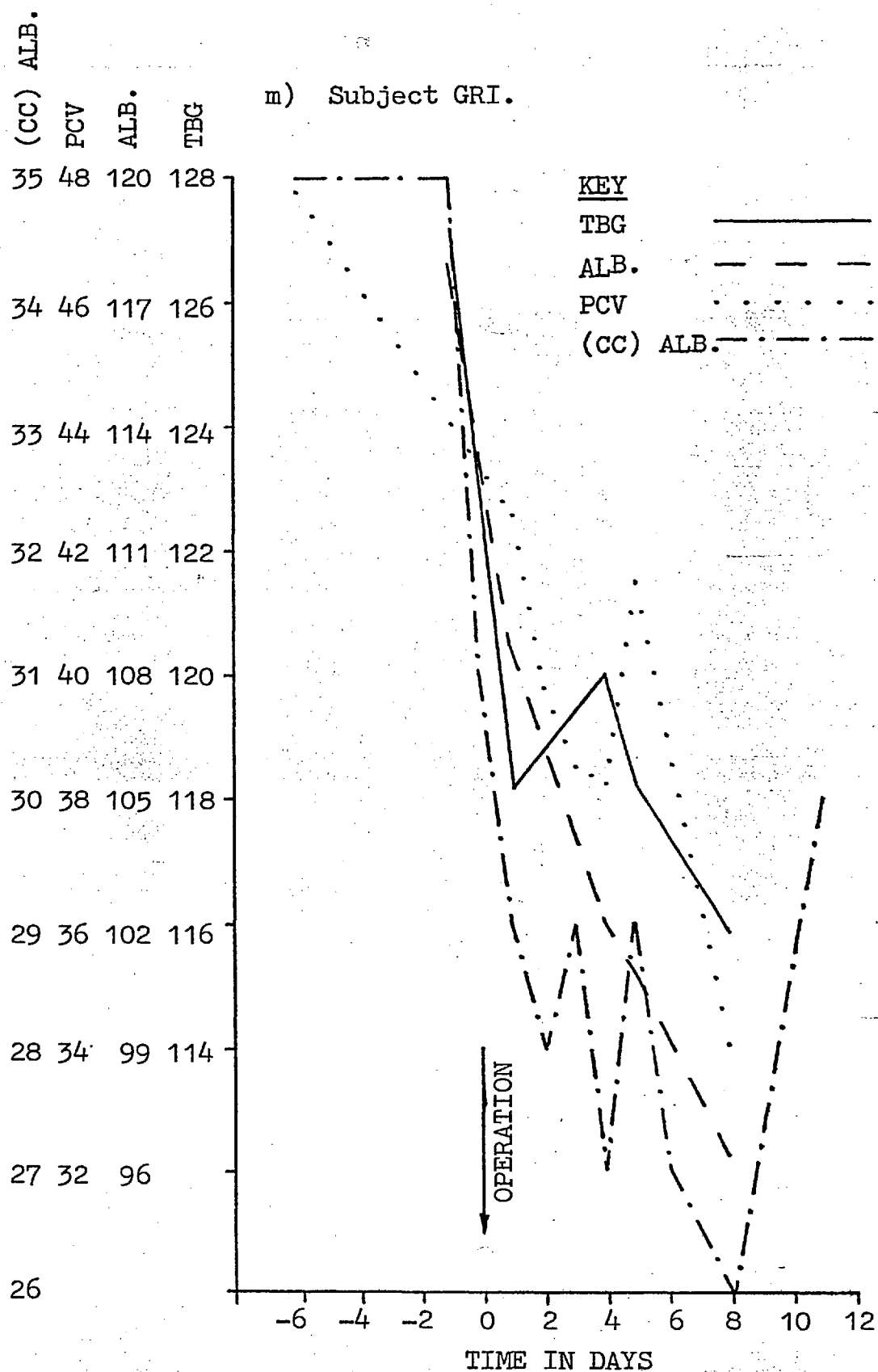


FIGURE 57

CHANGES IN TBG CONCENTRATION
FOLLOWING SURGERY



4.3.1.9.1. DISCUSSION OF TBG CHANGES DURING SURGERY

Maximum Binding Capacity (M.B.C.) measurements for indirect estimation of TBG have usually found no significant change in TBG concentration in patients after surgery (Johnston, 1972; Kirby, Clarke, & Johnston, 1973; Surks & Oppenheimer, 1964), though changes in TBPA concentration have been detected. The well documented increase in free thyroxine after surgery has been associated with the TBPA fall and with increased thyroxine output by the thyroid (Kirby et al., 1973; Harland, Orr & Richards, 1972), but since TBG is the major thyroxine binding protein in serum (circa 75%) smaller changes in its concentration would significantly affect the free hormone level. A TBG change has been postulated by Kirby et al. (1973) who indicated that the rise they found in free T_4 was not explainable in terms of their measured TBPA decrease alone, but that alteration of TBG binding occurred, which their method was unable to measure. The changes in TBG found in this study were diverse and not always accompanied by changes in albumin or packed cell volume, an observation supported by Harvey (1971), and indicating that the TBG changes are not only explainable by changes in plasma volume.

The different patterns of TBG changes in the thirteen patients of the present study are presumably related to the severity of the initial conditions, the use of drugs and in particular fluid infusions or anti-diuretics, and the individual responses to stress, all of which are known to affect serum thyroxine and TBG concentrations (Harvey, 1971; Hays & Soloman, 1969; Woeber & Ingbar, 1963).

Although this study demonstrated real TBG changes in

patients after medium grade surgery, a more comprehensive study is required to establish exactly what the changes are, why they occur and what the affects are within the patient as a result of the consequent free thyroxine changes.

4.3.2. INTRODUCTION TO MODIFIED LINE

TECHNIQUE RESULTS

The Modified Line Technique as described in Section 4.2.3.4. has been used to investigate the relationship between total thyroxine concentration in serum and the percentage of this hormone bound to TBG. This investigation was performed to attempt to throw some light on the nature of the circulating hormone in vivo under different physiological conditions of hormone concentration.

4.3.2.1. THE EFFECT OF INCREASING TOTAL THYROXINE CONCENTRATIONS ON BINDING DISTRIBUTION

The Modified Line Technique was used to determine how the thyroxine concentration in serum affected the percentage binding of hormone to TBG. Radiolabelled thyroxine ($^{125}\text{I T}_4$) was equilibrated with serum samples in concentrations between 20 and 4200 nmol/l, although most studies were made within the usual clinical range of 20 to 350 nmol/l.

The major part of the study involved four sera, two of which were euthyroid and two hypothyroid. The two hypothyroid sera were chosen so that total hormone levels could be maintained below the normal concentration of 100 nmol/l, and sub-physiological hormone distribution studied. To compare naturally occurring hypothyroid serum with an artificially produced thyroxine deficient serum, a normal serum (I.D.) was stripped of endogenous hormone (Section 3.3.1.). To investigate the "fit" of a wider range of sera to the binding pattern, seven random euthyroid sera were included in the study.

Experimental Procedures

All samples were treated in like manner. The sera were equilibrated with radiolabelled thyroxine ($^{125}\text{I T}_4$) at room temperature for one hour and then mixed with phosphate pH 7.4 buffer and molten agarose to form the sample gel. Modified Line plates were then prepared and run in the phosphate system for 15 hours. The electrophoresed plates were cut up and counted (Section 4.2.3.4.).

The results were considered in terms of the total hormone content after the addition of radiolabelled hormone,

and the recovered counts on the samples were plotted as percentages of the total hormone recovered.

i) Subphysiological Thyroxine Level Distribution

The naturally occurring hypothyroid sera (A.S. and B.T.) were compared with the artificially produced thyroxine deficient serum (I.D.) at 20 nmol/l and 50 nmol/l concentrations of radiolabelled thyroxine. The B.T. serum (15 nmol/l) was compared with the stripped I.D. serum at a concentration of 20 nmol/l and the A.S. serum (24 nmol/l) was compared with the stripped I.D. serum at a concentration of 50 nmol/l of hormone.

At 20 nmol/L the activity centred about one main peak, TBG, in both the B.T. and stripped I.D. serum. The TBG bound 84% of the hormone in B.T. serum and 79% in the stripped I.D. serum. At 50 nmol/l the activity again centred on TBG which bound in the hypothyroid serum, A.S., 81.5% (81% & 82%) and stripped, I.D. serum, 78% (Figs. 58i, 58ii, 59i, 59ii, 60i, 60ii, 61i, 61ii, 61iii).

In the two naturally occurring hypothyroid sera (A.S. and B.T.) the remainder of the exogenous hormone was bound to a protein or proteins in the α_1 lipoprotein position between TBG and albumin. Albumin, TBPA and α_1 lipoprotein all carried trace amounts of hormone shown only by autoradiography of the plates when the counting time is considerably increased.

The stripped serum (I.D.) did not display the secondary α_1 lipoprotein peak, and the remaining activity was distributed more to the cathodal side of TBG. The stripping process involving the Aminex resin may have removed the large lipoprotein molecules thereby eliminating their binding potential. (see Discussion).

FIGURE 58

DISTRIBUTION OF RADIOLABELLED THYROXINE AT LOW TOTAL THYROXINE CONCENTRATIONS USING A NORMAL SERUM EITHER STRIPPED OF ENDOGENOUS HORMONE OR UNSTRIPPED.

a) Distribution of radiolabel with Exogenous Thyroxine concentration of 20 nmol /l.

i) Using a Stripped (I.D.) Serum.

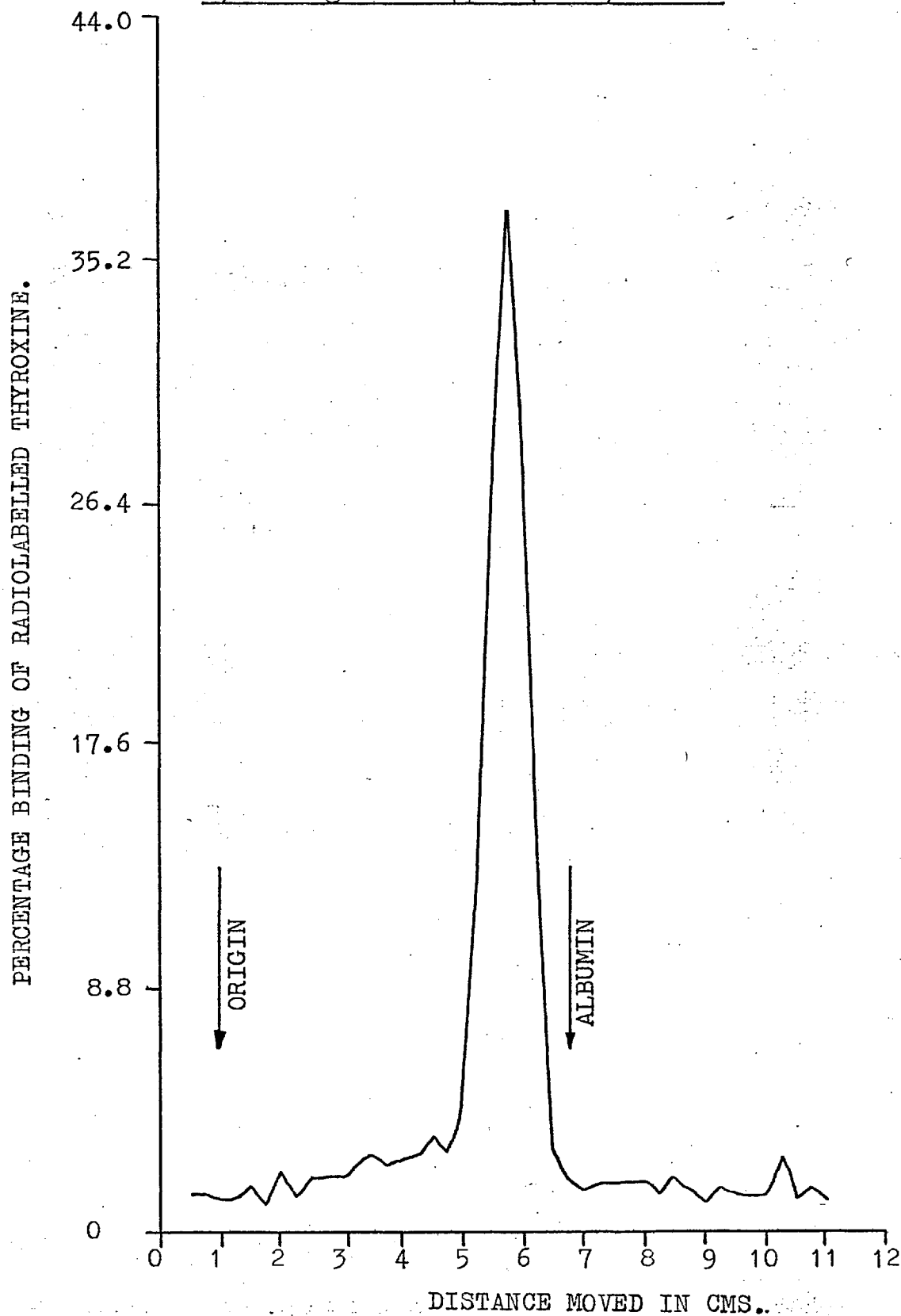


FIGURE 58

a) Distribution of Radiolabel with Exogenous Thyroxine
concentration of 20 nmol/l.

ii) Using an Unstripped (I.D.) Serum.

