STUDIES ON PLASMA THYROXINE-BINDING PROTEINS

A THESIS

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R. L. SUTHERLAND

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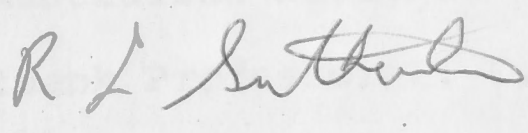
STATEMENT

The experiments reported in this thesis were carried out by myself.



R. L. SUTHERLAND

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ABSTRACT

A competitive binding technique is described for the estimation of the thyroxine (T_4) binding properties of proteins in samples of dilute blood plasma and lymph. The assay is performed in physiological media at physiological temperature and pH. When the total plasma T_4 concentration is known it is possible to obtain estimates of the following parameters: the number of functionally different T_4 binding proteins (T_4BPs), their individual association constants and binding capacities for T_4, the amount of total T_4 which is bound to each binding species, and the concentration of unbound T_4.

Both human and sheep serum were shown to have three functionally different T_4BPs. The mean association constants for the binding of T_4 to the three human T_4BPs were 9.5×10^9, 1.6×10^8 and 3.1×10^5 M^{-1} for T_4-binding globulin (TBG), T_4-binding prealbumin (TBPA) and serum albumin respectively. The corresponding sheep proteins; TBG, TBP-2 and albumin, had mean association constants of 8.9×10^9, 1.4×10^8 and 3.5×10^5 M^{-1} respectively. Human TBG had a mean T_4 binding capacity of 21.3 µg/100 ml of plasma while that of sheep TBG was 12.8 µg/100 ml. The other specific T_4BPs (TBPA in the human and TBP-2 in the sheep) had mean T_4 binding capacities of 307 and 359 µg/100 ml respectively.

Two functionally different T_4BPs were identified in rat serum. The specific T_4BP had T_4 binding properties

similar to those of TBPA and TBP-2, i.e. a mean association constant for T_4 of 3.64×10^8 M^{-1} and a mean T_4 binding capacity of 317 µg/100 ml. Rat albumin had an association constant of 6.3×10^5 M^{-1}.

Isotope tracer experiments in sheep with indwelling lymphatic cannulae demonstrated that T_4 [^{125}I] disappeared from the circulation and appeared in lymph at a greater fractional rate than human serum albumin [^{131}I] which was injected simultaneously into the circulation. The steady state lymph/plasma concentration ratios of the three T_4BPs, albumin and total T_4 did not differ significantly in any sample of lymph studied. Thus the more rapid fractional rate of movement of T_4 from blood to lymph was attributed mainly to the transfer of the unbound T_4 moiety across capillary endothelium.

Thyroidectomy and oestrogen administration resulted in a decline in the concentration of plasma TBG in ewes.

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CHAPTER 1

INTRODUCTION

Many intracellular and extracellular proteins are capable of binding steroid and thyroid hormones. The physico-chemical nature of the interaction varies according to the protein and hormone under consideration but can be divided arbitrarily into two distinct types: specific and non-specific binding. Specific binding is characterised by a high affinity of the protein for the hormone or ligand, a low concentration of binding sites, and a high degree of specificity of the binding site for the ligand. Non-specific binding involves low affinity, high capacity binding with no ligand specificity. The plasma proteins corticosteroid-binding globulin (CBG) and thyroxine-binding globulin (TBG) are examples of specific binding proteins. Corticosteroid-binding globulin specifically binds cortisol, corticosterone and progesterone while TBG binds thyroxine (T_4) and triiodothyronine (T_3). These hormones are bound with high affinity having association constants of the order of 10^9 litres/mole (M^{-1}). This enables appreciable proportions of the hormone to be bound even when present at very low concentrations. The binding sites are saturated at hormonal concentrations which although higher than the normal physiological levels are nevertheless of the same order of magnitude. Albumin, on the other hand, exhibits non-specific binding since a large number of steroid and thyroid hormones are bound with low affinity (association constants of the order of 10^5 M^{-1}) and the protein is unsaturable at hormone concentrations several orders of magnitude greater than those existing <u>in vivo</u>.

The interaction of hormone and protein involves non-covalent reversible binding, the binding sites are assumed to be independent of each other and the system obeys the law of mass action. Such reactions are not confined to hormone-protein interactions but are characteristic of a large number of biological systems. Since the discovery of the binding of acid dyes to serum proteins, a broad spectrum of low molecular weight substances have been observed to bind reversibly to a number of different proteins contained within the cells, tissues and fluids of the body. Included in this group of low molecular weight ligands are: acid dyes, fatty acids, enzyme substrates, steroid and thyroid hormones, prostaglandins, bile acids, metal ions, vitamins and a number of drugs. Antigen-antibody and hapten-antibody interactions are characterized by the same type of reversible binding and appear to obey the law of mass action.

Ligand-protein interactions have been studied in a number of scientific disciplines, consequently the experimental approach has varied considerably. Enzymologists were quick to realize that the interaction of enzyme and substrate was an equilibrium reaction. With the advent of Michaelis-Menten kinetics a method was available to quantitate several aspects of these interactions. It has taken several decades however, for similar approaches to be adopted by other disciplines studying phenomena based on the same physico-chemical principle. Even today some

authors in the field of endocrinology fail to appreciate the dynamic equilibrium which exists between a number of hormones and the proteins to which they bind at the cellular and extracellular levels.

In recent years a large number of specific hormone-binding proteins have been purified and their binding characteristics determined *in vitro*. While such studies have yielded valuable biochemical information the results obtained may have little relevance to binding *in vivo* and have helped little in attributing a functional role to such proteins. A more profitable approach to the understanding of the role of protein-binding in hormone distribution and metabolism *in vivo*, appears to be the use of techniques which quantitate binding in complex mixtures of binding proteins, e.g. plasma, under conditions mimicking those *in vivo*. These techniques in conjunction with whole animal experiments designed to quantitate changes in binding function under varying physiological conditions should prove valuable in assigning a functional role to specific hormone-binding proteins. An approach of this type has been adopted in the present study which documents some aspects of thyroid hormone-plasma protein interactions. Since these interactions are typical of many non-covalent ligand-protein interactions, the phenomena described in this dissertation are likely to be typical of many other binding systems and the techniques described may be useful in the study of such systems.

As the ultimate aim was to study aspects of the physiology of thyroxine-binding proteins (T_4BPs) *in vivo* the initial experimentation involved the development of an *in vitro* technique which could yield close estimates of the T_4 binding constants existing *in vivo*. The principle of the assay system, its advantages and disadvantages over other systems, the methods and accuracy of data analysis, as well as a description of *in vitro* conditions which influence the T_4 binding parameters are discussed in Chapters 3, 4 and 5. Albumin binding values derived from assays of whole plasma described in Chapters 4 and 5 were checked by using purified albumin preparations. These results are summarized in Chapter 6.

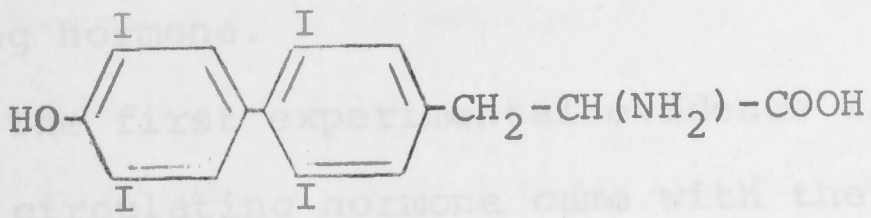
The second part of the study describes sheep experiments which were conducted to assess the role of T_4BPs in thyroid hormone distribution and metabolism. Chapter 7 describes experiments carried out to study the role of T_4BPs in the transfer of thyroid hormones from blood to extracellular fluid in sheep with indwelling lymphatic cannulae. Results presented in Chapter 8 illustrate the effect of thyroid hormones and some sex steroids on the concentration of circulating T_4BPs in sheep.

CHAPTER 2

THE THYROXINE-BINDING PROPERTIES OF PLASMA PROTEINS. A REVIEW.

Historical

The association of iodine with thyroid gland function was established in 1896 when Baumann demonstrated the presence of iodine in a protein fraction from thyroid tissue. On hydrolysis, this fraction yielded a substance, iodothyrin, which was capable of relieving the symptoms and signs of myxoedema produced as a result of thyroid insufficiency. It therefore seemed probable that the active principle of the thyroid gland contained iodine (Baumann, 1896a, b). Almost two decades later Kendall isolated this active iodine containing principle, which he called thyroxine (T_4), and found it contained as much as 65% iodine (Kendall, 1919; Kendall and Osterberg, 1919). Kendall's work was later repeated by Harington (1926a) who, with a modified extraction procedure, was able to obtain a one hundred fold increase in the extraction efficiency of T_4 from thyroid tissue. His subsequent synthesis of thyronine (4-(4'-hydroxyphenoxy) phenylalanine) (Harington, 1926b) established the structure of T_4 except for the position of the iodine atoms in the aromatic nuclei. However, the fact that both T_4 and diiodotyrosine (DIT) gave reactions characteristic of ortho-diiodophenols, together with the likelihood that T_4 was synthesized in the thyroid from two molecules of DIT, indicated that T_4 would have the following formula (Harington and Barger, 1927):



Thyroxine: α amino-β-[3:5-diiodo-4-(3′:5′-diiodo-4′ hydroxy phenoxy) phenyl] propionic acid.

Degradation and synthesis studies proved this to be correct (Harington and Barger, 1927) and it was later shown that L-thyroxine was the active principle (Harington and Salter, 1930; Foster, Palmer and Leland, 1936).