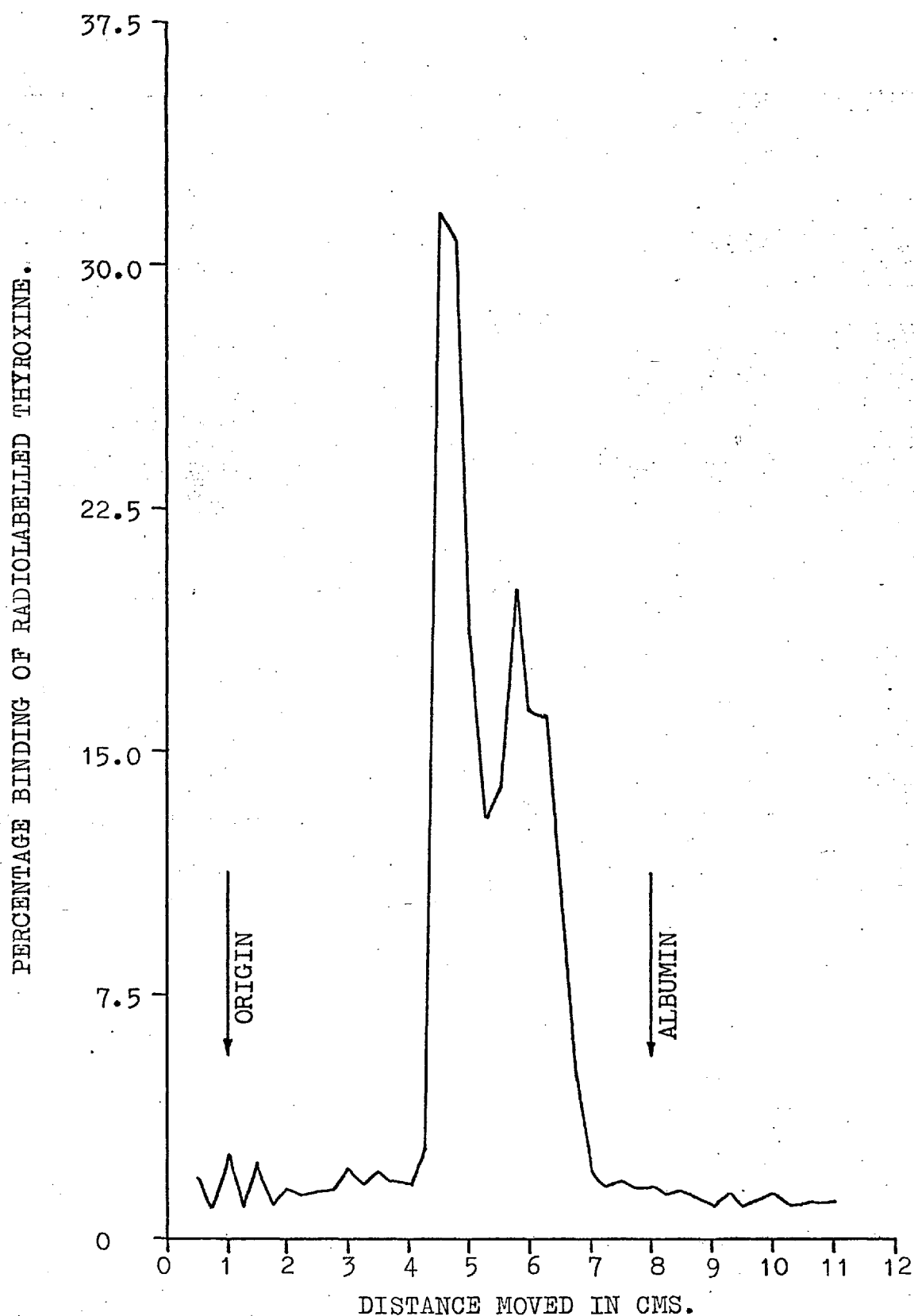


FIGURE 59

b) Distribution of Radiolabel with Exogenous Thyroxine
concentration of 50 nmol/l.

i) Using a Stripped (I. D.) Serum.

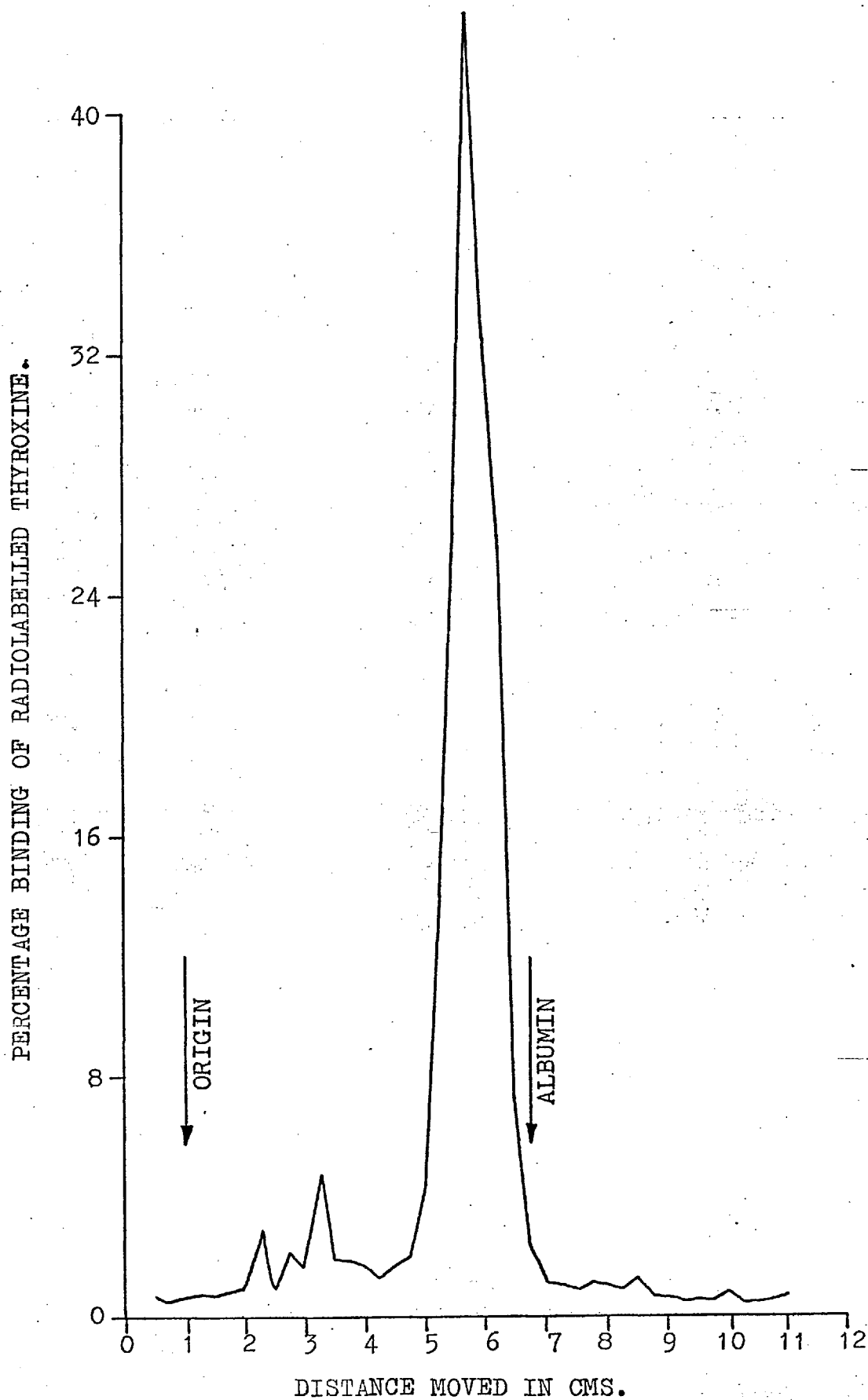
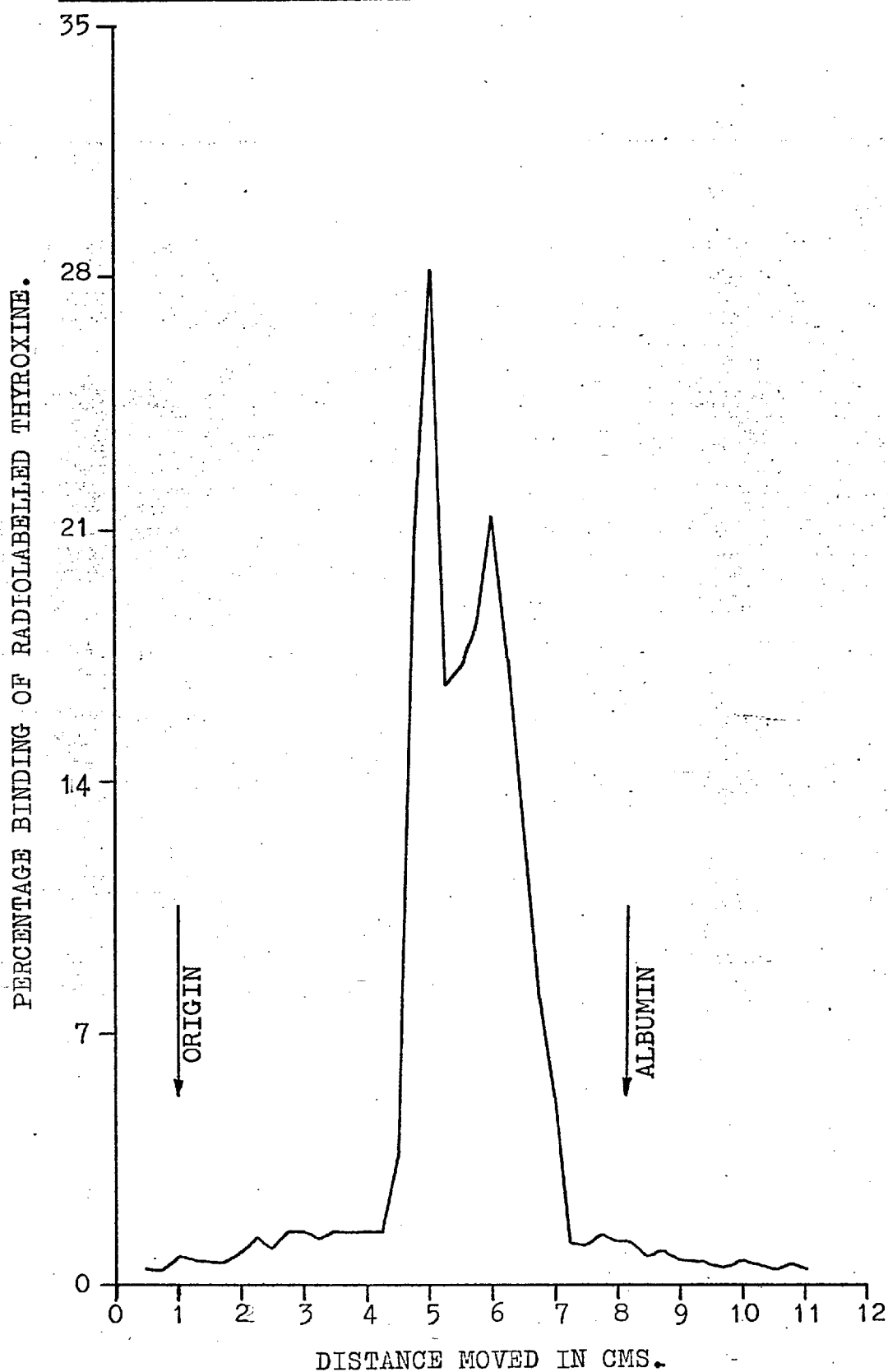


FIGURE 59

b) Distribution of Radiolabel with Exogenous Thyroxine
concentration of 50 nmol/l.

ii) Using an Unstripped (I.D.) Serum.



ii) Physiological Hormone Level Distribution

The sera used for this study fell into two groups;

- a) those which initially contained a subphysiological level of thyroid hormone, and
- b) those which initially contained a physiological level of thyroid hormone.

Both sets of sera were equilibrated with radiolabelled thyroxine to give final, total, hormone concentrations between 100 and 125 nmol/l.

a) Three sera with endogenous thyroxine concentrations below 25 nmol/l were chosen. These sera were the two hypothyroid sera B.T. (15 nmol/l) and A.S. (24 nmol/l) and the stripped serum I.D. Each of these sera were equilibrated with a physiological level of radiolabelled thyroxine to give final, total concentrations of 115 nmol/l (B.T.), 124 nmol/l (A.S.) and 100 nmol/l (I.D.) and each was run under similar conditions in the phosphate pH 7.4 system. After electrophoresis the plates were cut up and counted, and the results plotted graphically (Figs. 60i, 60ii, 61 & 31b).

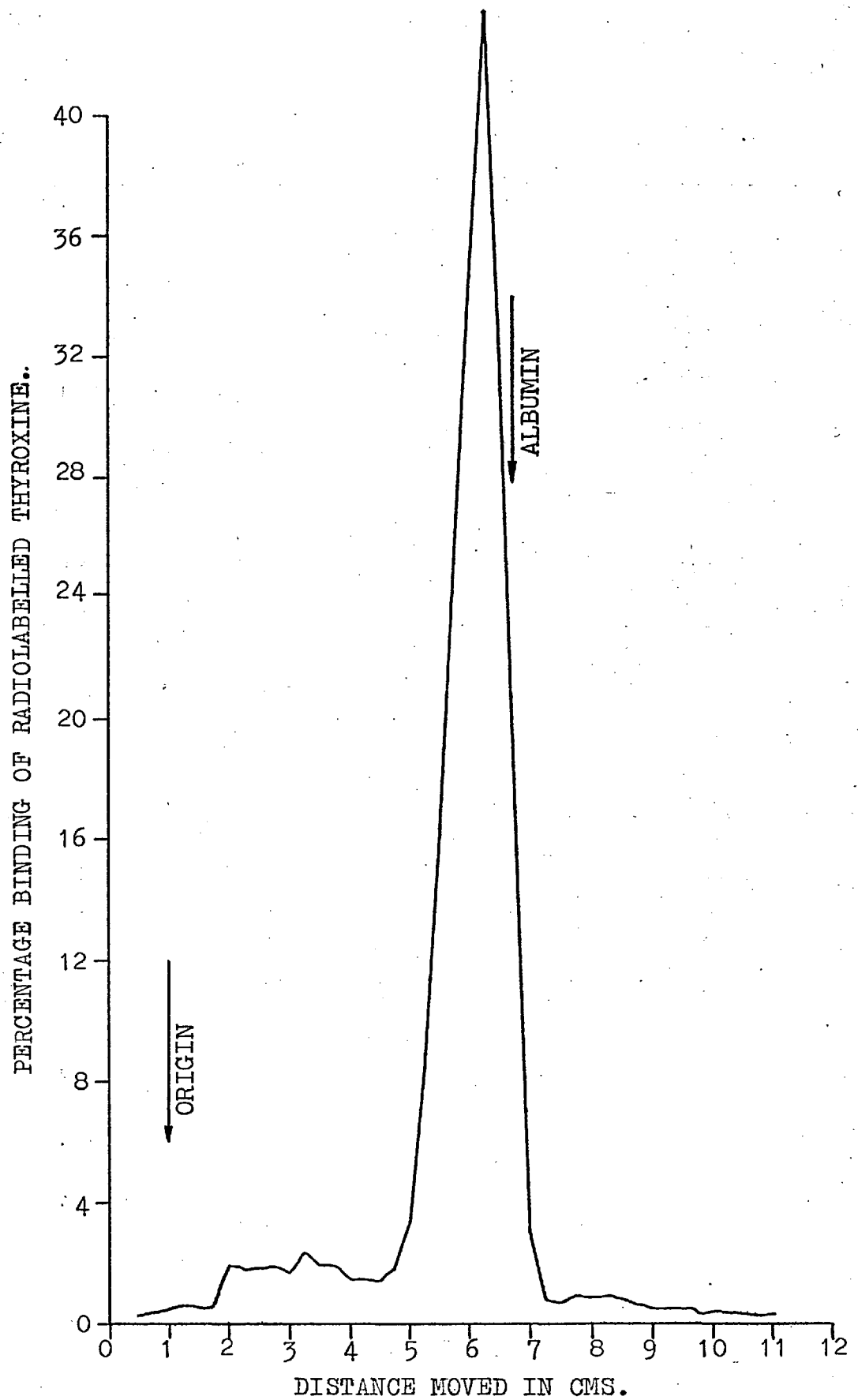
On each plate 100% of the radiolabel was recovered and the major binding protein, TBG bound 76% (I.D.), 65% (A.S.) and 61% (B.T.) of the hormone. The I.D. serum, although it possessed a lower TBG concentration (116%) than the other two sera (A.S. 130%, B.T. 151%), as shown by Modified Rocket analysis, showed a higher TBG binding. This was probably due to the thyroxine stripping method, possibly removing α_1 lipoprotein from the I.D. serum, which binds thyroxine at physiological levels of hormone, and because the two hypothyroid sera contained higher levels of total hormone.

b) Five serum samples (I.D., P.P., T.F.E., O.H., O.F.S.) were selected with normal physiological levels of thyroid

c) Distribution of Radiolabel with Exogenous
Thyroxine concentration of 100 nmol/l.