The discovery of iodine compounds in the blood quickly followed their demonstration in the thyroid gland, but their intimate association with serum proteins (Gley and Bourcet, 1900) led to erroneous interpretations. Some workers suggested plasma iodine was present as thyroglobulin (Hektoen, Carlson and Schulhof, 1923; Carlson, Hektoen and Schulhof, 1924; Hektoen and Schulhof, 1925; Hicks, 1926; Barnes and Jones, 1933). However, the failure of Lerman (1940) to detect thyroglobulin in the blood of euthyroid and hyperthyroid individuals, when sensitive serological tests were used, put an end to this theory. Other workers postulated the existence of a circulating peptide or polypeptide containing both T_4 and DIT (Harington, 1935; Salter, 1940; Williams and Whittenberger, 1947). In a re-examination of previous evidence, Harington (1944) concluded that the peptide concept was an unnecessary complication and that T_4 itself was probably the

circulating hormone.

The first experimental evidence in favour of T_4 as the circulating hormone came with the work of Trevorrow (1939). This study showed that the thyroid hormone of blood behaved the same way towards protein precipitants, dialysis and ethanol extraction as did added T_4. It was not until nine years later that Taurog and Chaikoff (1948) demonstrated unequivocally that the circulating hormone labelled with ^{131}I, and added T_4, showed identical behaviour in the presence of protein precipitants and organic solvents. This work was confirmed by Leblond and Gross (1949) and additional evidence in favour of T_4 as the circulating hormone was presented in the same year when Laidlaw (1949) identified T_4 on paper chromatograms of rat plasma. Numerous studies employing a variety of techniques in a number of mammalian species have confirmed that over 90% of the circulating organic iodine in the normal animal is T_4 (Rall, Robbins and Lewallen, 1964; Robbins and Rall, 1967).

The observation that blood iodine, despite its precipitability with plasma proteins (Gley and Bourcet, 1900) and inability to pass an ultrafilter (Trevorrow, 1939) or a dialysis membrane (Silver, 1942) was readily extractable in certain organic solvents (Trevorrow, 1939; Riggs, Lavietes and Man, 1942; Burger and Member, 1943; Taurog and Chaikoff, 1948), lead to the postulate that circulating T_4 is bound in

some way to one or more plasma proteins. Early attempts to identify the binding of T_4 to plasma proteins using salting out procedures showed that iodine was present in most protein fractions. The highest iodine content was found in the albumin and α-globulin fractions but the latter fraction had the highest iodine to nitrogen ratio (Basset, Coons and Salter, 1941; Riggs et al., 1942; Taurog and Chaikoff, 1948; Salter, 1949; Silver and Reiner, 1950). The application of zone electrophoresis to the problem provided clear evidence for a specific T_4-binding protein in serum (Gordon, Gross, O'Connor and Pitt-Rivers, 1952). These workers found that when serum labelled with a small quantity of radio-thyroxine was electrophoresed on paper using barbital buffer at pH 8.6, the majority of T_4 migrated with the α-globulins. The remainder of the label was bound to albumin. Such findings were soon confirmed, but it was found that T_4 was bound to a protein with an electrophoretic mobility between α_1 and α_2-globulins (Robbins and Rall, 1952; Larson, Deiss and Albright, 1952; Winzler and Notrica, 1952; Larson, Deiss and Albright, 1954). This α-globulin fraction, then termed thyroxine-binding protein, is now known as thyroxine-binding globulin (TBG).

Subsequent electrophoretic work at similar alkaline pH employing Tris-maleate (Ingbar, 1958; Tata, Widnell and Gratzer, 1961) and several other buffer systems (Ingbar, 1960; Robbins and Rall, 1960; Blumberg and Robbins, 1960; Sterling and Tabachnick, 1961a; Tata et al., 1961) showed that some of the hormone migrated

with one of the prealbumins, now termed thyroxine-binding prealbumin (TBPA). The inability to identify binding to this protein in barbital buffer was explained by the observation that barbiturate ions compete with T_4 for binding sites on TBPA (Tata et al., 1961; Ingbar, 1963; Woebar and Ingbar, 1964).

It is now generally accepted that in human serum under normal physiological conditions, T_4 is bound to three proteins; TBG, TBPA and albumin. This is not necessarily true of all mammalian species. Large interspecies variations have been demonstrated in the electrophoretic mobility of T_4BPs in the plasma of a large number of species (Robbins and Rall, 1957, 1960; Refetoff, Robin and Fang, 1970). Studies using sheep serum have demonstrated T_4 binding in the α-globulin and albumin regions using a number of electrophoretic media and buffer systems (Robbins and Rall, 1957, 1960; Annison and Lewis, 1959; Annison, 1960; Farer et al., 1962; Refetoff et al., 1970). The subsequent discussion will be primarily concerned with human T_4BPs, but species differences will be considered where relevant.

The subject of thyroid hormone-plasma protein interactions has been discussed in great detail in several expansive reviews (Pitt-Rivers and Tata, 1959; Robbins and Rall, 1957, 1960, 1967; Ingbar and Freinkel, 1960; Ingbar, 1960; Rall et al., 1964; Ingbar and Woebar, 1968). It is not the purpose of this treatise to describe in detail the development of knowledge on the properties and functions of plasma T_4BPs,

but rather to give a precise summary of the present state of knowledge. For a more detailed discussion of the development of current knowledge the reader is referred to the above review articles.

Thyroxine-binding globulin (TBG)

Some controversy arose as to the homogeneity of TBG when two TBG bands were identified in human serum using starch-gel electrophoresis (Tata et al., 1961; Blumberg, Farer, Rall and Robbins, 1961; Thorson, Tauxe and Taswell, 1966; Inada and Sterling, 1970; Refetoff et al., 1970). Although both components showed TBG-like behaviour, only one band was present when an albuminaemic serum was used, suggesting that the splitting represented a protein-protein interaction on a single species of TBG protein (Werner and Nauman, 1968; Inada and Sterling, 1970; Refetoff et al., 1970). Despite this evidence, only one TBG band was found by other groups using a similar buffer system and technique of electrophoresis (Britton, Webster, Ezrin and Volpé, 1965) and agar gel (Diguilio, Michalak, Weinhold, Hamilton and Thomas, 1964) or polyacrylamide gel electrophoresis (Marshall and Levy, 1966). These results suggest that TBG is a single molecular entity, but the possibility of interaction between TBG and albumin cannot be ruled out. Studies with purified TBG, using reverse flow electrophoresis in glycine-acetate buffer at pH 8.6, show that purified TBG has a different mobility to TBG

of whole serum. On the other hand a TBG-human serum albumin (HSA) mixture had a mobility similar to that of whole serum TBG (Sterling, Hamada, Takemura, Brenner, Newman and Inada, 1971). This was not confirmed by Marshall and Pensky (1971) who demonstrated that purified TBG and TBG of whole serum had identical mobility on cellulose acetate electrophoresis in Tris-acetate buffer at pH 8.9. More recently a slow moving TBG band has been isolated by cellulose acetate electrophoresis of TBG fractions prepared on T_4 substituted Sepharose columns (Marshall, Pensky and Green, 1972). This slow band has been attributed to the loss of sialic acid residues from the native molecule.

Although TBG has been identified for over twenty years, its purification has proven difficult to achieve due to its very low plasma concentration. Several groups of workers have now reported the isolation of highly purified TBG (Giorgio and Tabachnick, 1968; Marshall and Pensky, 1969, 1971; Pensky and Marshall, 1969; Sterling et al., 1971; Green, Marshall, Pensky and Stanbury, 1972a).

Thyroxine-binding globulin is known to be an acidic glycoprotein with an isoelectric point of about pH 4 (Robbins, Peterman and Rall, 1955; Ingbar, Dowling and Freinkel, 1957; Sterling et al., 1971) and contains sialic acid and other carbohydrates (Ingbar, 1958; Blumberg and Warren, 1961; Seal and Doe, 1962; Giorgio and Tabachnick, 1968; Sterling et al., 1971). Its amino

acid composition has been assessed in a number of laboratories but the estimates show little agreement (Seal and Doe, 1964; Giorgio and Tabachnick, 1968; Sterling et al., 1971). Estimates of the molecular weight of TBG have also varied widely. Early studies on human TBG showed it to be a relatively small molecule with a sedimentation coefficient of 3.3 - 3.5 S and an estimated molecular weight of the order of 50,000 (Peterman, Robbins and Hamilton, 1954; Tata, 1961; Seal and Doe, 1962). Robbins and Rall (1957) pointed out, however, that such sedimentation coefficients would give molecular weights in the range 30,000 - 1,000,000 depending on the shape of the molecule. Later studies on highly purified TBG reported a sedimentation coefficient of 3.4 - 3.9 S and a molecular weight of 54,000 - 65,000 (Seal and Doe, 1964; Giorgio and Tabachnick, 1968; Marshall and Pensky, 1969, 1971). A recent publication suggests that TBG is a much smaller molecule having a sedimentation coefficient of 3.0 S and an estimated molecular weight of 36,500 (Sterling et al., 1971). While these latter authors thought it unlikely that the purified TBG was a dissociation product of the native TBG molecule it seems that like the composition, the molecular weight of TBG has yet to be measured unequivocally.

The fact that the interaction between T_4 and TBG is one of reversible equilibrium is evident from numerous studies (Robbins and Rall, 1957, 1960, 1967; Rall et al., 1964; Andreoli, Robbins, Rall and Berman, 1965). Robbins and Rall (1957) calculated the association

constant to be of the order of 10^{10} M^{-1}. Experimental values in the range 1.7 - 4 x 10^{10} M^{-1} have been reported which substantiates the concept that TBG has an exceedingly high affinity for T_4 (Woebar and Ingbar, 1968; Hamada, Nakagawa, Mori and Torizuka, 1970; Sterling et al., 1971; Green et al., 1972a, b). The binding of T_4 to TBG is temperature dependent (Green et al., 1972a) however exposure to temperatures of 60° for prolonged periods abolishes binding due to denaturation of TBG (Takemura, Hocman and Sterling, 1971). Various estimates of the association constant are summarized in Table 2.1.

Table 2.1. Reported estimates of the association constant between TBG and T_4.

Association Constant (M^{-1})	Reference
7.9×10^9	Robbins and Rall, 1957
8.2×10^9	Oppenheimer and Surks, 1964
4×10^{10}	Robbins and Rall, 1967
1.7×10^{10}	Woebar and Ingbar, 1968
2.2×10^{10}	Hamada et al., 1970
2.3×10^{10} (23°)	Green et al., 1972a, b
1.7×10^{10} (37°)	

Thyroxine is bound at the rate of one molecule of T_4 per TBG molecule (Seal and Doe, 1962; Marshall and

Pensky, 1971; Sterling et al., 1971; Green et al., 1972a) and is strongly bound at moderately alkaline as well as neutral pH; the affinity, however, is greatly reduced in acid conditions (Robbins and Rall, 1960; Korsgaard Christensen, 1961; Marshall and Pensky, 1971). The last study, using purified TBG, demonstrated that binding of T_4 to TBG was maximal over a pH range of 6.4 - 10.4. Below pH 6.4 the strength of the T_4-TBG interaction began to decline and at pH 4.2 binding nearly disappeared (Marshall and Pensky, 1971). It has been postulated that changes in the degree of ionization of the imidazole group of histidine residues on TBG, and in the phenolic hydroxyl group of T_4 may cause the pH dependence of T_4-TBG interactions (Marshall and Pensky, 1971).

The structural requirements on the part of the ligand are very strict since closely related analogues of T_4 are bound much less strongly than T_4 (Larson and Albright, 1955; Robbins and Rall, 1955, 1957). Thyroxine-binding globulin has a lower affinity for T_3 than for T_4 (Tanaka and Starr, 1959; Korsgaard Christensen, 1960). Some workers have suggested that T_3 is bound with about one third of the affinity of T_4 (Tata, 1960; Robbins and Rall, 1967) while others conclude that in vivo binding of T_3 by TBG is much lower than this (Zaninovich, Farach, Ezrin and Volpé, 1966).

The binding capacity of TBG for T_4 in normal human plasma has been estimated to be 18 - 22 µg T_4/100 ml

of plasma (Robbins and Rall, 1957; Digiulio et al., 1964; Inada and Sterling, 1967; Ingbar and Woebar, 1968; Hamada et al., 1970; Refetoff, Hagan and Selenkow, 1972). This binding capacity is considerably altered by several physiological and pathological conditions. Estimation of the concentration of TBG in normal human plasma indicates a much wider range of 1 - 3.4 mg/100 ml (Giorgio and Tabachnick, 1968; Marshall and Pensky, 1971; Sterling et al., 1971; Levy, Marshall and Velayo, 1971; Chopra, Solomon and Ho, 1972). This apparent discrepancy between estimates of binding capacity and TBG concentration are probably due to the wide variety of techniques used to estimate the latter parameter. While some estimates of TBG concentration are derived from the binding capacity others have involved competitive binding and radioimmunoassay techniques (Levy et al., 1971; Chopra et al., 1972).

A summary of various estimates of some of the physical properties of TBG is presented in Table 2.2.

Thyroxine-binding prealbumin (TBPA)

The higher plasma concentration and the better electrophoretic separation of TBPA has made its purification a somewhat simpler procedure than the purification of TBG. Thyroxine-binding prealbumin has now been isolated in pure form in several laboratories (Oppenheimer, Surks, Smith and Squef, 1965; Purdy, Woebar, Hollaway and Ingbar, 1965; Raz and Goodman, 1969; Branch, Robbins and Edelhoch, 1971). Centrifugal analysis suggested human

Table 2.2. Reported estimates of the physical properties of TBG.

$S_{20,w}$ ($\times 10^{-13}$ Sec.)	M.Wt. ($\times 10^{3}$)	Association Constant ($\times 10^{10}$ M^{-1})	Maximum Moles T_4 bound per Mole TBG	Plasma Conc. (mg/100 ml.)	Reference
3.3					Peterman et al., 1954
3.5	40-50			1-2	Tata, 1961
3.3	50±2		1		Seal and Doe, 1962
3.6	59				Seal and Doe, 1964
3.92	58	0.1		1.6	Giorgio and Tabachnick, 1968
3.91	54				Marshall and Pensky, 1969
3.4	63-65		1	1.5	Marshall and Pensky, 1971
3.0	36.5	1.0	0.7	1.0	Sterling et al., 1971
				3.4	Levy et al., 1971
		1.7-2.3	0.85(T_4) 0.91(T_3)		Green et al., 1972a, b
				2.85	Chopra et al., 1972

TBPA had a sedimentation coefficient of 4.58 S and a molecular weight of 73,000 (Oppenheimer et al., 1965). A later study indicated that the molecular weight was only 50,000 and the authors postulated that the discrepancy between the two estimates was due to the presence of retinol-binding protein in the early preparation (Raz and Goodman, 1969). More recently Branch et al (1971) have shown that TBPA has a molecular weight of 54,000 and is a stable tetramer of identical subunits. The protein also binds the retinol-binding protein but binding of T_4 and retinol-binding protein are independent (van Jaarsveld, Edelhoch, Goodman and Robbins, 1973).

The affinity of TBPA for T_4 is weaker than the corresponding binding to TBG and is strongly pH dependent. Binding is most avid at pH 8.6 but decreases with a reduction in pH to the physiological range (Ingbar, 1963; Lutz and Gregerman, 1969). Some workers using paper electrophoresis have shown that T_4 binding by TBPA is virtually absent at pH 7.4 (Myant and Osorio, 1960; Korsgaard Christensen and Litonjua, 1961; Osorio, 1967). However recent work employing agarose gel electrophoresis and immunoadsorption suggests TBPA is responsible for about 15% of T_4 binding at pH 7.4 in normal serum (Woebar and Ingbar, 1968; Lutz and Gregerman, 1969).

Thyroxine-binding prealbumin has only one T_4 binding site per molecule (Woebar and Ingbar, 1964; Oppenheimer et al., 1965; Oppenheimer, Martinez and Bernstein, 1966; Pages, Robbins and Edelhoch, 1973). The requirements for hormone binding to this site are

quite different from those for binding to the TBG site. D-thyroxine and T_3 are bound very weakly (Ingbar, 1963; Ross and Tapley, 1966; Davis, Handwerger and Gregerman, 1972; Larsen, 1972; Pages et al., 1973) whereas acidic side chain analogues such as tetraiodothyroacetic acid are bound more firmly to TBPA than to TBG (Tata et al., 1961; Ingbar, 1963; Woebar and Ingbar, 1964; Ross and Tapley, 1966).

Despite its lower affinity for T_4, as shown by an association constant of 0.16 - 2.3 x 10^8 M^{-1} (Woebar and Ingbar, 1968; Raz and Goodman, 1969; Hamada et al., 1970; Pages et al., 1973), TBPA can bind at least ten times as much T_4 as TBG due to its higher plasma concentration of about 30 mg/100 ml (Oppenheimer et al., 1965, 1966). Although estimates of the binding capacity of TBPA for T_4 range from 40 - 340 µg/100 ml (Digiulio et al., 1964; Britton et al., 1965) it is generally agreed that the normal mean binding capacity in human serum is 230 - 270 µg/100 ml (Oppenheimer et al., 1963, 1966; Inada and Sterling, 1967; Hamada et al., 1970; Gordon, Kleinerman, Ehrenfeld and Ehrenfeld, 1971). Certain types of non-thyroidal illness have profound effects on the level of circulating TBPA (Ingbar and Woebar, 1968).

Albumin

The binding of thyroid hormones to albumin has been studied intensively due to the ready availability of this protein in a highly purified state. Earlier work

on human albumin (Sterling and Tabachnick, 1961b; Sterling, Rosen and Tabachnick, 1962) and bovine albumin (Lein, 1952) indicated the existence of four T_4 binding sites per molecule with an association constant of 0.79 - 1.1 x 10^5 M^{-1}. Later studies employing fluorescence quenching (Steiner, Roth and Robbins, 1966) or equilibrium dialysis (Steiner et al., 1966; Tabachnick, 1967) indicated that there was a single strong binding site with an affinity constant of 1.6 ± 0.4 x 10^6 M^{-1} at pH 7.4, 26°, and an undetermined number of much weaker sites. These results are close to the value of 2.5 x 10^6 M^{-1} obtained earlier by an enzymic assay (Tritsch, Rathke, Tritsch and Weiss, 1961). Bovine (BSA) and human serum albumin have similar T_4 binding properties (Tritsch et al., 1961; Steiner et al., 1966) but chicken serum albumin has an eightfold higher affinity at the primary site (Tritsch and Tritsch, 1965).

Because of its high plasma concentration serum albumin has an extremely high capacity for T_4, the upper limit being governed by the solubility of T_4. The affinity of albumin for T_3 is about 1/4 - 1/6th of that for T_4 at pH 7.4 (Tabachnick, 1964a; Steiner et al., 1966). This lower affinity for T_3 is probably due to the decreased ionization of the phenolic hydroxyl group of that hormone (Sterling and Tabachnick, 1961b; Steiner et al., 1966).

As is the case with TBG and TBPA the interaction of thyroid hormones with albumin is temperature and pH dependent. Thyroxine is bound more tightly to albumin at

5° than at 38° (Sterling et al., 1962). Investigation of the pH dependence of thyroid hormone binding to serum albumin has not yielded uniform results. Tabachnick (1964a) suggests that the affinity for T_4 is constant over the pH range 6.8 - 9.3 while outside this range binding declines rapidly. In contrast, Korsgaard Christensen (1961) found that T_4 binding to BSA increased with increasing pH between 6.5 and 9.4. Other workers reported an increase in the association constant between pH 6 and 8 but found little change between pH 8 and 9 (Tritsch et al., 1961; Steiner et al., 1966).