240

i) Using a Stripped (I.D.) Serum.



- c) Distribution of Radiolabel with Exogenous
Thyroxine concentration of 100 nmol/l.
ii) Using an Unstripped (I.D.) Serum.

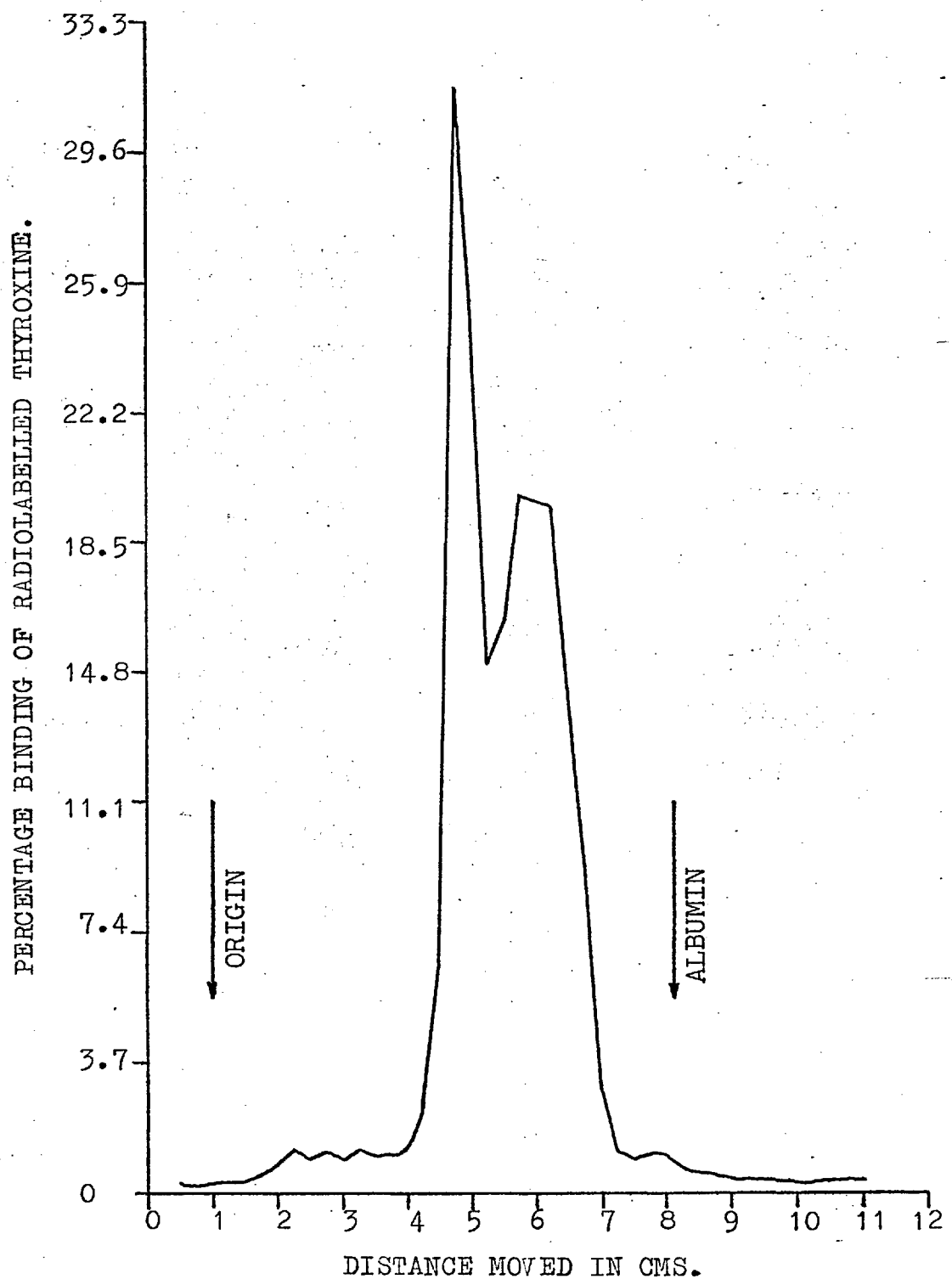


FIGURE 61

d) Distribution of Radiolabel using an Hypothyroid Serum with an Endogenous Thyroxine Concentration of 15 nmol/l.

i) Distribution of Radiolabel using a Total Thyroxine Concentration of 35 nmol/l.

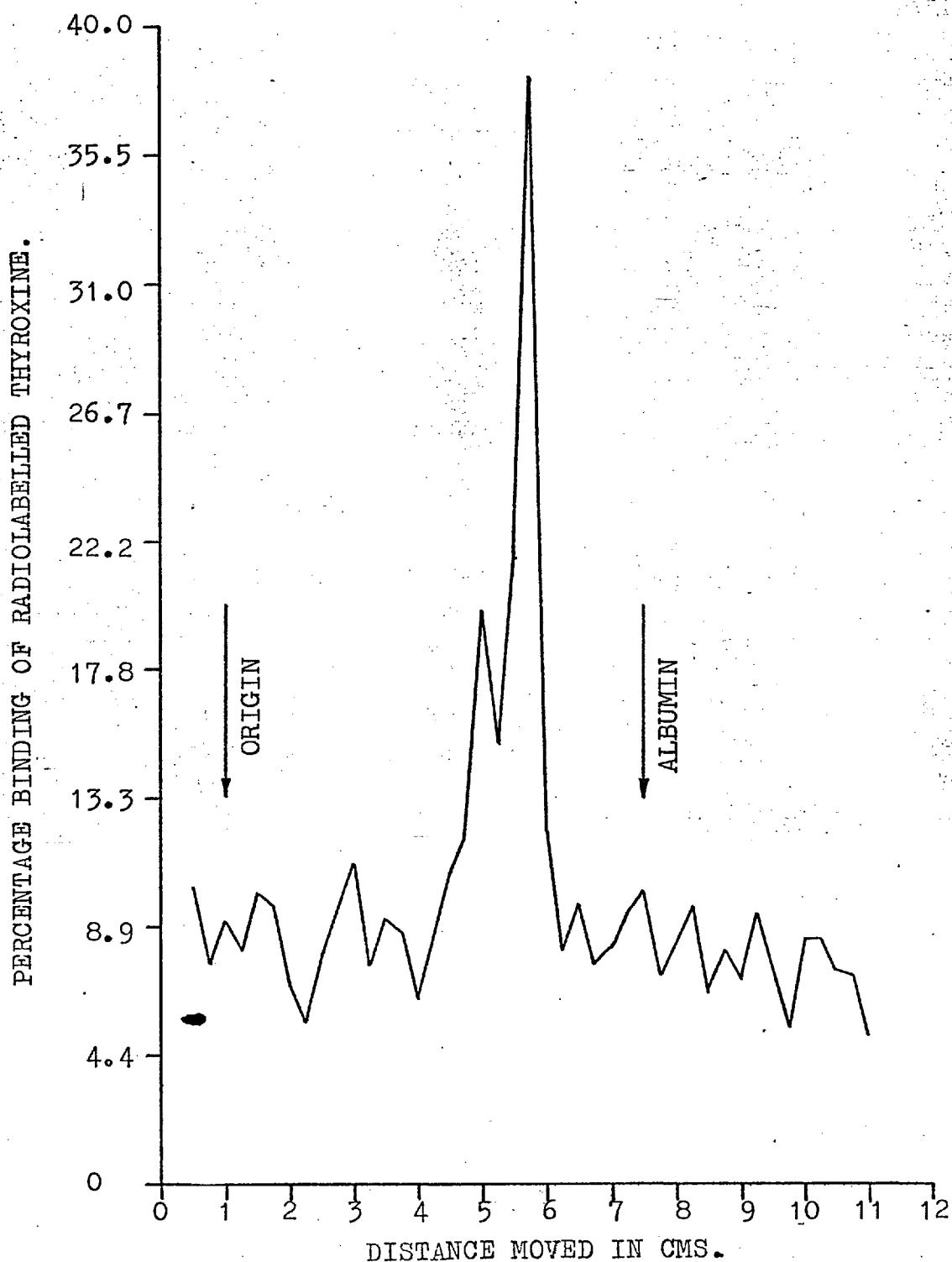
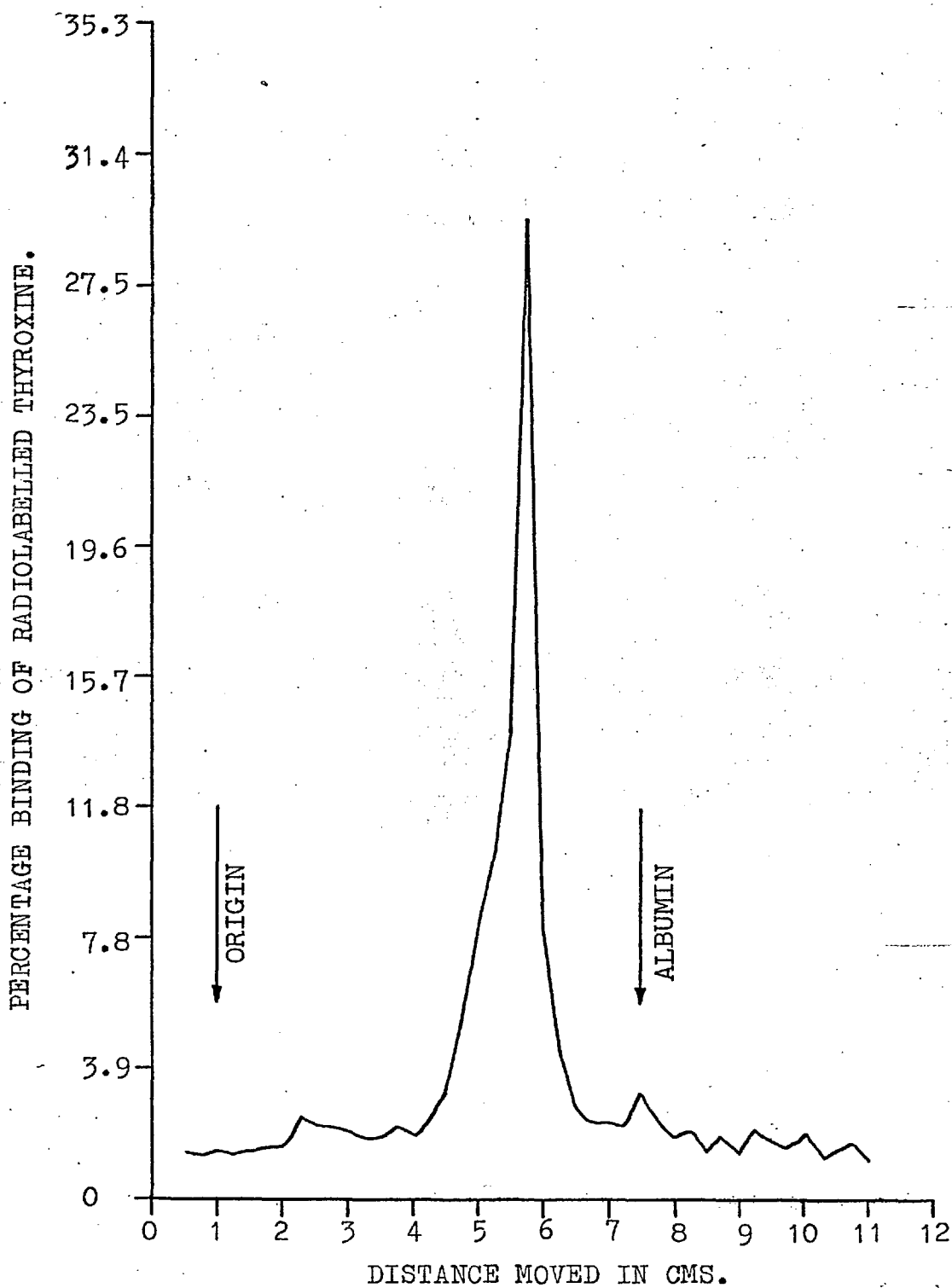


FIGURE 61

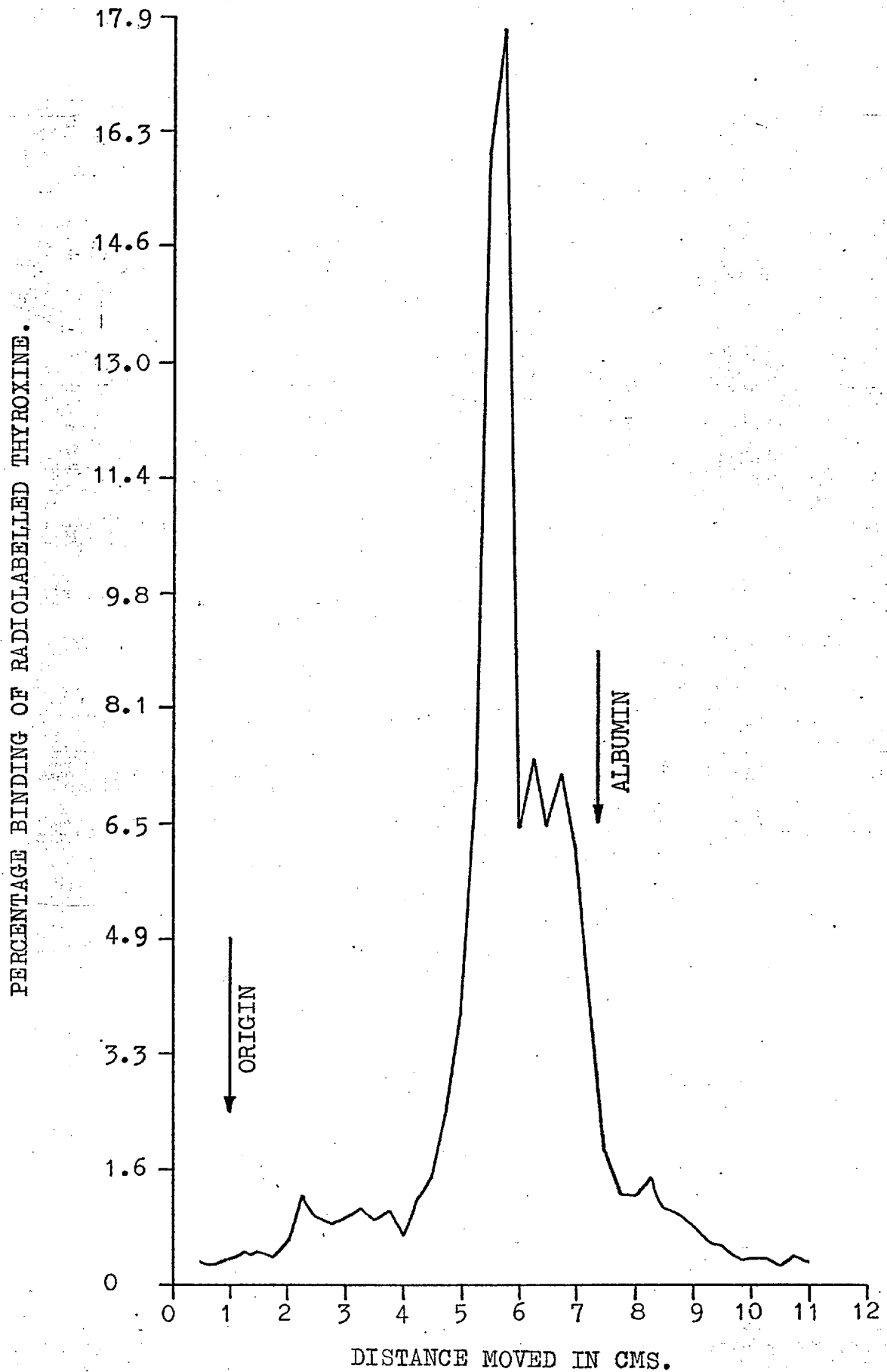
d) Distribution of Radiolabel using an Hypothyroid Serum with an Endogenous Thyroxine Concentration of 15 nmol/l.

ii) Distribution of Radiolabel using a Total Thyroxine Concentration of 115 nmol/l.



d) Distribution of Radiolabel using an Hypothyroid
Serum with an Endogenous Thyroxine Concentration
of 15 nmol/L.

iii) Distribution of Radiolabel using a Total Thyroxine
Concentration of 515 nmol/L.



hormone and each was equilibrated with 20 nmol/l of radio-labelled thyroxine.

Using the unstripped I.D. serum, ten plates were run at different times and the results were collected and a mean value calculated and plotted on the graph (Fig. 65). On each of these plates 100% of the radioactivity was recovered and the TBG bound a mean of 53% of the labelled hormone.

The serum of P.P. after equilibration with radiolabelled thyroxine and electrophoresis was found to have 63% of the activity associated with TBG. The sera of T.F.E., O.H. and O.F.S. were found to have similar hormone binding to TBG and the mean values of duplicate determinations were 65% (T.F.E.), 58% (O.H.) and 61% (O.F.S.). On each of these plates 100% of the original radiolabel was recovered.

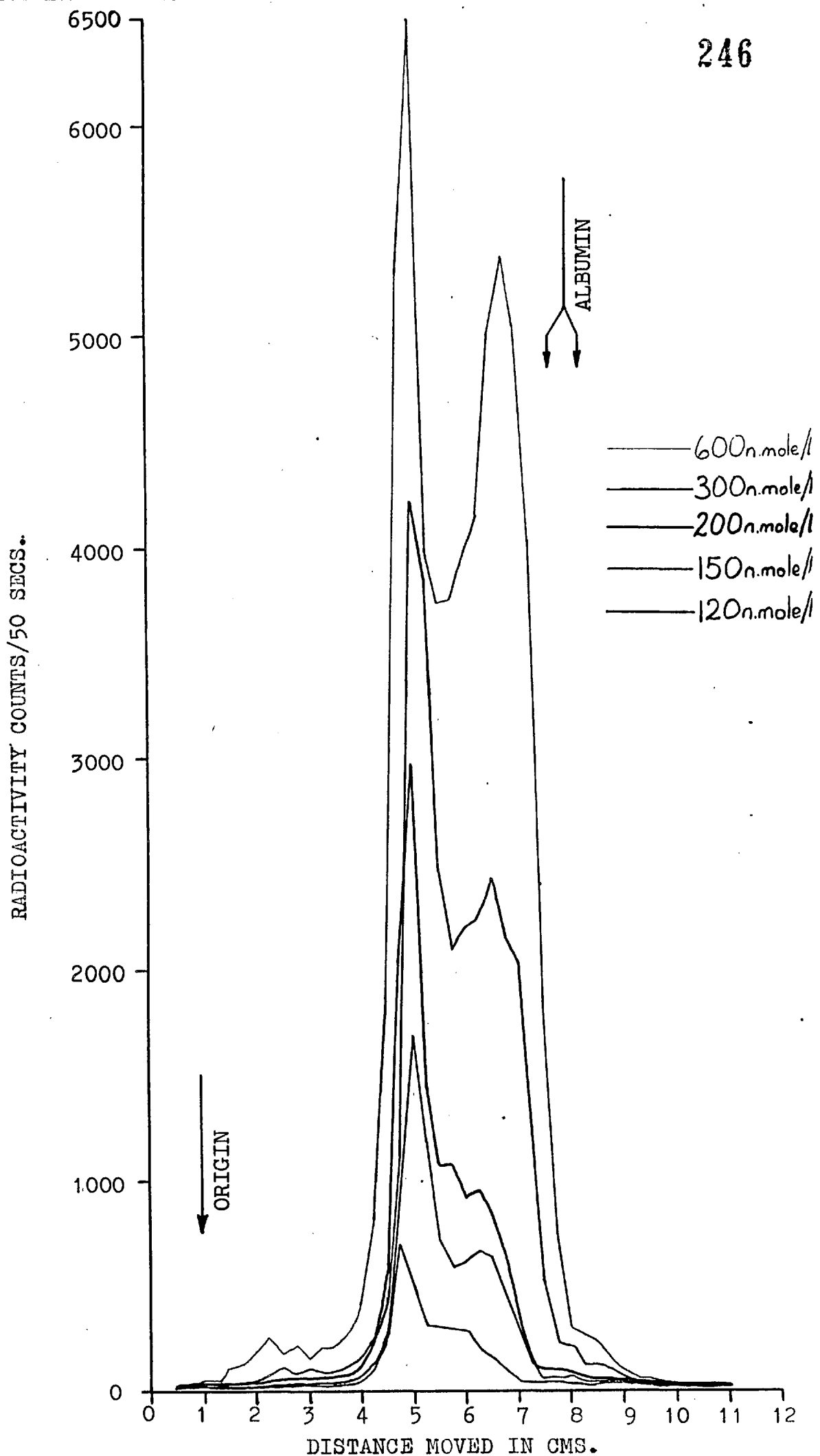
The results are shown in Table 16 and plotted graphically (Figs. 62, 65 & 66).

iii) Hyperthyroid Hormone Level Distribution

The usual maximum level of thyroxine found clinically is in the region of 300-350 nmol/l which is 3-3.5 times the accepted mean normal thyroxine concentration. Radiolabelled thyroxine was equilibrated with six sera to give total hormone concentrations up to and just above this level giving a range of total hormone from 150 to 600 nmol/l.

Two euthyroid subjects' sera were equilibrated with radiolabelled hormone to give final concentrations of 150, 200, 300 and 600 nmol/l. On each plate 100% of the radio-labelled thyroxine was recovered. However the percentage binding of hormone to TBG differed both between the individual sera and between the levels of exogenous hormone added.

FIGURE 62 : DISTRIBUTION OF RADIOLABELLED THYROXINE CN IN-
CREASING THE TOTAL HORMONE CONCENTRATION (120-600 nmol/l)
USING THE SAME EUTHYROID SERUM (P.P.)



The TBG of subject P.P. bound 57% at 150 nmol/l, 58% at 200, 48% at 300 and 33% at 600 nmol/l (Figs. 62 & 66). At the same total hormone levels the TBG of subject I.D. bound 37%, 42%, 32% and 16% of the radiolabelled thyroxine. Whilst the P.P. results were single determinations, the I.D. results at 150 and 200 nmol/l were means of respectively 3 and 7 plates, but the 300 and 600 nmol/l plates using I.D. serum were single determinations (Table 16).

Two hypothyroid sera were investigated after being equilibrated with radiolabelled thyroxine to give a total hormone concentration of 525 nmol/l. At these levels 100% of the label was recovered and the TBG bound 46% of the hormone in B.T. serum and a mean of 36% (35, 36 and 37%) in A.S. serum (Figs. 31 & 63).

Two euthyroid females' sera with elevated TBG, resulting from administration of oestrogen, were also studied. Both sera retained 100% of the labelled hormone, and the TBG binding percentages were 73% for J.M. at 150 nmol/l and 57% for D.N. at 200 nmol/l.

At each level of total hormone studied in each serum, binding to TBPA, albumin and α_1 and β lipoprotein increased progressively.

iv) Supra-Hyperthyroid Hormone Level Distribution

A euthyroid serum (P.P.) was studied at levels of thyroxine above the hyperthyroid range at 800, 1100, 1600 and 2100 nmol/l. Each plate resulted in 100% recovery of hormone and the TBG bound respectively 21%, 23%, 20% and 14% of the radiolabelled hormone (Table 16 & Fig. 64). The secondary binding proteins steadily increased their role in hormone carriage until the radioactivity peak on the protein or proteins anodal to TBG dwarfed the TBG peak on the graph

FIGURE 3 : DISTRIBUTION OF RADIOLABELLED THYROXINE ON
INCREASING THE TOTAL HORMONE CONCENTRATION (75-525 nmol/l)
USING THE SAME HYPOTHYROID SERUM (A.S.)

248

RADIOACTIVITY COUNTS $\times 10^{-3}/100$ SECS.

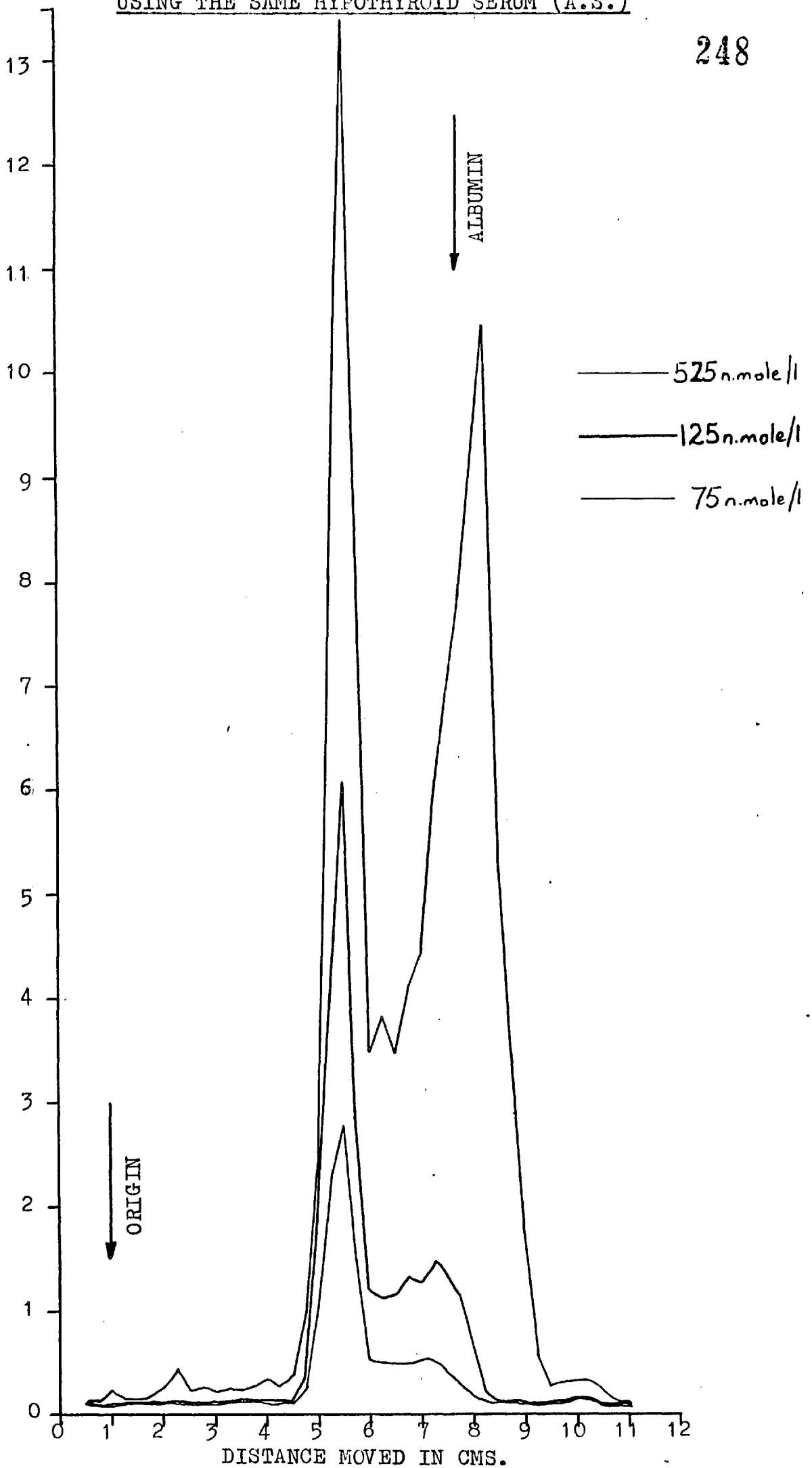
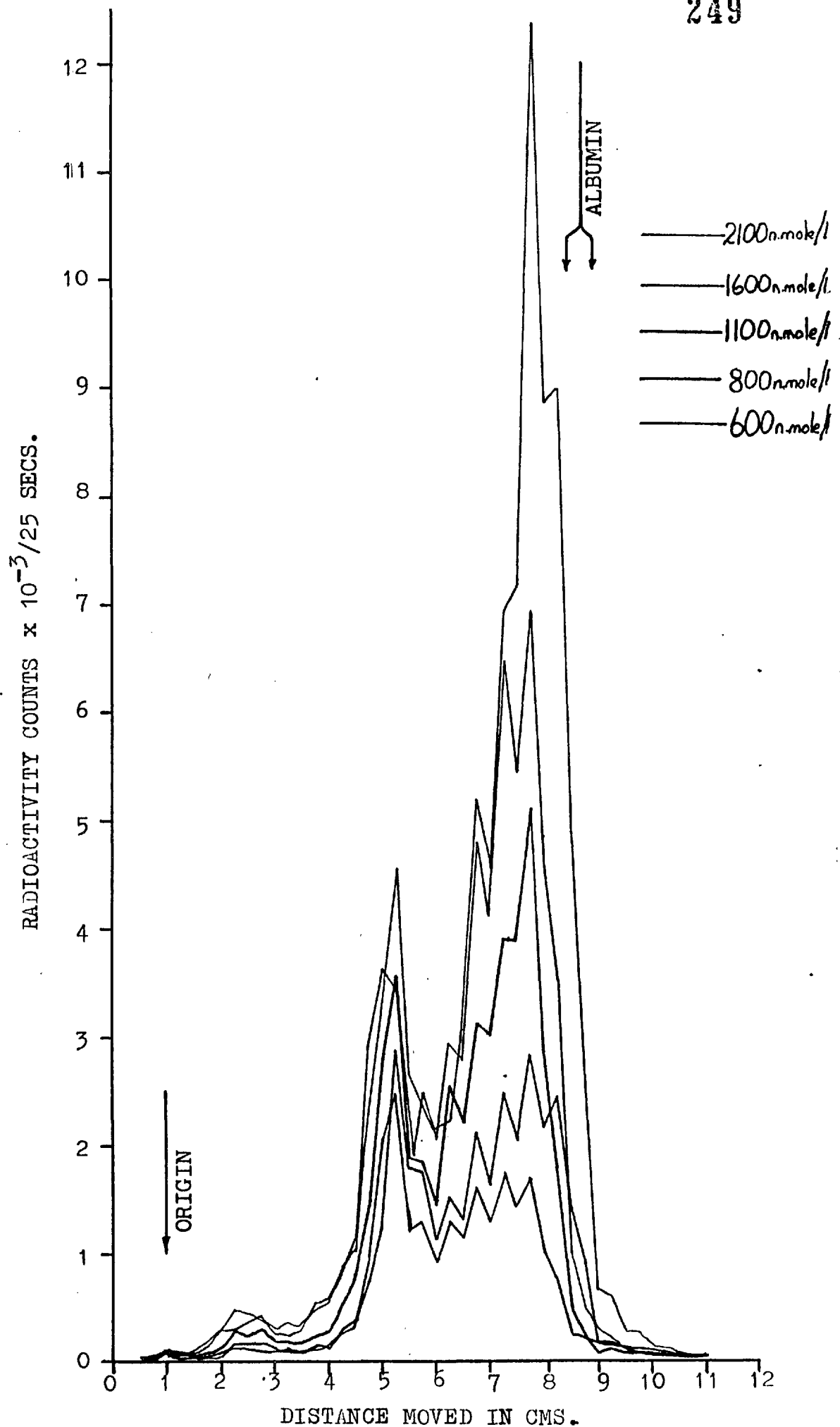