The T_4 binding sites on albumin are considered relatively specific for T_4 since it has been shown that the phenolic hydroxyl group, the iodine atoms in the 3' and 5' positions, the diphenyl ether group and the carboxyl group are all essential for high affinity binding (Sterling, 1964; Tabachnick and Giorgio, 1964). The positively charged ε-amino groups of lysine on albumin appear to form part of the thyroid hormone binding site (Tabachnick, 1964a; Sterling et al., 1962).

Fatty acids and some other anions are known to reduce hormone binding (Sterling et al., 1962; Tabachnick, 1964b; Tabachnick, Downs and Giorgio, 1965; Tabachnick, 1967). Sodium chloride at a concentration of 0.1 M reduces T_4 binding by 50% at pH 7.4 (Tabachnick, 1967).

Reported values for the association constants of T_4 with serum albumin are summarized in Table 2.3.

Table 2.3. Reported estimates of the association constant between serum albumin and T_4

No. of Binding Sites	Association Constant (M^{-1})	Experimental Conditions	Reference
4	7.9×10^4	BSA, 25° pH 9.75	Lein, 1952
4	1.1×10^5	HSA, 38° pH 7.4	Sterling and Tabachnick, 1961b
1	2.5×10^6	HSA & BSA, 23° pH 7.4	Tritsch et al., 1961
1	2.0×10^7	Chicken SA, 23° pH 7.4	Tritsch and Tritsch, 1965
4	1.07×10^5	HSA, 30° pH 7.4	Sterling et al., 1962
4.2	1.63×10^5	" fatty acids extracted	"
2	2.75×10^5	HSA, 30° pH 7.4	Tabachnick, 1964a
2	3.3×10^5	pH 9.3	"
2	3.75×10^5	HSA, 30°, pH 7.4 fatty acids extracted	Tabachnick, 1964b
1	1.6×10^6	HSA, BSA, 26° pH 7.4	Steiner et al., 1966
0.85	1.4×10^6	HSA, 30° pH 7.4	Tabachnick, 1967

Distribution of endogenous T_3 and T_4 between TBG, TBPA and albumin

The partition of T_3 and T_4 between their individual serum carrier proteins *in vivo* is dependent on the concentration of each binding protein, its respective association constant for T_3 and T_4, the concentration of T_3 and T_4, and the concentration of any plasma constituent that may compete for binding sites on the proteins. Studies employing conventional electrophoretic techniques have only been able to give approximations of the *in vivo* situation since the distribution of radioactive T_3 and T_4 between the carrier proteins is not only strongly influenced by the pH, the ionic composition and the ionic strength of the buffer employed, but also by the type of medium used, the amount of tracer T_3 and T_4 added, and the temperature at which the assay is performed (Tata *et al.*, 1961; Marshall and Levy, 1966; Gordon and Coutsoftides, 1969; Davis and Gregerman, 1970, 1971).

Early estimates indicated that at pH 8.6 TBG was responsible for 50-60% of T_4 binding while a large proportion (32-37%) was bound to TBPA (Tata *et al.*, 1961; Oppenheimer *et al.*, 1963). Recent evidence however suggests that under physiological conditions TBPA is only responsible for 15% of the total bound T_4 (Woebar and Ingbar, 1968; Hamada *et al.*, 1970). There is general agreement that serum albumin accounts for only 10-15% of T_4 binding under normal circumstances (Tata *et al.*, 1961; Oppenheimer *et al.*, 1963; Woebar and Ingbar, 1968; Gordon and Coutsoftides, 1969; Hamada *et al.*,

1970). It appears likely that the role of TBG in T_4 binding *in vivo* is quantitatively much more important, and that of TBPA much less important, than was previously thought (Woebar and Ingbar, 1968; Hamada *et al.*, 1970).

The only estimate of the distribution of T_3 between its carrier proteins is from results of polyacrylamide gel electrophoresis performed at pH 9.0 (Davis *et al.*, 1972). Whilst the results are unlikely to portray the true *in vivo* situation it appears that TBG binds over 50% of endogenous T_3. Thyroxine-binding prealbumin seems to be only a very minor T_3 binding protein while a significant portion (30-40%) is bound to albumin (Davis *et al.*, 1972).

Unbound thyroid hormones

As the major proportion of plasma T_3 and T_4 is strongly bound to plasma proteins it is not surprising that the concentration of unbound (free) thyroid hormones in plasma is very low. The term unbound T_4 is used in preference to free T_4 since free hormone refers to unconjugated hormone in the steroid literature and thus does not refer to that portion of circulating hormone that is not protein bound. The concentration of unbound hormone is so low that direct measurement of unbound T_4 has been technically very difficult. The earliest estimation of unbound T_4 was from mathematical calculations employing data obtained from electrophoretic studies and studies using purified serum albumin. This estimate indicated that the average concentration of unbound T_4 in the plasma

of the euthyroid human was 4.7 ng/100 ml or approximately 0.06% of the total plasma T_4 (Robbins and Rall, 1957).

Because of the difficulty in direct measurement of unbound T_4 a parameter, the "free thyroxine index", was introduced as a proportional estimate of unbound T_4. Such indices are obtained by multiplying the total T_4 concentration by a factor which is inversely proportional to the concentration of the unoccupied T_4 binding sites. Various workers have employed such factors as the T_4 dialysis rate (Korsgaard Christensen, 1959; Gimlette, 1965), the erythrocyte T_3 uptake (Hamolsky, Stein and Freedberg, 1957) and the resin T_3 uptake (Mitchell, Harden and O'Rourke, 1960; Gimlette, 1967). These techniques give good correlations with clinical findings and more sophisticated modifications of the resin method are widely employed clinically (Maclagan and Howorth, 1969; Hamada et al., 1970).

The first measurement of unbound T_4 was described by Sterling and Hegedus (1962). Several variations of their basic method, which involves measurement of the dialysable portion of serum T_4 after labelling with tracer quantities of T_4 (^{125}I or ^{131}I), have now been described. The concentration of unbound T_4 is the product of this dialysable fraction and the total serum T_4 level. The major technical problem with this method has been the presence of radioactive dialysable impurities in the dialysate. These compounds cause an overestimate of unbound T_4 and a number of methods have been employed to remove them. Paper chromatography, Dowex 50 column

chromatography, and gel filtration on Sephadex G-25 columns have all been used to separate T_4 and iodide in the dialysate (Sterling and Hegedus, 1962; Liewendahl and Lamberg, 1965). Labelled T_4 in the dialysate has been precipitated by $MgCl_2$ (Sterling and Brenner, 1966) and by TCA following addition of whole serum (Oppenheimer et al., 1963). Ingbar et al. (1965) have added serum to the dialysate and then removed labelled iodide by further dialysis in the presence of Amberlite IRA-400 resin.

Ultrafiltration methods have also been described for the measurement of unbound T_4 but suffer from the same problems of radioactive impurities in the ultrafiltrate (Oppenheimer and Surks, 1964; Schussler and Plager, 1967; Thorson, Wilkins, Schaffrin, Morrison and McIntosh, 1972). Schussler and Plager (1967) suggest that more accurate results are obtained if the isotope is purified prior to use. However, Thorson et al. (1972) obtain similar results with purified and unpurified T_4 [^{131}I].

Although all methods give internally consistent results there are considerable differences between laboratories. Table 2.4 summarizes the estimates of unbound T_4 concentration in normal human serum.

The effect of dilution of serum on the measurement of unbound T_4 has been discussed by many authors. Oppenheimer and Surks (1964) found that dilution had no effect on the concentration of free T_4. This has been refuted by others (Ingbar et al., 1965; Sterling and Brenner, 1966; Lee and Pileggi, 1971). Spaulding and Gregerman (1972) have attributed the decrease in unbound

Table 2.4. Reported estimates of the concentration of unbound T_4 in normal human serum

% Unbound T_4	Unbound T_4 Concentration (ng/100 ml)	Reference
0.06	4.7	Robbins and Rall, 1957
0.110 ± 0.016	10.0 ± 2.9	Sterling and Hegedus, 1962
0.042 ± 0.004	2.34 ± 0.28	Oppenheimer et al., 1963
0.050 ± 0.009	4.03 ± 1.08	Ingbar et al., 1965
0.046 ± 0.005	2.76 ± 0.5	Sterling and Brenner, 1966
0.026 ± 0.004	2.1 ± 0.4	Schussler and Plager, 1967
0.054 ± 0.008	5.67 ± 0.09	Liewendahl and Lamberg, 1969
0.038 ± 0.004	2.5 ± 0.4	Fang and Selenkow, 1970
0.031 ± 0.004	2.64 ± 0.26	Hamada et al., 1970
	1.9 ± 0.2	Lee and Pileggi, 1971
0.056 ± 0.007	4.5 ± 1.1	Liewendahl, Tatterman and Lamberg, 1971
0.046 ± 0.010	4.28 ± 0.78	Thorson et al., 1972

T_4 concentration with dilution to changes in the concentration of phosphate and chloride ions during dialysis while Schussler and Plager (1967) suggested that the presence of dialysable radioactive contaminants could account for these dilution effects. The latter work showed that removal of labelled contaminants reduced the ultrafiltrable fraction in undiluted serum by about 50%. Moreover, when a 1:10 dilution of plasma was made with phosphate buffer the ultrafiltrable fraction did not decrease (Schussler and Plager, 1967).

Since it has been demonstrated that pH and temperature are important determinants of the binding of T_4 to plasma proteins it is not surprising that these factors greatly influence the level of unbound T_4 measured *in vitro* (Fang and Selenkow, 1970; Lee and Pileggi, 1971; Thorson *et al.*, 1972; Spaulding and Gregerman, 1972).