FIGURE 64

DISTRIBUTION OF RADIOLABELLED THYROXINE ON INCREASING
THE TOTAL HORMONE CONCENTRATION (600-2,100 nmol/l)
USING THE SAME EUTHYROID SERUM (P.P.).

249



(Fig. 64).

A sample of I.D. serum was also equilibrated with a high concentration of thyroxine to give 4300 nmol/l. The percentage recovery was 100%, but TBG bound only 9% of the radioactivity.

When all the values were plotted on the same graph, a general trend was observed indicating an inverse relation between total hormone content and percentage binding of thyroxine by TBG. However, superimposed on this general trend is another pattern which indicates two, and possibly three plateaux on the graphs of P.P. and I.D. sera when all values are plotted (Figs. 65 & 66). The first plateau appears between 150 and 200 nmol/l and the second much longer plateau between 800 and 1600 nmol/l. A possible third plateau appeared on only one of the sera (I.D.) as subphysiological total levels of hormone were only used with this serum. The plateau was between 20 and 100 nmol/l (Fig. 65). Although the percentage binding of hormone by TBG in the two sera is quite different, the similarity in the shape of the curves is striking.

In all sera where more than one level of hormone addition was studied it was observed that as the total concentration of thyroxine rose, the secondary binding proteins became more important as hormone carriers. The secondary proteins of particular importance were those in the α_1 lipoprotein and cathodal to albumin region (Figs. 62 & 64).

The major binding protein, TBG, did not appear to become saturated (M.B.C.) with thyroxine until the 16 to 21 times physiological level of hormone was added (Fig. 64). At these saturated levels the binding capacity of TBG was

FIGURE 65

GRAPH OF PERCENTAGE BINDING OF RADIOLABELLED THYROXINE TO TBG PLOTTED AGAINST TOTAL HORMONE CONCENTRATION USING THE SAME EUTHYROID SERUM (I.D.).

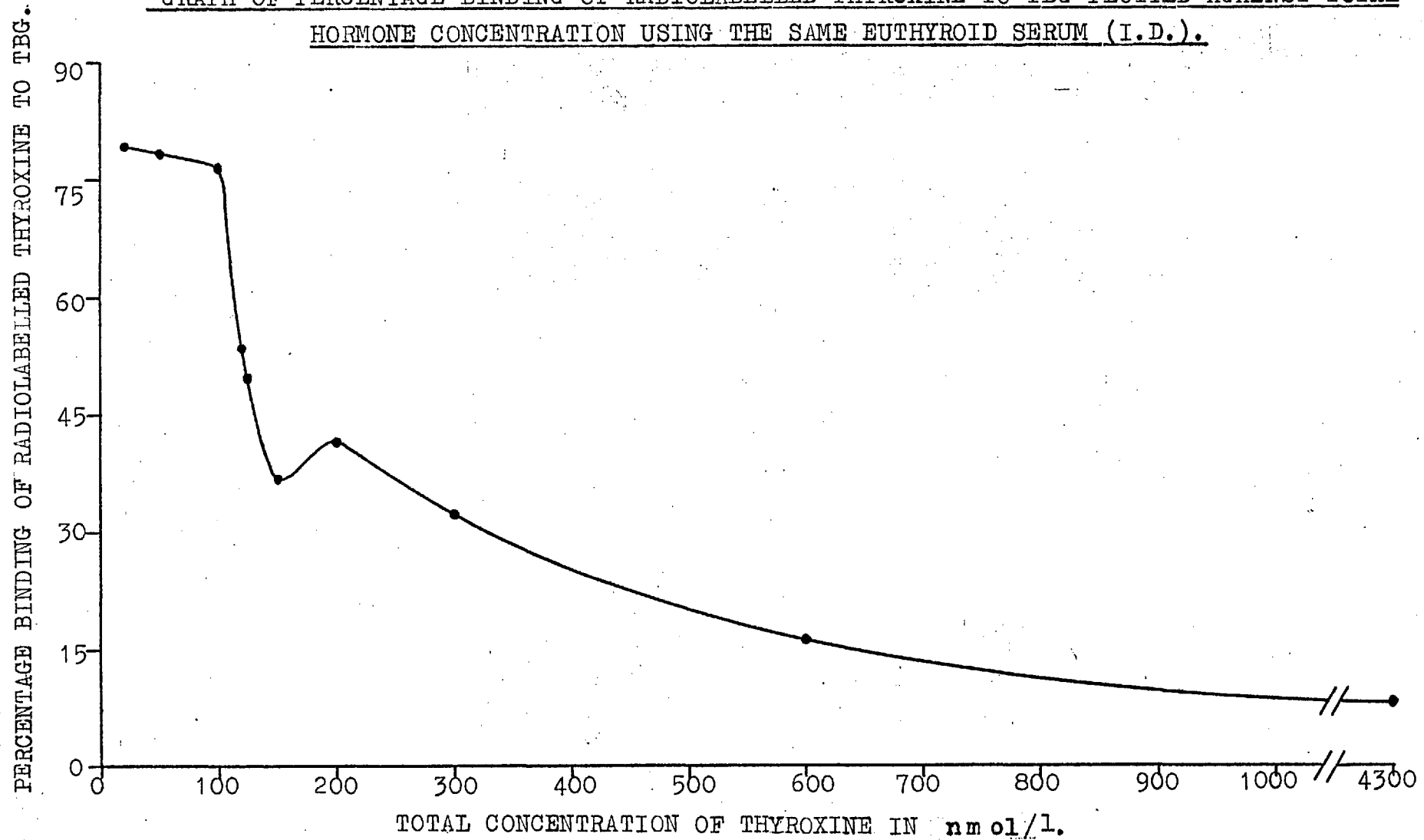
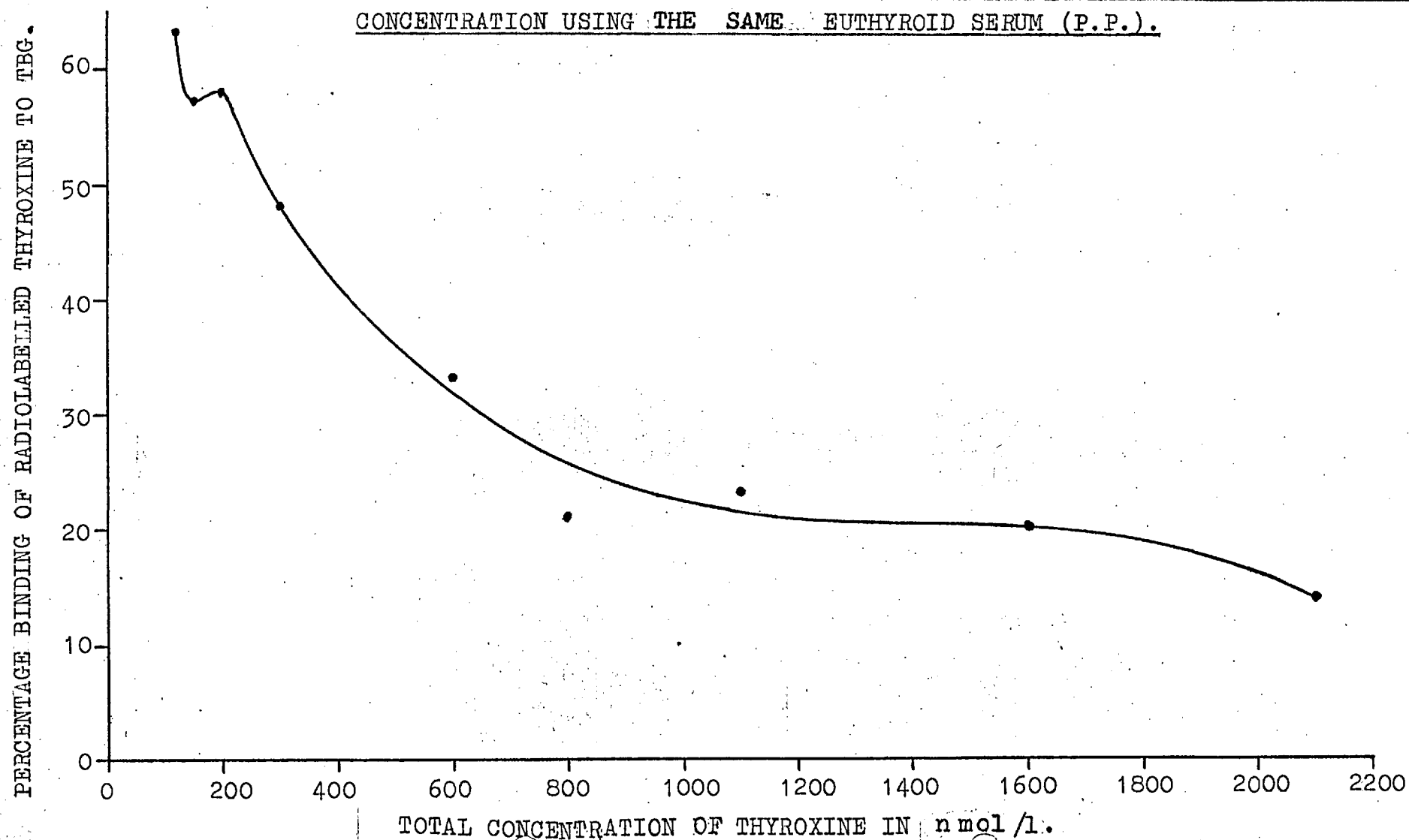


FIGURE 66

GRAPH OF PERCENTAGE BINDING OF RADIOLABELLED THYROXINE TO TBG PLOTTED AGAINST TOTAL HORMONE CONCENTRATION USING THE SAME EUTHYROID SERUM (P.P.).



estimated, and at:-

16 times physiological (1600 nmol/l) binding capacity

= 280 nmol/l (22.4 g T₄%).

21 times physiological (2100 nmol/l) binding capacity

= 285 nmol/l (22.8 g T₄%),

which are well within the normal range of TBG estimation

using the Maximum Binding Capacity technique (Clark, 1967;

Ingbar & Freinkel, 1960; Sutherland & Simpson-Morgan, 1975;

Shimizu, Shishiba, Yoshimura & Shizume, 1972; Davis et al.,

1972; Woeber & Ingbar, 1974).

4.3.2.1.1. DISCUSSION OF BINDING

DISTRIBUTION OF THYROXINE ON INCREASING TOTAL CONCENTRATION

Studies of percentage binding of thyroxine show that TBG is the major binding protein accounting for between 60% and 75% of the hormone depending on the type and pH of the buffer and the electrophoretic medium used (Oppenheimer et al., 1963; Woeber & Ingbar, 1974). These studies used trace additions of radiolabelled thyroxine and determined the distribution of this trace amongst the binding proteins assuming it to mirror the endogenous hormone distribution (Taurog & Chaikoff, 1948). The present study investigated not only the distribution of trace amounts of hormone amongst serum proteins of euthyroid individuals, but extended the range of hormone additions to include hypo- and hyperthyroid levels of hormone. The resulting plot of percentage binding of thyroxine to TBG against total hormone concentration displays an interesting pattern.