Although the electrophoretic pattern of T_4 binding proteins varies greatly between species it is interesting to note that the concentrations of unbound T_4 in the plasma of most mammalian species studied fall within the range reported for normal humans (Refetoff *et al.*, 1970). Due to uncertainties in the measurement of total T_3 concentration in human serum, it has not been possible to arrive at a precise value for unbound T_3. Since T_3 is bound less strongly to TBG the dialysable fraction is 10-15 times greater than that of T_4 (Ingbar *et al.*, 1965; Larsen, 1972). This corresponds to a normal unbound fraction of 0.26 - 0.46% (Nauman, Nauman

and Werner, 1967; Larsen, 1972). If one uses a mean value of 0.36% and a mean radioimmunoassay value for total T_3 of 1.2 ng/ml the mean concentration of unbound T_3 would be 430 pg/100 ml, about one-fifth of the absolute concentration of unbound T_4 in human serum (Larsen, 1972).

Factors affecting thyroid hormone-plasma protein interactions

A number of physiological, pathological and pharmacological factors alter the interaction of thyroid hormones with plasma proteins. Two types of mechanisms are apparent, i.e. those that involve competitive interactions for the T_4 binding sites on proteins, e.g. diphenylhydantoin, and those which cause an alteration in the concentration of binding sites by affecting protein synthesis and turnover, e.g. pregnancy and oestrogenic agents. The influence of such factors on thyroid hormone binding to human plasma has been considered in depth in two expansive reviews (Robbins and Rall, 1967; Ingbar and Woebar, 1968) and the findings are summarized in Table 2.5.

Species differences may be important, for example diphenylhydantoin has no effect on hormone binding to rat plasma (Hershman, 1963) but greatly reduces T_4 binding to TBG in human plasma (Wolff, Standaert and Rall, 1960), while pregnancy which induces a significant increase in TBG concentration in the human (Robbins and Rall, 1967) has no effect on TBG concentration in sheep (Annison, 1960).

Table 2.5. Factors affecting thyroid hormome-plasma protein interactions in human plasma

Factors causing a decrease in hormone binding	
(A) to TBG	
1	age
2	androgenic and anabolic steroids
3	masculinizing disorders
4	idiopathic decrease in TBG
5	excess glucocorticoids
6	acrogmegaly
7	diphenylhydantoin
(B) to TBPA	
1	salicylates
2	dinitrophenol
3	some cases of nonspecific illness e.g. chronic illness or surgical stress
Factors causing an increase in hormone binding	
(A) to TBG	
1	pregnancy
2	oestrogenic agents
3	feminizing tumours
4	acute hepatocellular disease
5	acute intermittent porphyria
6	idiopathic increase in TBG
(B) to TBPA	
1	androgen

The physiological role of plasma hormone binding proteins

As a result of their interaction with plasma proteins, thyroid and steroid hormones acquire macromolecular properties which profoundly alter their distribution and metabolism. These interactions appear to retard the transfer of hormones to extravascular sites and although direct experimental evidence is lacking there is a possibility that the hormonal supply to tissues *in vivo* may be limited by the rate of dissociation of the hormone protein complexes (Robbins and Rall, 1967).

It is apparent from numerous studies that the unbound hormone is freely available to tissues where it can induce a metabolic response and undergo degradation (Ingbar and Woebar, 1968; Westphal, 1971). When considering the thyroid "status" of individuals it is well documented that hyper-, hypo-, and euthyroidism are correlated with the unbound rather than the total T_4 concentration (Sterling and Hegedus, 1962; Ingbar *et al.*, 1965; Thorson *et al.*, 1972). It is also evident that abnormally high (Beierwaltes and Robbins, 1959) and low (Ingbar, 1961) TBG levels are entirely compatible with euthyroidism. In these instances, T_4 production and disposal proceed at normal rates. Thus binding of thyroid hormones to proteins does not affect either hormone action or its steady state production but rather determines the level of total T_4 in blood. It thus appears that the T_4-protein complex in blood and extracellular fluid serves as a hormone reservoir or buffer in that it

tends to prevent rapid changes in unbound T_4 concentration resulting from abrupt changes in T_4 secretion and degradation.

Other roles have been postulated for hormone binding proteins. Robbins and Rall (1967) suggest one function of T_4BPs may be to establish an unbound T_4 concentration gradient from the cells and extracellular fluid of the thyroid gland to the blood stream. A similar role has been postulated for the steroid hormone binding proteins which are also thought to be involved in the active role of transporting hormones into their respective target tissues (Baulieu, Raynaud and Milgrom, 1970).

CHAPTER 3

MATERIALS AND METHODS

Experimental animals

Sheep. Male and female Merino and Merino cross sheep of various breeds and ages were used throughout this study. Prior to experimentation animals were maintained on pasture in small holding paddocks. During the experiments the sheep were housed indoors in individual pens or metabolism cages and received lucerne chaff, oats and water ad libitum.

Rats. Adult male rats weighing approximately 250 g were obtained from a Wistar derived colony maintained by the laboratory. Water and a commercial rat diet were fed ad libitum.

Rabbits. Adult male rabbits used for the production of antisera and as a supply of normal rabbit sera were obtained from the outbred colony maintained by the laboratory. They were housed in individual cages and fed a diet of commercial rabbit pellets.

Human plasma samples

Normal human plasma was obtained from expired blood bank whole blood. Plasma was recalcified with 1 M $CaCl_2$, the clot disrupted and removed by centrifugation. After dialysis overnight against 0.05 M phosphate, 0.10 M NaCl, pH 7.4 the serum was frozen in 5 ml portions. Plasma from hypothyroid, thyrotoxic and pregnant patients was obtained from the Department of Nuclear Medicine, The Prince of Wales Hospital, N.S.W. through the kindness of Dr I. P. C. Murray.

Anaesthetics

Anaesthetic ether B.P. (Drug Houses of Australia, Brooklyn, Victoria) was used to anaesthetise rats.

Thiopentone sodium B.P. ("Intraval" Sodium, May and Baker, Ltd) was used as a 5% solution in distilled water for the induction of anaesthesia in sheep. Subsequently Halothane B.P. (Fluothane, I.C.I. Ltd) was used for maintenance of anaesthesia in a closed circuit.

Pentobarbitone sodium B.P. ("Nembutal", Abbot Laboratories Pty Ltd) was used for some anaesthetic procedures.

Biological reagents

Anticoagulant. Heparin (157 I.U./mg, pyrogen free, Evans Medical Australia) was used as anti-coagulant for lymph and blood samples.

Radio-Isotopes. L-Thyroxine [^{125}I] and L-3,3',5-Triiodothyronine [^{125}I] in 50% propylene glycol, with an initial specific activity of 40-45 mCi/mg, containing 4-5 µg hormone/ml were supplied at 2 monthly intervals by the Radiochemical Centre, Amersham, England.

Sodium iodide [^{131}I] and [^{125}I] (carrier free) were obtained as required from the Atomic Energy Commission, Lucas Heights, New South Wales and the Radiochemical Centre, Amersham, England.

Thyroid and steroid hormones. L-Thyroxine sodium salt (BDH Chemicals Ltd, Poole, England) and the corresponding salt of L-3,3',5-Triiodothyronine (Schwarz Bioresearch

Inc., Orangeburg, N.Y., U.S.A.) were dissolved separately in an appropriate volume of 50% propylene glycol containing a few drops of 0.1 M NaOH, to give a final concentration of 1 mg/ml. These stock solutions were diluted by weight with 50% propylene glycol to produce serial 10 fold dilutions which were used as working standards.

Stilboestrol, 5 mg/ml (Knoll Laboratories Pty Ltd, Sydney) and testosterone propionate, 50 mg/ml (Troy Laboratories Pty Ltd, N.S.W.) in arachis oil were used for intramuscular injection.

Serum albumin. Human serum albumin, 100% pure on electrophoresis was supplied by Hoechst Australia Ltd, Sydney. Sheep fraction V (95% pure) and rat fraction V were obtained from Schwarz Bioresearch Inc., Orangeburg, N.Y., U.S.A. Crystallised rabbit albumin, 100% pure by electrophoresis was obtained from Mann Research Laboratories, N.Y., U.S.A.

Scintillation fluid. Naphthalene 60 gm, 2,5-diphenyl oxazole (PPO, scintillation grade, Packard) 4.0 gm, and 100 ml methanol were dissolved in 1,4-dioxane to give a final volume of 1 litre.

Biological solutions. Physiological saline was a 0.9% w/v solution of sodium chloride in distilled water.

Tris (hydroxymethyl) aminomethane and barbital buffers of varying molarity and pH were prepared by titrating an appropriate amount of Tris (Sigma Chemical

Company, St Louis, U.S.A.) or sodium barbital to the desired pH with 1 M HCl.

Sodium phosphate buffers of varying molarity and pH were prepared as described by Green and Hughes (1955).

Statistical methods

All statistical analyses and mathematical computations were carried out using a PDP 8/I computer (Digital Equipment Corporation) running a 7 user FOCAL system 'LIBRA'.

Analysis of variance used the program VAIM which calculated the means and sum of squared deviations about the means of any number of groups of observations. For only one group, it calculated the mean, variance, standard deviation, and standard deviation of the mean (standard error), and it tested whether the mean was significantly different from zero. For two or more groups, it calculated the mean and sums of squared deviations about the mean of each group and the overall mean. The variance, standard deviation, and standard error of the overall mean were calculated from the aggregate within-group variation. It tested whether or not the group means differed significantly from each other and if the overall mean differed significantly from zero. These tests were made according to standard methods of analyses of variance.

Program RIIM was used for linear regression analysis. It fitted the line of best fit of the form,

$$Y = a + bx$$

for paired observations x_1y_1, x_2y_2, ... x_ny_n. It calculated the values of a and b, the values fitted for each x value, the mean values of x and y, the variance of a and b, the variance and standard deviation of y, and the standard error of the mean of y. It tested whether a and b were significantly different from zero. When replicate values of y were available for the various x values, it also tested whether the relationship between y and x deviated significantly from linearity. These analyses were made using standard methods of least squares analysis.

A large number of other programs were used to solve specific problems, where relevant such programs will be described in detail.

The majority of graphs in this thesis were drawn by a Hewlett-Packard 7200A Graphic Plotter connected to the PDP 8/I computer. Data points and axes were plotted using two FOCAL programs, PFIM and PLIM, chained together. A number of different programs were used to draw the graphical solutions to models fitted to the experimental data points.

Surgical procedures

All sheep undergoing surgery were starved for the preceding 12 hours. Anaesthesia was induced by intravenously administered thiopentone sodium at a dose rate of 0.1 gm per 5.0 kg body weight. The sheep were then intubated with a cuffed Magill endotracheal tube and

anaesthesia was maintained with a mixture of halothane and oxygen administered in closed circuit by means of a Boyle's anaesthetic machine. All operations were carried out under strict aseptic conditions.

Lymphatic cannulations. Chronic lymphatic fistulae were established in sheep in the afferent and efferent vessels of the popliteal lymph node, the afferent testicular lymphatics, the lumbar lymphatic trunk, the intestinal lymphatic trunk, the efferent hepatic lymph duct, the deep cervical lymph duct and the efferent lymphatics from the prefemoral and prescapular lymph nodes. The surgical procedures have previously been described by Lascelles and Morris (1961), Hall and Morris (1962) and Morris and McIntosh (1971). Lymph was collected into plastic bottles or plastic centrifuge tubes tied to a plastic holder sutured to the animal's skin. Powdered heparin was used as an anticoagulant. Lymph so collected was centrifuged for 10 minutes at 2,000 rpm to remove the cells. Cell free lymph was separated and frozen at -20° until required. Occasionally small volumes of lymph were collected without anticoagulant, the lymph was allowed to clot and the clot gently disrupted. After approximately 6 hours at room temperature the serum was poured off, centrifuged at 2,000 rpm for 10 minutes and stored at -20°.

Samples of afferent hepatic and afferent renal lymph were kindly supplied by Dr G. G. MacPherson.

Blood vessel cannulations. Chronic indwelling arterial and venous cannulae were placed in either the

carotid artery or external jugular vein of sheep. The cannulae were kept patent by flushing out with heparinized sterile saline and care was taken to maintain sterility at all times.

Blood was also obtained from sheep by venipuncture of the external jugular vein. Both serum and plasma were prepared as described above.

Thyroidectomy. The thyroid glands of adult sheep were ablated via a mid-line incision extending from the laryngeal cartilage to the 10th tracheal ring. The gland was dissected free of its connective tissue attachments, leaving the recurrent laryngeal nerve *in situ*. The gland was removed following ligation of the thyroid arteries and veins.

Cannulation for terminal bleeding. Adult male rats were anaesthetized with ether and the abdominal aorta was cannulated. Blood was collected into tubes containing powdered heparin and plasma was separated by centrifugation.

Protein estimations

The total protein and albumin concentrations of blood plasma and lymph were measured by the method of Lowry, Rosebrough, Farr and Randall (1951) as modified by Debro, Tarver and Korner (1957). Bovine serum albumin (0-150 µg) was used for preparation of the standard curves.

Immunization procedure

Rabbits were injected in multiple intramuscular sites with 1 ml of whole serum or approximately 5 mg of serum albumin fractions emulsified in 1 ml of Freund's complete adjuvant (Difco Bacto Laboratories, Michigan, U.S.A.). A second course of injections was given 3 weeks later and the rabbits bled at weekly intervals from the external marginal ear vein. Blood was allowed to clot and serum removed, centrifuged and then frozen at -20°.

Immunoelectrophoresis

Agar coated glass slides were prepared using a 1% solution of agar (1.0 gm of special Agar-Noble, Difco Bacto Laboratories, Michigan, U.S.A. dissolved by boiling in 25 ml of 0.1 M barbital buffer, pH 8.6 and 75 ml of distilled water containing 1:10,000 merthiolate). Samples were electrophoresed for 1 hour in agar plates with a current of 50 mA and a voltage of 250 V using an LKB 6800 A apparatus (LKB Produktor, Sweden). The antisera were allowed to react for 12-24 hours at room temperature in humid chambers. Slides were then washed in 1% saline for 6 hours, a fresh 1% saline solution for 16 hours and finally rinsed in distilled water. The gel was covered with strips of wet blotting paper and dried at room temperature. When dry the blotting paper was moistened, removed and the slides gently washed in tap water.

The slides were then stained for 10-60 seconds with amido black (9 gm of amido black in 1,500 ml of

rinsing solution) while maintaining a constant rocking motion and then rocked to and fro for 2 minutes in each of 4 separate volumes of rinsing solution (1,500 ml of methanol, acetic acid and distilled water in the ratio by volume 9:2:9). After drying the slides were trimmed and labelled.

Double diffusion

Double diffusion in agar was carried out in buffered agar on glass slides as described above. Samples (5 µl) were placed in wells punched 5 mm apart in the gel and were allowed to react with antiserum for 12-24 hours, after which the slides were processed as described in the preceding section.

Column chromatography

Samples of serum, lymph or serum albumin labelled with $T_4[^{125}I]$ and/or serum albumin $[^{131}I]$ were fractionated on G-200 and DEAE (A-50) Sephadex columns.

G-200. Descending gel filtration was carried out using 2.0 x 90 cm glass columns. Fifteen grams of Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) were allowed to swell in 0.05 M phosphate, 0.10 M NaCl buffer, pH 7.4 containing 0.02% sodium azide for 2-3 days at room temperature. After washing 3 times in the buffer to remove fines, the slurry was packed into the column under gravity. The column was used with the LKB 4900A RecyChrom System at 4°. Samples

of serum, lymph (1.0 - 1.2 ml) or serum albumin (50 - 60 mg in 1.0 ml of buffer) were loaded onto the column and eluted with the above buffer. A constant flow rate of 5 ml/hour was maintained by using a peristaltic pump and effluent fractions of 3 ml were collected using an automatic fraction collector. The effluent was continually monitored for protein at 254 nm using a 3 mm light path continuous flow cell. Pooled protein fractions were concentrated by dialysis at 4° in 10% polyethylene glycol containing 0.9% sodium chloride.

DEAE Sephadex. Ten grams of DEAE-A50 Sephadex (Pharmacia) was allowed to swell in distilled water for 2-3 hours, then washed 2-3 times with distilled water and the starting buffer and finally packed into a 2.5 x 50 cm glass column. The column was equilibrated with the starting buffer, 0.01 M phosphate pH 8.0. The sample, 500 mg of sheep fraction V in 5 ml of starting buffer, was introduced on to the top of the column and eluted with a phosphate linear gradient system (Fahey, McCoy and Goullian, 1958). A flow rate of 15 ml/hour was obtained by hydrostatic pressure and consecutive 5 ml volumes of eluate were collected. The optical densities of eluate fractions were read at 280 nm using a Hitachi 101, spectrophotometer.

The purity of isolated albumin was tested by immunoelectrophoresis and double diffusion in agar using rabbit anti-sheep serum, rabbit anti-sheep fraction V, and monospecific rabbit anti-sheep albumin. Pure fractions

from gel filtration were pooled, dialysed for 48 hours against several changes of distilled water, lyophilized and refrigerated until use.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed in the E-C470 Vertical Gel Electrophoresis Cell (E-C Apparatus Corporation, Philadelphia, Pennsylvania). A water cooled 3 mm thick slab of 5% Cyanogum-41 (95% acrylamide and 5% bisacrylamide) was used and the buffer system was Tris-Na_2 EDTA-boric acid at pH 8.4 (Peacock, Bunting and Queen, 1965). Samples were prepared as either 2% or 4% protein solutions in 40% sucrose and 20 µl portions were loaded into each slot. The separation occurred in a uniform electric field of 300 volts at 100-150 mA for 2 hours at room temperature. All samples were electrophoresed in duplicate. Following electrophoretic separation the gel was divided in half longitudinally; one half was stained for protein while the other was retained for autoradiography.

The gels were stained with a 0.25% solution of amido black for 30 minutes. The gel was then washed several times in the rinsing solution of methanol, water, acetic acid (5:5:1) to remove excess stain.

Protein labelling with radio-iodide

Protein samples were labelled with ^{131}I or ^{125}I according to the method of Helmkamp, Goodland, Bale, Spar and Mutschler (1960). A 2 mg/ml solution of

lyophilized protein was made up in 0.20 M borate buffer pH 8.0. Sodium iodide ^{131}I or ^{125}I was diluted to 2 mCi/ml with 0.05 M NaOH. Borate buffer (4.0 ml) and an appropriate amount of ^{131}I (usually 100 µCi) were mixed and allowed to stand in an ice bath. Iodine monochloride (5 µl of a 0.02 M solution) was added, mixed and rapidly jetted into 4.0 ml of the protein solution. The non-protein bound radio-iodide was removed by passing the protein solution over a column of Dowex 1-X4 ion exchange resin in the chloride form.

With 100% incorporation of iodine the above reagent mixtures would lead to 1 atom of iodine per molecule of protein (M.Wt. = 70,000). The normal incorporation rate was of the order of 55 - 65%.

Radioactivity measurements

Samples containing both ^{125}I and ^{131}I labelled compounds were counted in a two channel Packard Gamma Scintillation Spectrometer, Model 578. Windows were chosen such that ^{131}I counted independent of ^{125}I while a constant proportion of ^{131}I (16%) counted in the ^{125}I channel. Suitable volumes were chosen such that the efficiency of counting did not change between samples.

Thyroxine [^{125}I] in the T_4BP assay was counted either in a Beckman LS200 liquid scintillation counter or in a Packard well-type external crystal scintillation counter.

Autoradiography

Electrophoretograms of rat fraction V and rat plasma which had been labelled with $T_4[^{125}I]$ were held in close contact with Kodak rapid processing medical X-ray film (RP/554). After a suitable exposure period the film was developed using Kodak liquid X-ray developer, type 2 (Kodak (Australasia) Pty Ltd, Coburg, Victoria).