Three sera were studied at subphysiological levels of thyroxine, two of which were hypothyroid and one euthyroid from which the thyroxine had been stripped. Although each serum contained significantly different TBG concentrations, as measured using the modified Rocket technique, the percentage binding of thyroxine was very similar. At 20 and 50 nmol/l the percentage binding by TBG was respectively between 84% and 79% and between 82% and 78% (Table 16). The mean percentage binding by TBG therefore decreased only 1.5% during a change in total hormone from 20 to 50 nmol/l. The one individual serum that was investigated at both hormone levels also showed this 1% change in hormone binding

by TBG (I.D.). The serum I.D. contained a TBG of 116% whilst B.T. contained a TBG of 150%. However, during the stripping technique involving the I.D. serum and with Aminex resin, the lipoprotein molecules were almost certainly removed and their physiological role at normal levels of hormone thus eliminated, leaving relatively more hormone for TBG to bind. The I.D. sample stripped of thyroxine did not display a lipoprotein peak by the counting or autoradiographic methods. The percentage of thyroxine bound by TBG at 20 and 50 nmol/l concentrations was slightly higher than the values published by Woeber & Ingbar (1974) and by Oppenheimer et al. (1963), but this difference would be expected since the total amount of hormone was greater in these other studies.

At physiological levels of total hormone the same three sera (AS; BT; ID) were used together with five other random euthyroid sera. The result from these collected sera showed a definite and interesting pattern. Only one sample actually contained the mean normal level of total thyroxine (100 nmol/l), the stripped I.D. serum which displayed a percentage binding of 76%, a significantly higher result than the remaining sera. Although this serum contained a low TBG concentration, 116% as measured using the modified Rocket Technique, it was equilibrated with the lowest total thyroxine concentration, and lacked α_1 lipoprotein. The remaining sera all contained total hormone concentrations between 115 and 125 nmol/l which is slightly higher than the normal mean thyroxine concentration (100 nmol/l). In this range of hormone concentration the TBG of the various sera carried 53% - 68% of the hormone. The two individual

sera (I.D., P.P.) studied in this range of total thyroxine concentration indicated a precipitous fall in percentage binding by TBG (Figs. 65 & 66), and although duplicate experiments show a reproducibility to $\pm 2.5\%$, when the different plates were run using the I.D. serum at the total thyroxine concentration of 120 nmol/l, the range of percentage binding to TBG was 41% to 66%. This excessively wide interassay range may be explained by the pipetting error involved in aliquoting the radiolabelled hormone. Just very small errors in pipetting in this region of the graph could make significant changes to the percentage binding in a similar way to that of pH change in a buffer when acid or alkali is added outside of the buffering region. Thus if slightly more hormone was equilibrated, the percentage recovery would fall, but if slightly less hormone were added, although the total hormone concentration would fall, the percentage bound to TBG would rise. In this region of the I.D. curve, a pipetting inaccuracy of 10 nmol/l would change the TBG percentage binding by more than 20% (Fig. 65). The other sera (TFE, OH & OFS) all show percentage binding of thyroxine by TBG, to occur near the 60% region (65%, 58% & 61%) which is well within the range of hormone binding shown by the first four sera studied (AS, BT, PP, ID).

At the hyperthyroid levels of thyroxine the same four sera (AS, BT, PP & ID) were studied but a divergence of percentage binding became apparent. At 150 nmol/l of thyroxine, the I.D. serum, which contained the lowest TBG concentration, showed a percentage binding of 37%. The P.P. serum at this hormone concentration displayed a percentage binding of 57% although it contained only 4% more TBG in the serum than did I.D. serum. The P.P. and I.D. percentage binding curves

above 150 nmol/l of hormone show very close parallelism up to 600 nmol/l and possibly after, though only one point on the I.D. curve was plotted after this hormone concentration. Between 150 and 200 nmol/l of thyroxine both the P.P. and I.D. curves showed increased percentage binding by TBG, the P.P. percentage rising by only 1% whilst the I.D. serum rose by 5%. The I.D. serum results were means of respectively 3 and 7 determinations while the P.P. results were single determinations. Above 200 nmol/l the percentage binding by TBG fell uniformly until the 600 to 800 nmol/l hormone concentration when they showed a plateau formation. Between the two hormone values of 200 and 600 nmol/l, the P.P. and I.D. serum displayed exactly parallel curves with the I.D. curve displaced 17% below the P.P. curve. The A.S. and B.T. sera were examined at 500 nmol/l of thyroxine and the TBG bound respectively 36% (35%, 36%, 37%) (A.S.) and 46% (B.T.) of the radioactivity. The A.S. value of 36% was almost identical with the extrapolated value for P.P. serum TBG at the same hormone level although the TBG content of P.P. serum was 10% lower than that of A.S. The percentage binding of hormone to the TBG of B.T. was 10% higher than for A.S., but the TBG concentration in the B.T. serum was also significantly higher (21%) as determined using the Modified Rocket Technique.

The P.P. serum was studied at hormone levels above 600 nmol/l and between 800 and 1600 nmol/l indicated a relative plateau of percentage binding, but at 2100 nmol/l, the percentage binding again fell.

The overall picture of thyroxine binding to TBG is one of a series of plateaux and connecting sections of relatively rapid change in percentage binding. The positions of

the first two of these plateau are interesting. The first is apparent only with the I.D. serum since this serum alone was used for multiple determinations in the plateau region below the normal mean physiological level. Thus below 100 nmol/l, more than 75% of the hormone was found bound to TBG. The function of this plateau is obscure, but at the low concentration of hormone at which it occurs, TBG is by far the most significant determinant of free thyroxine and by largely maintaining its percentage binding, the secondary binding proteins have only a relatively small percentage of the total hormone to carry, which presumably enables better buffering of free hormone concentrations. Immediately after this plateau, between 100 and 150 nmol/l the percentage binding of TBG falls precipitously. In the I.D. serum the percentage binding of thyroxine to TBG falls from 76% at normal hormone levels (100 nmol/l) to 37% at 150 nmol/l, whilst the P.P. serum TBG falls from 63% at 120 nmol/l to 57% at 150 nmol/l. Although the absolute change in the P.P. serum is small (6%), the rate of change is more than twice as rapid as anywhere else on the TBG curve, using this serum. The second plateau in both I.D. and P.P. serum falls between 150 and 200 nmol/l of thyroxine and the percentage binding by TBG actually rises during this phase. Again the function of the plateau is obscure, although the hyperthyroid range of total T_4 concentration is entered at this hormone level (150 - 200 nmol/l). The percentage binding plateau of TBG between these hormone levels could therefore indicate a stronger binding of hormone in this range when the free hormone levels start to become excessive to normal requirements. After this second plateau region the TBG percentage binding falls evenly, and in par-

allel fashion between I.D. and P.P. sera, to six times normal hormone levels which is through the hyperthyroid range of thyroxine concentration. After this second decreasing phase of hormone binding by TBG, the decline again plateau's to become apparently asymptotic with the X axis so that at the grossly supra-physiological level of 2100 nmol/l of thyroxine, P.P. serum TBG bound 14% of the hormone whilst with a thyroxine concentration of 4300 nmol/l, I.D. serum TBG bound 9%.

The P.P. graphs of increasing total hormone concentration also indicate the distribution of radiolabelled thyroxine amongst the remaining, secondary, proteins. As TBG becomes more saturated with hormone, α_1 lipoprotein and the TBPA and albumin start to bind more hormone. As the hormone level rises still further however, TBG may be seen to become saturated, by the constant area beneath the graphical peak, while other proteins around the TBG region become more important in percentage binding (Figs. 62 & 64).

5.

CONCLUSION

Two immunoelectrophoretic methods have been developed to study TBG, the major thyroxine transporting protein in serum. The first of these methods was developed to investigate the concentration of TBG in serum using a simple, direct and relatively inexpensive procedure, whilst the second method was developed to simultaneously and directly estimate serum TBG concentration and the percentage binding of thyroxine by TBG.

The methods were developed so that they could be reliably used with radiolabelled hormone under conditions resembling as closely as possible the in vivo "milieu". The methods were extensively evaluated and were found in both intra-assay and interassay studies to be highly reproducible.

The development of these two techniques resulted in two studies. The first technique discussed, used the Modified Rocket Technique in the quantitation of TBG in sera. Although some of the results were consistent with previous work, a large proportion of results in this section were not.

The normal range of TBG values was found to vary with both age and sex, but whilst the individual's sex has been previously shown to result in a statistically different TBG, age has rarely been included in the data of these studies. The conclusions drawn from the present work therefore indicate that either a wide "normal" range of TBG concentrations must be tolerated, or that sex and age TBG ranges must be separately determined by further studies.

The TBG concentration of patients with thyroid disease also showed some deviation from the normally accepted ranges. The hypo- and hyper thyroid females' TBG concentrations

showed significantly lower means ($P < 0.005$) than the normal female means, but the male groups did not differ significantly from their normal means. A sex difference has not previously been published and it may account for the differing results found by investigators between the hyper- and hypothyroid groups.

The general elevating effect of oestrogen on serum TBG levels is well known and documented and with which this study agrees. However, the day to day effect of oestrogen on TBG levels is less well known. From this study, however, the results were striking in that they showed a complete reversal of TBG 28 day cyclicity when compared with women following a physiologically controlled menstrual cycle. This obvious serum change may well reflect a general change in a woman's physiology during oestrogen administration and might be considered as a biochemical disadvantage of oestrogen therapy. The effects of starting and stopping oestrogen therapy were also investigated. It was found that the initial stimulatory effect of oestrogen on increasing serum TBG concentrations was more rapid than the decrease in this protein's concentration after cessation of oestrogen. The significance of this difference is obscure, but is presumably related to the half-lives of oestrogen and TBG, and to the speed of stimulation of TBG biosynthesis.

Post-operative patients undergoing medium grade surgery were shown to have a significant initial decrease of TBG concentration. The current literature considers TBG change following surgery as debatable, but the accepted increase in free thyroxine is certainly supported by the present study. Also, the much smaller changes in TBG concentration, compared with those of TBPA, needed to result in the same free

hormone changes supports TBG as the major factor in these free thyroxine changes.

The patients suffering from liver disease were found to have similar TBG changes to those studied during other investigations, which report non-specific changes in TBG concentration during hepatic disease generally although infectious-hepatitis has been associated with elevated TBG.

A family with a TBG anomaly was also studied. Certain male members of the family were found to have immeasurable TBG levels in common with the reports from other studies. However, during this study the Modified Rocket method was used to determine whether any immunologically recognizable TBG was present in the apparently "a-TBG" serum. No serum protein immunologically characterisable as TBG was found which seems to clarify the currently held view in the literature that in these individuals TBG is thought to be absent, but until now not proven so.

To quantitatively determine hormone binding to TBG in this study, the second method, the Modified Line Technique, was used. This second method has also been shown to have good reproducibility. It has been employed in the simultaneous quantitation of TBG in serum and in the measurement of percentage binding of thyroxine to TBG. The Modified Line Technique, in contrast to the standard method, was shown to result in total hormone recovery, indicating its validity in quantitative estimation of hormone distribution.

This second technique was used to investigate the effect of buffer ion and pH on thyroxine binding and again the results conclusively demonstrated that the more physiological milieu resulted in a greater binding of thyroxine to TBG and to the secondary binding proteins.

The percentage binding of thyroxine to TBG was also extensively investigated with the technique using increasing total concentrations of radio-labelled hormone. The resulting binding pattern was unexpected showing TBG to have a titration-like curve of a tribasic acid. Three plateaux were found on this curve separated by regions of relatively rapid changes in binding quantity. The first two plateaux are significantly situated in the just sub-physiological to hypothyroid range (about 20-80 nmol/l) and in the high physiological to hyperthyroid hormone range (about 150-200 nmol/l). In the range of physiological hormone levels (about 75-150 nmol/l) the percentage binding of thyroxine to TBG undergoes its greatest change. The significance of these plateaux has yet to be discovered, but further investigation with more determinations of percentage binding in the hormone range of 0-200 nmol/l could result in significant additions to the knowledge of the physiology of circulating thyroxine.

In conclusion, two modified techniques have been developed and used to quantitate TBG in serum and to quantitate percentage binding of thyroxine to TBG. The techniques are easy to use and inexpensive to set up and run. The results are highly reproducible and while the first method, the Modified Rocket Technique, is applicable to routine laboratories for the direct and reliable estimation of TBG, the second, the Modified Line Technique, is suggested as a research tool for the physiologist, biochemist, or pharmacologist, who is interested in studying the interactions of hormones, or drugs with hormones, under essentially physiological conditions.

6.

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7.

APPENDIX IDATA TABLES

DATA FOR MALE NORMALS USED IN SECTION 4.3.

GROUPED IN AGE CATEGORIES

[illegible]

DATA FOR MALE & FEMALE NON-THYROID SICK
PATIENTS USED IN SECTION 4.3. GROUPED
IN AGE CATEGORIES

FEMALE:

	20-29	30-39	40-49	50-59	60-65	66+	
	132	147	150	125	127	134	
	126	145	139	125	134	125	
	138	137	130	137	130	134	
		102	140	137	108	127	
		165	134	156		131	
		139	132	133		114	
			154	137		124	
			137	124		131	
			154	136		118	
				128		142	
				124			
				123			
				134			
				124			
				145			
				135			
				147			
							<u>TOTAL</u>
n	3	6	9	17	4	10	49
Mean	132	139	141	133.5	125	128	134
S.D.	6	21	9.3	9.4	11.5	8.2	11.8

MALE:

	20-29	30-39	40-49	50-59	60-65	66+	
	-	135	-	125	129	120	
		122		130	142	138	
				132	138	119	
				127	125	132	
				124	137	135	
						144	
						122	
						135	
						132	
							<u>TOTAL</u>
n	-	2	-	5	5	9	21
Mean	122	128.5	-	127.6	134.2	130.8	130.6
S.D.	-	9.2	-	3.4	7.0	8.6	7.2

DATA FOR MALE & FEMALE HYPOTHYROIDIS USED INSECTION 4.3. GROUPED IN AGE CATEGORIESFEMALE:

	20-29	30-39	40-49	50-59	60-65	66+	
	-	130	144	127	134	122	
		128	133	130	118	116	
		111	133	126	142	141	
				123	137	111	
				135	154	113	
				135	151	124	
				133	136	137	
						151	
							<u>TOTAL</u>
n	-	3	3	7	7	8	28
Mean	-	123	137	130	139	127	131
S.D.	-	10.4	6.4	4.7	12.0	14.5	11.6

MALE:

	20-29	30-39	40-49	50-59	60-65	66+	
	-	136	-	135	124	153	
					170	128	
					156	114	
					150		
							<u>TOTAL</u>
n	-	1	-	1	4	3	9
Mean	-	136	-	135	150	132	141
S.D.	-	-	-	-	19.3	19.8	17.8

DATA FOR MALE & FEMALE HYPERTHYROIDS USED INSECTION 4.3. GROUPED IN AGE CATEGORIESFEMALE:

	20-29	30-39	40-49	50-59	60-65	66+	
	121	137	130	128	148	145	
	119	131	131	114	136	136	
	133	125	118	138		116	
			102	146		125	
				142			
							<u>TOTAL</u>
n	3	3	4	5	2	4	21
Mean	124	131	120	134	142	131	130
S.D.	7.6	6.0	13.5	12.8	8.5	12.7	11.8

MALE:

	20-29	30-39	40-49	50-59	60-65	66+	
	-	112	-	140	124	116	
							<u>TOTAL</u>
n	-	1	-	1	1	1	4
Mean							123
S.D.							12.4

DATA FOR OESTROGEN ADMINISTERED AND
PREGNANT WOMEN USED IN SECTION 4.3.