Estimation of the thyroxine-binding properties of plasma proteins

Principle of measurement of unbound and protein-bound T_4. Thyroxine is bound reversibly by Sephadex. When a constant amount of Sephadex is in contact with a constant volume of buffer solution containing T_4, at equilibrium the amount of T_4 included within the Sephadex (I), comprising both Sephadex-bound T_4 and T_4 dissolved within the included buffer, is related linearly to the amount of unbound T_4 (U) in the excluded volume (EV).

$$I = \alpha U \qquad (1)$$

When T_4 binding proteins are added to such a system, they are confined to the excluded volume if the porosity of the Sephadex is appropriate, and a greater proportion of the total T_4 is present in the excluded volume than when no binding proteins are present. The T_4 in the excluded volume (E) consists of unbound T_4 and protein-bound T_4 (B).

$$E = U + B \qquad (2)$$

If the total amount of T_4 present in the system (T), the

concentration of T_4 in the excluded volume ([E]), the excluded volume, and the constant α in equation (1) are all known, then the concentrations of unbound T_4 ([U]) and protein-bound T_4 ([B]) can be determined as follows:

$$T = I + U + B \qquad (3)$$

from (1)
$$T = (1 + \alpha)U + B \qquad (4)$$

solving (2) and (4) for U

$$U = (T - E)/\alpha \qquad (5)$$

$$[U] = (T/EV - [E])/\alpha \qquad (6)$$

$$[B] = [E] - [U] \qquad (7)$$

The partition of the total T_4 between the included and excluded volumes is measured most conveniently and accurately by using radioactively-labelled T_4. Equations (1) - (7) above are valid for both labelled and unlabelled T_4 and labelled T_4 is distinguished in the following by an asterisk. If the total amount of radioactivity added to the system (T*) and the concentration of radioactivity in the excluded volume ([E*]) are measured, then the concentrations of unbound T_4 and protein-bound T_4 in the excluded volume are given by the following:

$$[U] = [U^*]\ T/T^*$$
$$= (T^*/EV - [E^*])\ T/\alpha T^* \qquad (8)$$

$$[B] = [B^*]\ T/T^*$$
$$= [E^*]\ T/T^* - [U] \qquad (9)$$

Serial additions of T_4 standards result in a progressive saturation of the T_4BPs in the EV. The corresponding concentrations of [B] and [U] can thus be calculated for each T_4 addition.

Measurement of radioactivity. Much preliminary experimentation was done using separate tubes to determine [U] and [B] for each amount of total T_4 added. One half gram portions of Sephadex G25 coarse grade, (Pharmacia) were weighed into 5 ml graduated stoppered tubes. Four millilitre samples of plasma or lymph diluted 1/160 with an appropriate buffer and containing the smallest convenient amount of $T_4[^{125}I]$, were added to each of the tubes, followed by the required amounts of T_4 dissolved in 50% propylene glycol. The gel swelled quickly and the contents of the tubes were mixed thoroughly for 10 minutes or longer at room temperature. The Sephadex was then allowed to settle. Approximately 1 ml of the supernatant, as judged by the graduations on the tubes, was aspirated from each tube in a single portion into a disposable transfer pipette, and transferred into a scintillator vial containing 10 ml of dioxane-based scintillator. A separate pipette was used for each tube and was rinsed several times with the scintillator in the vial after the sample was delivered. This procedure was essential to ensure quantitative transfer of the sample into the counting vial and to remove T_4 adsorbed onto the pipette during transfer. The adsorbed T_4 could otherwise be considerable and variable, especially when high proportions of T_4 in the supernatant were unbound. The exact amount of supernatant taken from each tube was determined by weighing the tubes before and after the samples were taken. One millilitre samples of the diluted

labelled plasma being studied were also pipetted into 10 ml portions of scintillator to determine the total amount of radioactivity added to the tubes.

The constant α in equation (1) was determined by the same procedure using buffer alone. However it was necessary to add the $T_4[^{125}I]$ (10 µl in 50% propylene glycol) directly to the tubes containing the Sephadex and buffer, and to the scintillator vials used to determine the total amount of radioactivity added. In the latter case, 1 ml of buffer was added to the scintillator to ensure the radioactivity was measured under comparable conditions.

Radioactivity was measured using an automatic 3 channel liquid scintillation counter. No corrections were necessary for differential quenching. The excluded volume was determined by measuring the concentration of added HSA $[^{131}I]$ in the supernatant.

It was possible to demonstrate the value of the principle by this procedure, and to determine optimal proportions of Sephadex and buffer and optimal dilutions of plasma for satisfactory analyses. However the procedure was considered too tedious for routine use because many tubes had to be used for each sample studied, the Sephadex had to be weighed before and after the samples of supernatant were taken for radioassay. Moreover it was difficult to study binding at temperatures other than room temperature. To overcome these difficulties, the following apparatus and procedure were developed.

Complete analysis of a single sample in the same vessel. It was found that when various volumes of a solution containing [^{125}I] labelled substances were added to a counting tube and counted in a well-type external crystal scintillation counter (Packard, Breda, The Netherlands), beyond a certain critical volume the count rate did not increase with the volume counted. Provided this critical volume was exceeded, relative concentrations of radioactivity in different solutions could be measured without accurately measuring the solutions into the counting tubes. At the same time full use was made of the counting potential of the external crystal detector.

An apparatus was devised to exploit this phenomenon in studying the binding of T_4 to proteins using Sephadex as a competitive binding agent as outlined above. Using this apparatus the radioactivity in the excluded volume could be measured each time the system came to equilibrium after additional T_4 was added.

The vessel used is shown in Figure 3.1. It consists of a flat-bottomed round flask of about 85 ml capacity, fitted with B10 and B29 ground glass socket. A stopper was machined from Teflon or Nylon to fit in the larger socket, and bored so that a polypropylene counting tube could be fitted tightly into it to make a water-tight seal.

To carry out an assay, 3.75 g of Sephadex G25 was weighed accurately into the flask. Plasma to be

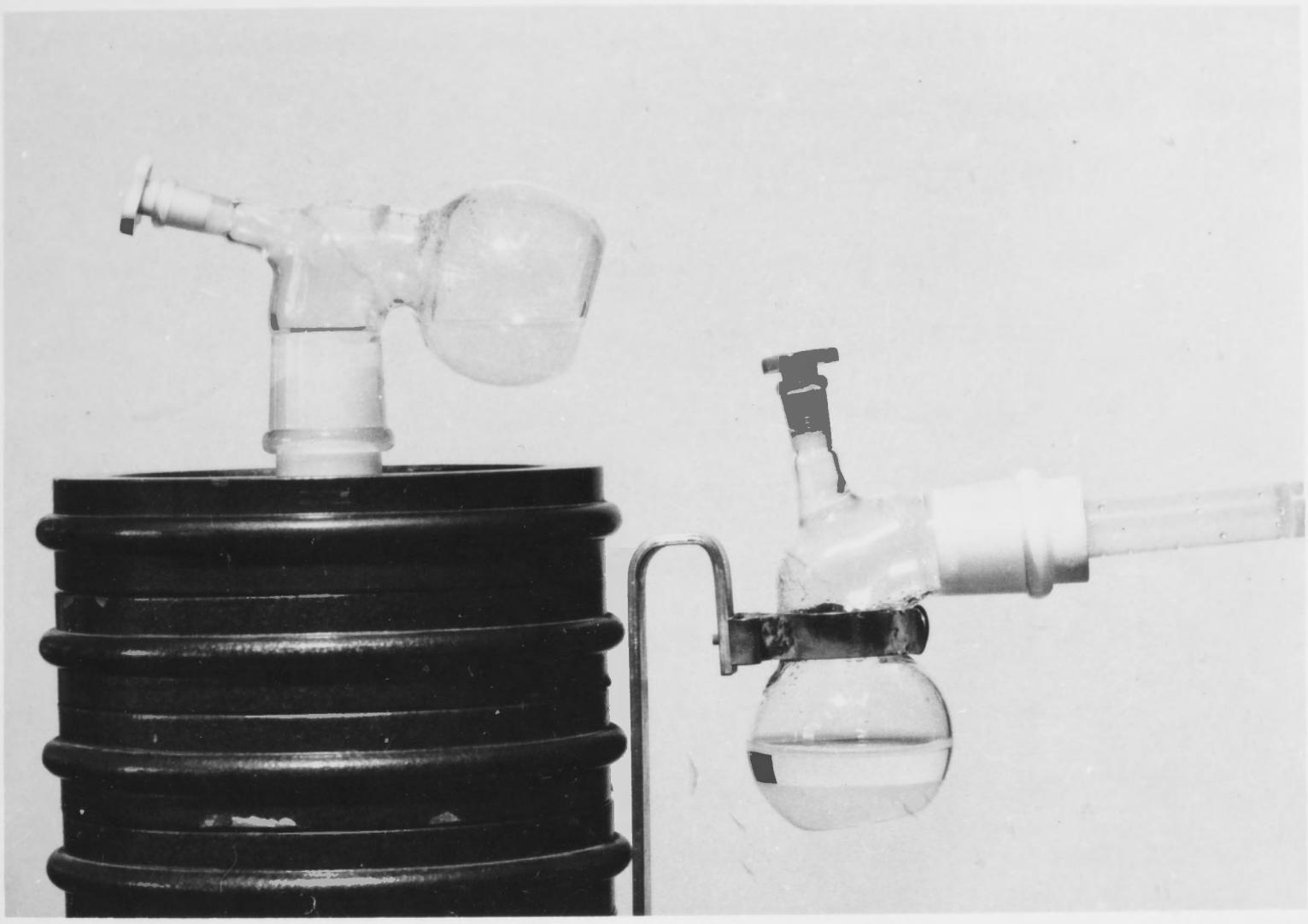


Figure 3.1. The apparatus used for the thyroxine-binding protein assay. One flask is in the upright position for shaking in the Warburg apparatus at 37°. The separation of settled Sephadex and the supernatant is clearly demonstrated. The supernatant can then be poured into the counting tube which is placed in the scintillation detector as shown.