Oestrogen Administered	Pregnant Women		
	Age Groups		
	20-29	30-39	
186	154	176	
149	178	170	
175	171	164	
160	162	157	
147	181	139	
141	157	159	
150	156	131	
162	128		
159	155		
159	179		
144	165		
156	181		
161	175		
146	182		
179	161		
162	168		
147	151		
165	140		
164	168		
142	143		
157	138		
	160		
	133		
	151		
	141		
	150		
	159		
			<u>TOTAL</u>
n	21	27	7 34
Mean	157.7	158.8	156.6 158
S.D.	12	15.4	16.2 15.3

DATA FOR FOETAL SERA USED IN SECTION 4.3.

GROUPED ACCORDING TO FOETAL SEX AND

MATERNAL AGE.

	<u>Male Foetus</u>		<u>Female Foetus</u>	
	20-29	30-39	20-29	30-39
	172	149	156	156
	176	130	155	142
	167	146	162	139
	159	183	154	176
	157	144	155	144
	150	161	138	130
	165	128	150	138
	169	140	163	141
	159	114	158	138
	161	141	148	146
	135		152	
	144		117	
	144		119	
	138		130	
	137		137	
	137		133	
	146		137	
	151		128	
	127			
	156			
n	20	10	18	10
Mean	152.5	143.6	144	145
S.D.	13.8	18.9	14.4	12.8

	<u>TOTAL</u>	<u>TOTAL</u>
n	30	28
Mean	149.5	144.4
S.D.	15.9	13.6

	<u>GRAND TOTAL</u>
n	58
Mean	147
S.D.	15

DATA FOR AMNIOTIC FLUIDS USED IN SECTION 4.3.AMNIOTIC TBG

54
81
88
88
88
78
61
66
68
71
68
73

n 12
Mean 74
S.D. 11.2

7.

APPENDIX II
PUBLISHED WORK

17. A Routine Method for the Estimation of Thyroxine Binding Globulin in Human Serum

I.P. DRYSDALE, D.B. RAMSDEN AND R. HOFFENBERG

Division of Clinical Investigation,

Clinical Research Centre, Harrow

and Department of Medicine, University of Birmingham.

INTRODUCTION

There are two commonly used methods of estimating thyroxine binding globulin in serum :

- a) based on saturation analysis (1,2)
- b) indirectly by the T_3 uptake method (3) which reflects unoccupied binding sites.

Both measure hormone binding capacity not concentration. Within the past five years a few reports have described methods for the direct measurement of TBG concentration. First of these was that of Freeman and Pearson (4) who used a Laurell two dimensional immuno-electrophoretic method to precipitate the proteins in the form of Gaussian or near Gaussian peaks whose area was proportional to antigen concentration. TBG was identified by saturating the precipitate with radioactive T_4 , washing off excess T_4 , then submitting the plate to autoradiography. Whilst this method has the advantage of simultaneously allowing measurement of the concentration of many other serum proteins, it is too cumbersome and costly in antiserum and other reagents for routine assay of TBG alone.

Several variants of this technique have been published which use serum prelabelled with radioactive T_4 and Laurell "monorocket" immuno-electrophoresis in which the antigen is electrophoresed directly into the antiserum-containing gel (5,6).

Radioimmunoassay for TBG has been developed by Levy *et al.* (7) but their method requires stringently pure TBG and a monospecific anti-TBG, which are not easily prepared.

In view of the above points the most readily available and cheapest system would appear to be the modified monorocket technique, since this requires little specialist equipment and materials. In this paper therefore we describe the technical details and validation of such a method for estimation of TBG concentration in serum.

EXPERIMENTAL

Materials

The following materials were obtained from the sources indicated below :-

Agarose "Indubiose-A37", Industrie Biologique Francaise, from Micro-Bio Laboratories, Pembridge Road, London, W11.

Anti-whole human serum from either Wellcome Reagents Ltd., Beckenham, Kent or Dakopats, Mercia Ltd., Sandown Road, Watford.

¹²⁵I-Thyroxine from the Radiochemical Centre, Amersham, Bucks.
TEAE-Cellulose from Sigma Chemicals Ltd., Croydon, Surrey.
Kodirex from Kodak U.K. Ltd.

Glass plates from the Chance Glass Co. Ltd., Birmingham.

The reference sera were gifts from Blood Products Laboratories Lister Institute, Elstree, Herts. or National Institute for Biological Standards, Hampstead, London (Batch No. 68/449).

All other chemicals were obtained from either B.D.H. Ltd. or Hopkins and Williams Ltd. and were of "Lab. reagent" or "General purpose reagent" quality.

Methods

Purification of Agarose. To a hot solution (80°C) of agarose (2% w/v) was added TEAE-cellulose powder (10g/l). The mixture was stirred vigorously for 60 min then filtered, the temperature being maintained at 80°C throughout. The filtrate was divided into 200 ml lots and stored at 4°C until required.

Laurel "Monorocket" immunoelectrophoresis. "Monorocket" immunoelectrophoresis was performed as described by Laurell (8) with a number of modifications. These were :-

- (a) the buffer used was sodium phosphate (0.025 M with regard to phosphate) of pH 7.4.
- (b) Agarose purified as described above was used to reduce excessive electroendosmosis which otherwise occurs at this pH.
- (c) Since no specific anti-TBG was available, antiwhole human serum was employed instead.
- (d) TBG was identified by prelabelling the sample with ^{125}I -T₄ and submitting the immunoelectrophoretogram to autoradiography before staining.
- (e) Antigen wells were not punched directly into the antiserum containing gel but into an agarose strip (2.5 cm wide) containing only buffer. This strip will subsequently be referred to as the "buffer gel".

The buffer gel was placed on one edge of a glass plate and antiserum containing gel poured on to the remainder of the plate and allowed to set. Antigen wells were then punched into the buffer gel approximately 0.5 cm from the outer edge,

so that the antigens were electrophoresed through 1.5 to 2 cm of buffer gel before coming into contact with antibodies. Normally glass plates (11 cm x 11 cm) were used and each contained either 2.5 ml of Wellcome antiserum or 1.5 ml of Dakopats allowing eight TBG estimations per plate, two of which were always reference sera. With large plates a greater number of samples could be run simultaneously and correspondingly larger amounts of antiserum were used. Before electrophoresis, serum (50 μ l) was equilibrated with $^{125}\text{I-T}_4$ solution (200 $\mu\text{Ci/ml}$ and 4 $\mu\text{g/ml}$) for 1 hour at room temperature. The amount of radioactive T_4 added was sufficient to bring the T_4 concentration to about 5x physiological (arbitrarily accepted as 8 $\mu\text{g/100 ml}$). Equilibrated serum (2 μ l) was added to each antigen well. After electrophoresis excess antiserum was removed by placing a damp filter paper onto the surface of the gel. The plate and paper together were then dried in a stream of warm air. When both were thoroughly dry the filter paper was peeled off and discarded. A thin sheet of plastic was placed between the X-ray film and the immunoelectrophoretogram which was submitted to autoradiography for 7 days. After this the X-ray film was developed and the immunoelectrophoretogram stained for protein using amido black solution.

R E S U L T S

Figure 1 shows a typical autoradiograph of human serum prelabelled with $^{125}\text{I-T}_4$ and submitted to quantitative monorocket immunoelectrophoresis. One precipitin peak, TBG, clearly possesses bound $^{125}\text{I-T}_4$ although from the stained plate many protein peaks are visible. Other serum proteins are known to bind thyroxine but under the conditions described

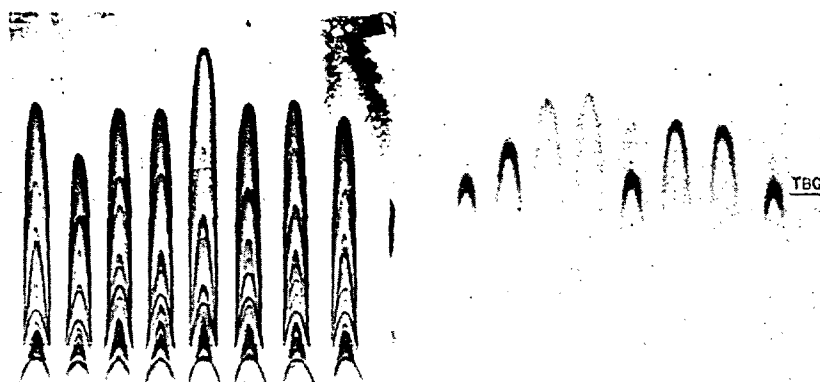


Fig. 1. Typical "monorocket" immunoelectrophoretogram. Autoradiograph shows one highly radioactive peak corresponding to TBG which is not visible on original plate when stained for protein with amido black, although many other serum proteins are visible. Broken line on autoradiograph indicates the position of buffer gel.

either insufficient antiserum was used to precipitate them on the immunoelectrophoretogram or they bound too little $^{125}\text{I-T}_4$ to cause an intense image to be formed on the X-ray film.

The advantage of using a buffer gel for consistent results is illustrated in Fig. 2. Here a wedge-shaped buffer gel was used so that some antigen wells are punched into antibody containing gel and others into the buffer gel at gradually increasing distances from the antiserum containing gel. Where the antigen well was directly surrounded by antiserum, $^{125}\text{I-T}_4$ was displaced almost entirely from the sample, and where the antigen well was only partially surrounded by

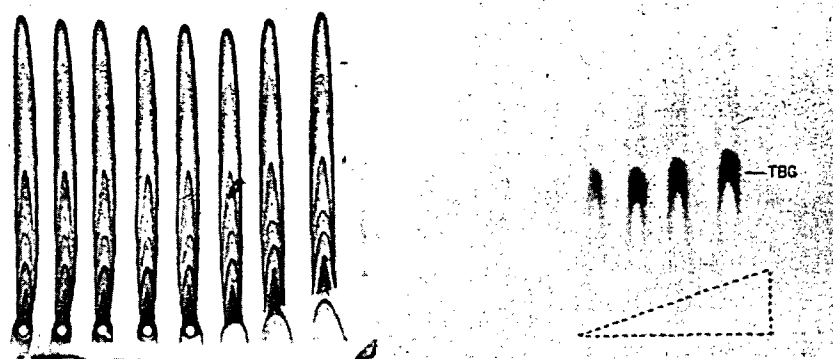


Fig. 2. 'Monorocket' immunoelectrophoretogram with wedge-shaped buffer gel. The autoradiograph shows that binding of $^{125}\text{I-T}_4$ by sample serum was retained only when the antigen well was separated from antiserum-containing gel by the buffer-gel. Equal mounts of sample and radioactivity were placed in each antigen well. Broken line on autoradiograph indicates the position of buffer gel.

antibody slightly more efficient binding occurred. Optimal binding was found when the antigen well was some distance from the antiserum. The factor in the antiserum responsible for the displacement of T_4 was not dialysable and was present in commercial preparations of purified sheep anti-human serum protein immunoglobulins. This factor was also present in specific rabbit or sheep antisera to a range of human serum proteins e.g. caeruloplasmin, transferrin, IgA, IgM, IgG, Albumin, α_1 -antitrypsin.

Since no pure preparation of human TBG was available the

17 Estimation of Human TBG

267

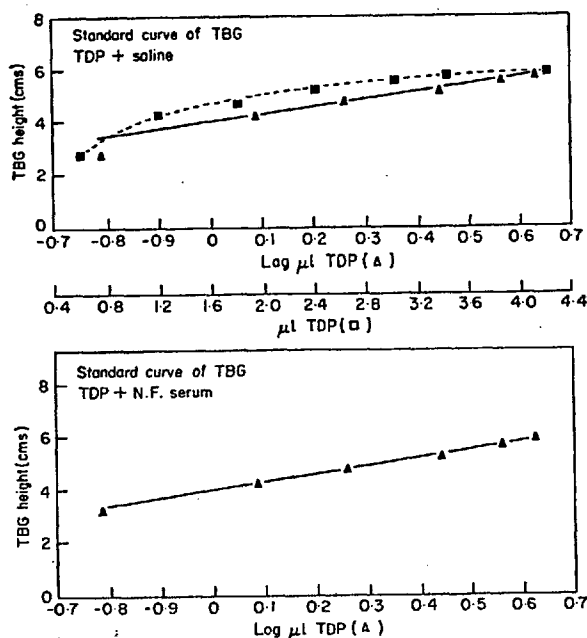


Fig. 3. Upper graph shows a typical dilution curve when working standard serum (TDP) was diluted with saline. The broken line is a plot of height of TBG in cm against sample volume in μl . The solid line is a plot of height of TBG peak against log sample volume. The lower graph is a dilution curve obtained when the working standard serum was diluted with a serum with a very low TBG concentration. The parallelism of the two solid lines indicates that the ratio of albumin to TBG did not influence peak height of TBG.

system was calibrated using a standard reference serum (Nat. Institute for Biol. Standard Serum No. 68/449). Because only limited quantities of this could be obtained a second standard serum, which was part of a thousand donor pool (TDP) was used throughout as an internal reference and working standard. All results referred to in this communication are quoted in terms of percentage of working standard which was diluted with acid : citrate : dextrose at collection. All concent-

Table 1.

Reproducibility Data

		Number of Observations	Mean	S.D.	$\frac{\text{S.D.} \times 100}{\text{Mean}}$
Inter-assay	Standard Serum	50	4.06 cm	0.12 cm	3.0
	Normal Serum	50	113.6%	2.19%	1.9
Intra-assay	Standard Serum	10	3.83 cm	0.026 cm	<1
	Normal Serum	10	121 %	0.8%	<1

Units of Standard Serum = peak height in cm

Units of Normal serum = $\frac{\text{peak height of normal} \times 100}{\text{peak height of standard}}$

Reproducibility of measurement of the TBG concentration of a working standard serum and a normal serum.

rations of normal serum are therefore greater than 100%. Inter-laboratory comparison may be made by reference to the Standard Reference Serum (68/449, National Institute for Biological Standards) which had a TBG concentration 1.21 times greater than the working standard. Figure 3 shows a typical calibration curve of the peak height of the TBG peak against volume of working standard for the range 0.6 μ l to 4.2 μ l serum. Although the plot of peak height v concentration is not linear, probably because area under the precipitin peak is a truer estimate of concentration, this proved to be the most convenient way of expressing results. Linearity was more nearly approached when peak height was plotted against log volume. It has been suggested that TBG may undergo some protein-protein interaction with albumin which may influence its electrophoretic mobility (9). Whilst the above method is relatively independent of mobility such interaction could affect the precipitation by antibody. To test for this, a serum from a subject with very low thyroxine binding globulin concentration was diluted with increasing amounts of the working standard serum. This had the effect of keeping albumin and other serum proteins approximately constant with an increasing concentration of TBG. The results were compared with those obtained when increasing amounts of working standard were added to saline. The parallelism of the two lines indicated that any protein-protein interactions which did occur did not affect this system.

An important feature of any assay is its reproducibility. To test this a normal serum as well as the standard serum was run on a number of plates. The results are shown in Table 1.

In the relatively short period of time that the assay has been functioning, we have not collected serum from an adequate number of healthy euthyroid subjects to obtain a comprehensive table of values of TBG concentration for ten year age groupings. However the results obtained so far for both males and females suggest that there is a noticeable increase from the 20-29 age-group to the 30-39 group which then either remains constant or increases slightly until the age of sixty. Table 2 also shows the average TBG concentration expressed as a percentage of the working standard for males and females irrespective of age. There is a significant difference between the means for males and for females ($P < 0.05$).

Table 2. *Serum TBG Concentration of Healthy Euthyroids*

	Male			Female		
	No.	S.D.	Mean	No.	S.D.	Mean
20 - 29	22	6.6	123	11	5.3	128
30 - 39	23	6.1	130	8	6.8	130
40 - 49	16	5.3	132	13	3.8	132
50 - 59	15	5.9	133	9	4.1	136
60 - 69	6	3.7	131	-	-	-
Total	82	6.9	129	41	5.5	132

Units = % TBG concentration of working standard serum.

Serum TBG concentration of normal males and females.

For a "normal" hospital population of patients with disorders other than those of the thyroid the mean TBG concentration was similar to healthy normals although the standard deviation was greater (Table 3).

In both hyper- and hypothyroidism in females the mean TBG concentration was not significantly different from the

Table 3. *Serum TBG Concentrations of Hospitalised Euthyroids*

	Hospitalised Euthyroid		Normal	
	Male	Female	Male	Female
Number	17	27	82	41
S.D.	9.5	7.8	6.9	5.5
Mean	130	135	129	132

Units = % TBG concentration working standard serum

Serum TBG concentration of hospitalised euthyroid subjects and healthy euthyroid subjects.

Table 4. *Serum TBG Concentration in Hypo- and Hyperthyroidism*

	Hypothyroid		Normal Euth.		Hyper.
	Female	Male	Male	Female	Female
Number	23	7	82	41	16
S.D.	11.9	16.7	6.9	5.5	9.7
Mean	132	145	129	132	130

Units = % TBG concentration of working standard serum

Serum TBG concentration of hypo- and hyperthyroid subjects and healthy euthyroid subjects.

mean for euthyroid females although the scatter as shown by the standard deviation was somewhat greater perhaps because of the wider age-range (Table 4).

Sera from pregnant females had a mean value some 30% higher than the normal, whilst the mean for cord serum was approximately mid-way between these two values (Table 5).

The sensitivity and highly reproducible nature of the assay allowed detection of relatively small changes in TBG concentration. Figure 4a shows the values of TBG concentration

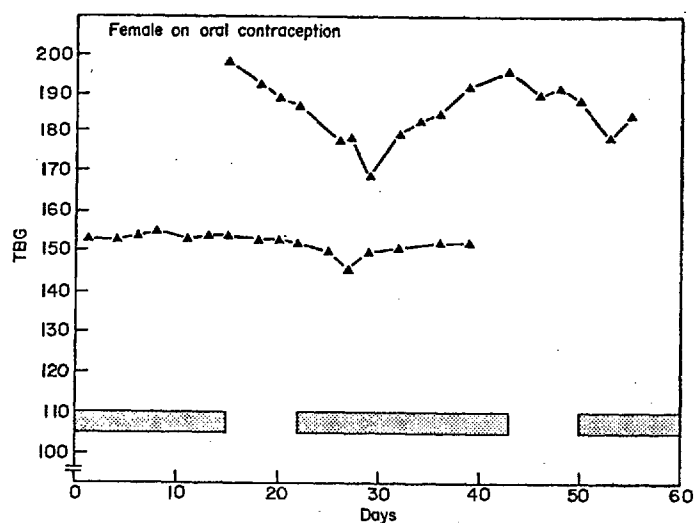


Fig. 4a

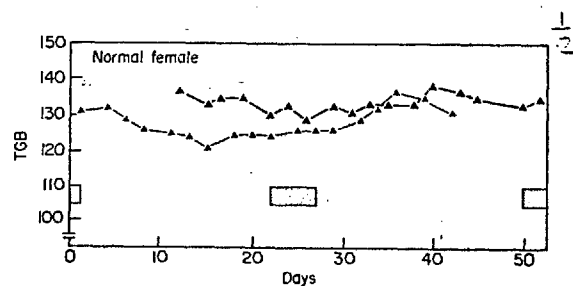


Fig. 4b

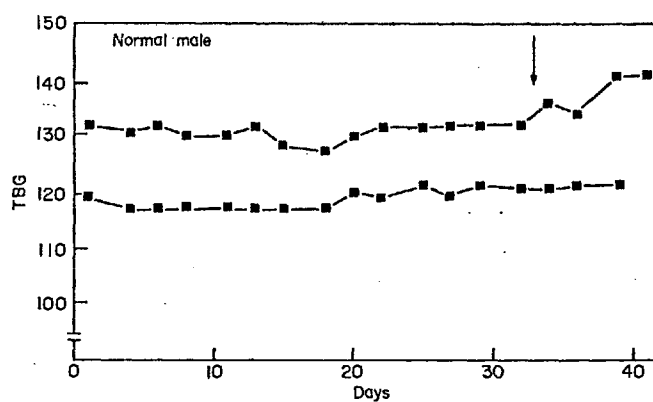


Fig. 4c

of sera taken at 2 or 3 day intervals through one cycle for two females taking oral contraceptives, two normal females (Fig. 4b) and over a similar time course two normal males of similar age (Fig. 4c).

The initial effect of oestrogen and its continuing effect after cessation of administration on TBG concentration is shown in Fig. 5. In one subject who had been taking a low oestrogen dose for more than three years the TBG level did not return to normal value until thirty days after stopping the pills. In a second subject on a two-month course of a high oestrogen dose the TBG level began to rise after an initial period of 3 days.

Thirteen patients were investigated before and after minor or medium surgery. In 12, within three days there was a mean decrease in TBG concentration of 6.8%, with a standard deviation of 3% of the preoperative level. After this the TBG either returned to the preoperative level or in some cases where the patient continued to be unwell remained low. In only one case did the TBG level rise immediately after surgery. A typical result for one patient is shown in Fig. 6.

DISCUSSION

The estimation of thyroxine binding globulin is usually made by measurement of binding capacity rather than actual

Fig. 4. Plot of serum TBG concentration against time. (a) for two females taking low oestrogen oral contraceptives, cross-hatched bars indicate times during which contraceptive was administered; (b) for two normal females, here cross-hatched bars indicate menstrual periods; (c) for two normal males, the arrow indicates the time at which one of the males developed a common cold.

Table 5. Serum TBG Concentration in Neonates (Cord) and Pregnant Females.

	Pregnant Females	Cord Serum	Normal Female
Number	34	66	41
S.D.	15.3	14.8	5.5
Mean	158	146	132

Units = % TBG concentration of working standard serum.

TBG concentration of serum from pregnant females, healthy euthyroid females and neonates (cord).

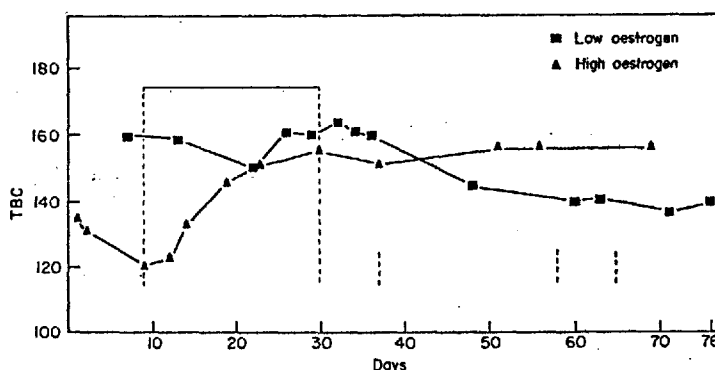


Fig. 5. Plot of TBG concentration against time for two females taking oral contraceptives. One female was on a high oestrogen contraceptive for two months only, the other had been on a low oestrogen contraceptive for more than three years, the last one month being studied. The extended broken lines joined by a solid horizontal line indicate time when contraceptive was taken by both subjects. The smaller vertical broken lines indicate alternating 21 and 7 day intervals. Only the subject on the high oestrogen dose was taking the contraceptives during the second 21 day period.

concentration of protein and therefore any conclusion from such measurements regarding concentration always possesses a degree of uncertainty. In this communication a method has

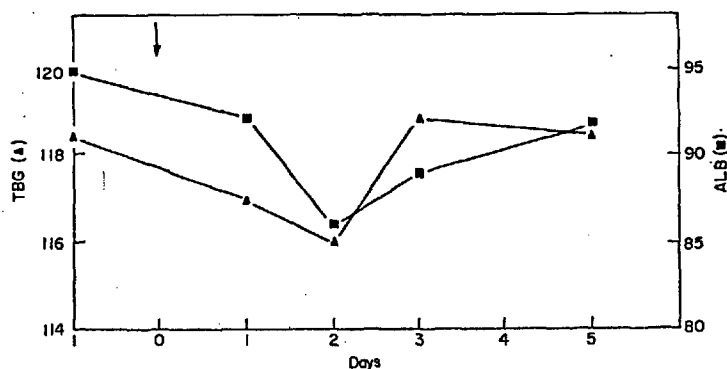


Fig. 6. A graph of TBG and albumin concentration against time for a typical patient immediately pre-operatively and for five days post-operatively. The arrow indicates approximate time of operation.

been described for the estimation of TBG concentration using a modified form of the Laurell "monorocket" immunoelectrophoretic technique combined with autoradiography. For consistently successful autoradiography in the method it was found necessary to ensure that the antigen well was punched into a strip of agarose gel which contained no antiserum. Why this buffer gel was essential is obscure but it is possible that the excess of animal TBG within the antiserum stripped the human proteins of T_4 if the two were allowed to come into contact. However when a buffer gel was used this appeared to allow sufficient physical separation for the animal TBG to be electrophoresed off the plate as the human migrated into the antiserum and competitive binding of T_4 was obviated.

It has been suggested that human TBG and albumin undergo some form of protein-protein interaction which is sufficiently

strong to influence the electrophoretic mobility of TBG (9). Although the assay method employed here is theoretically independent of electrophoretic mobility such interaction may affect the ability of antibodies to precipitate TBG. However the parallelism of the curves when standard human serum was diluted with either saline or serum from a patient with a very low concentration of thyroxine binding globulin suggests that if such interactions occur they are either disrupted by the conditions employed or have no significant interfering effect on precipitation by antibodies.

The results obtained using this method show a small but significant difference between the TBG concentration of a euthyroid male and female population. Comparison of hospitalised euthyroids with normal healthy euthyroid subjects showed no significant difference which is in agreement with previous workers using capacity measurements (10). For both hyper- and hypothyroid females there appeared to be no significant difference between the mean values and that for euthyroid females although the scatter as shown by the standard deviation was greater. Most workers using capacity measurements have suggested that there is no significant difference between hyperthyroid and euthyroid subjects (10,11,12) and that TBG is raised in hypothyroidism (13). Using radioimmunoassay Levy *et al.* (7) have shown a significant decrease in TBG concentration in hyperthyroid subjects and no significant difference in hypothyroidism when compared to euthyroid levels. The elevation in TBG concentration found in pregnancy and cord blood is well recognised and in agreement with the results of capacity measurements (14, 15).

The effect of oestrogens in raising TBG concentration

are clearly demonstrated and in agreement with previous observations using capacity measurements (16,17). The results obtained for normal females through one complete menstrual cycle show raised TBG concentration at about the time of ovulation when oestrogen values are known to be high, followed by a decline to a minimum before the onset of menstrual bleeding. In two females who were taking an oral contraceptive pill this picture was altered and the mean values were higher. Here the TBG level appeared to be regulated by exogenous oestrogen. Cessation was followed by a slow decline in TBG concentration reaching a minimum value only 14-16 days later, reflecting a continued action of oestrogen either on synthesis or breakdown of TBG. Whether the very different mean values in these two are due to differing normal response to oestrogen or the different composition of the contraceptives used is unclear since only two subjects were studied.

The continuing effect of oestrogens after cessation of administration was clearly demonstrated in one subject. The fact that even on a high oestrogen dose the lag period before a rise in TBG was observed was 3 days after commencement of administration in one person, would support the observation that in females taking low oestrogen oral contraceptives over a long period, the minimum TBG level occurs some while after the start of a fresh cycle.

A recent paper has claimed that the major thyroxine binding protein response to surgery is an interruption of TBPA synthesis, with no alteration in TBG levels, but this observation was based on the results of capacity measurements (18). Using the modified "monorocket" technique described here, consistent reduction in serum TBG concentration was

observed within 2 to 3 days of surgery. Whether this is due to a decrease in synthesis, increase in catabolism or a shift from an intra- to extra-vascular compartment is unknown. Since most circulating thyroxine is bound to TBG, relatively smaller alterations in TBG concentration could significantly affect free hormone values, which have been shown to rise after surgery, to an extent equal to the effects of larger alterations in TBPA concentration.

C O N C L U S I O N S

A method for the measurement of thyroxine binding globulin concentration in serum which involves immunoelectrophoresis combined with autoradiography is described. The method requires little specialised equipment and materials and is highly reproducible. The results obtained for a variety of pathological conditions are consistent with those of previous workers using techniques for estimation of thyroxine binding capacity.

Acknowledgements

We wish to thank Dr. R.L. Himsworth, Division of Clinical Investigation, Clinical Research Centre and Dr. B. Lewis, Foyal Postgraduate Medical School for helpful advice and discussions.

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