studied was diluted 1/150 with suitable buffer and T_4 [^{125}I] added to give a total initial concentration of exogenous T_4 of 0.1 ng/ml diluted plasma (0.01 - 0.02 µCi/ml). Ten millilitres of the diluted plasma was pipetted directly into the counting tube fitted in the large stopper, and 20 ml added directly to the Sephadex in the flask. After the radioactivity of the diluted plasma in the counting tube had been determined, the counting tube was attached to the flask and the diluted plasma transferred into the flask. The smaller socket was stoppered, and the flask with the counting tube horizontal, was shaken in a Warburg apparatus at 37° for 2 hours. The Sephadex was then allowed to settle while the flask remained in the bath, and the supernatant then poured into the counting tube which was inserted into the scintillation detector and the radioactivity in the supernatant measured. The supernatant was then poured back into the flask, the next required amount of T_4 added and the flask then shaken in the water bath for 7-10 minutes, when the Sephadex was again allowed to settle and the radioactivity in the supernatant measured as above. This procedure was repeated as many times as required. Thyroxine, dissolved in 50% propylene glycol, was added by means of micrometer burettes (Agla, Burroughs Wellcome, London).

The large stopper was machined so that it supported the weight of the flask on the top of the lead castle surrounding the scintillation detector during radioassay, with the bottom of the counting tube just clear of the bottom of the well. The shielding at the top

of the castle, and the geometry of the flask were such that radioactivity in the flask was not detected during radioassay and it was not necessary to remove the counting tube from the flask.

The constant α was determined by adding $T_4[^{125}I]$ (10 µl in 50% propylene glycol) directly to 30 ml of buffer and 3.75 g Sephadex in the assay flask, and assaying the supernatant after equilibration in the water bath as outlined above. The total amount of radioactivity added was measured by pipetting the same volume of $T_4[^{125}I]$ into 10 ml of buffer in a counting tube and measuring the radioactivity in the same apparatus. The mean α value (± standard deviation) was 7.362 ± 0.083 (n = 6) and was independent of the concentration of added cold T_4. Presumably there was some binding of T_4 to the glassware but as the count rate in the excluded volume did not change with added T_4 this binding to glass forms part of the competitive binding system and is accounted for by the calculation of alpha. Simulation studies have shown that substantial errors in the calculation of α result in unchanged estimates of the binding capacities of the specific T_4BPs.

The excluded volume was determined by the dilution of added HSA $[^{131}I]$ in the supernatant. Twelve separate estimates gave a mean (± standard deviation) value of 22.32 ± 0.53 ml. This resulted in a final dilution of plasma proteins in the excluded volume of 1:111.6 when the initial dilution in buffer was 1:150.

Calculation of results. In the detector used the actual critical volume above which the count rate does not increase, lies between 9 and 10 ml. Its true value is not required to calculate α, [U] or [B], and is of no importance provided the volume of fluid counted exceeds this volume. Then the measured count rate for a solution can be used as a measure of the relative specific activity (cpm/unit volume) of the fluid. In the assay procedure, the actual count rate measured in the supernatant can be used in equations (8) and (9) for [E*], as it can also for calculating α according to equation (10) below. The total amount of radioactivity added, in equivalent units, is calculated by multiplying the count rate for the solution added by the total volume of solution in which the total added radioactivity was contained (viz 30 for the assay procedure, and 10 for measuring α). By substituting [E*] for [U*] in equation (6) α can be calculated according to:

$$\alpha = (T^*/EV - [E^*])/[E^*] \quad (10)$$

Analysis of errors. To reduce the counting error to less than 1% at least 100,000 counts were counted for each addition of T_4. Inspection of equations (8) and (9) indicates that if the precision of radioassay does not change during an assay, the precision of calculated values of [U] and [B] is likely to be related to the precision with which T_4 is added to the system which would be related to the amount of T_4 added.

Fitting the binding data. A considerable amount of evidence from electrophoretic studies suggests that T_4

is bound by a number of proteins. Therefore attempts were made to fit a model of the form of equation (11) to the binding data.

$$[B] = \Sigma\ C_i [U]/(K_i + [U]) \qquad (11)$$

This assumes that T_4 is bound according to the law of mass action by a number of non-interacting proteins with binding capacities C_i and dissociation constants K_i. Electrophoresis indicates the presence of 3 T_4BPs in human plasma: TBG, TBPA and albumin. Moreover the dissociation constant for T_4-albumin is much higher than that for T_4-TBG or T_4-TBPA (Robbins and Rall, 1967; Hamada et al., 1970). It should be possible therefore to study binding over a range of [U] which would contain sufficient information to calculate the binding parameters for TBG and TBPA, but [U] would remain small relative to the dissociation constant for T_4-albumin. The denominator $(K_i + [U])$ in the term in equation (11) corresponding to albumin would not change appreciably from K_i, and over this range of [U] albumin-bound T_4 (B_{alb}) would be given by:

$$[B]_{alb} = C_{alb}\ [U]/K_{alb} \qquad (12)$$
$$= K_n\ [U]$$

This is equivalent to the non-specific binding in the formulation of Baulieu and Raynaud (1970), but it should be pointed out that for any binding protein, even so-called specific binding proteins, there is a range of [U] over which such a relationship holds.

For human plasma the model fitted to the binding data is given by (13):

$$[B] = C_1[U]/(K_1 + [U]) + C_2[U]/(K_2 + [U]) + K_n[U] \quad (13)$$

The parameters C_1, K_1, C_2, K_2 and K_n were fitted to the experimental data using an iterative least squares method which has been well documented (Draper and Smith, 1966; Baulieu and Raynaud, 1970). These accounts have been presented in a formal mathematical manner, difficult for many non-specialist statisticians to follow. Therefore the practical application of these methods will be described.

Initial estimates of the non-linear parameters K_1 and K_2 were required to start the solution, and initial values for the linear parameters C_1, C_2 and K_n were set to zero. Corrections for C_1, C_2, K_1, K_2 and K_n (c_1, c_2, k_1, k_2 and k_n respectively) to improve the fit were calculated by fitting the multiple regression:

$$Y = c_1X_1 + c_2X_2 + k_1X_3 + k_2X_4 + k_nX_n \quad (14)$$

in which if $\hat{[B]}$ is the value of [B] calculated according to equation (13) using current values of C_1, C_2, K_1, K_2 and K_n the dependent variate Y is given by:

$$Y = ([B] - \hat{[B]})/T$$

and the independent variates X_1, X_2, X_3, X_4 and X_n are given by:

$$X_1 = [U]/(K_1 + [U])T \qquad X_3 = -C_1[U]/(K_1 + [U])^2T$$

$$X_2 = [U]/(K_2 + [U])T \qquad X_4 = -C_2[U]/(K_2 + [U])^2T$$

$$X_n = [U]/T$$

The term T in the denominators of each variate is a weighting function, since as outlined above, the likely error for any measured [B] is related to the total amount

of T_4 added. Use of these weighting functions also makes solution numerically possible when [B] and [U] vary over a wide range.

The current estimates of the parameters were corrected by adding to them their respective corrections, and the procedure repeated using the corrected estimates until the corrections were all less than some stipulated fraction of their last determined respective parameter. In practice a tolerance of 1% has proven suitable. If convergence did not occur, or if it occurred too slowly, the solution was stopped and the suitability of the model, or the starting values examined.

The actual solutions were obtained using a PDP 8/I computer. The routine used two separate FOCAL programs chained together and could handle a maximum of 18 pairs of data points. Complete solution takes about 5 minutes.

Calculation of unbound T_4 concentrations. The concentration of unbound T_4 in plasma or lymph was calculated for given values of total T_4, and the dissociation constants and binding capacities of the binding proteins. A FOCAL program was used to compute the appropriate root of the equation in [U]:

$$[U](1 + C_1/(K_1 + [U]) + C_2/(K_2 + [U]) + K_n) - \text{Total } T_4 = 0$$

by means of an iterative procedure. Amounts bound to the various proteins were calculated according to:

$$B = C[U]/(K + [U]) \text{ for specific binding proteins}$$

$$\text{or } B = K_n[U] \text{ for albumin (non-specific binding)}$$

where the various parameters have the same meaning as above.

Estimation of total T_4 concentration

Total plasma T_4 concentrations were measured by a modification of the method of Murphy and Pattee (1964). Serum or plasma (1.0 ml) was added dropwise to 2.0 ml of absolute ethanol, shaken and the precipitate removed by centrifugation. Duplicate portions (0.2 ml) of the supernatant were dried in 5 ml graduated test tubes under a stream of nitrogen at 37°. Sephadex G25 (0.500 gm) and 4.0 ml of a 1:150 dilution of a human plasma containing tracer T_4 [^{125}I] was added, the tubes were shaken for at least 10 minutes at room temperature and an accurately weighed portion (approximately 1 ml) of the supernatant was removed for counting as described above. A standard curve was prepared by adding in duplicate 0, 2, 5, 7, 10 and 15 ng quantities of T_4 to tubes containing Sephadex and the dilute plasma-tracer solution. Unknown T_4 concentrations were read from a standard curve of % T_4 [^{125}I] in the excluded volume versus T_4 concentration. The results were corrected for an ethanol extraction efficiency of 75.2% and expressed as µg/100 ml of plasma. A typical standard curve is shown in Figure 3.2.

A pooled sheep plasma sample was analysed 6 times in duplicate in 6 separate assays conducted over a period of 2 months. The mean (± SD) total T_4 value was 5.6 ± 0.4 which gave a coefficient of variation of 7.2%.

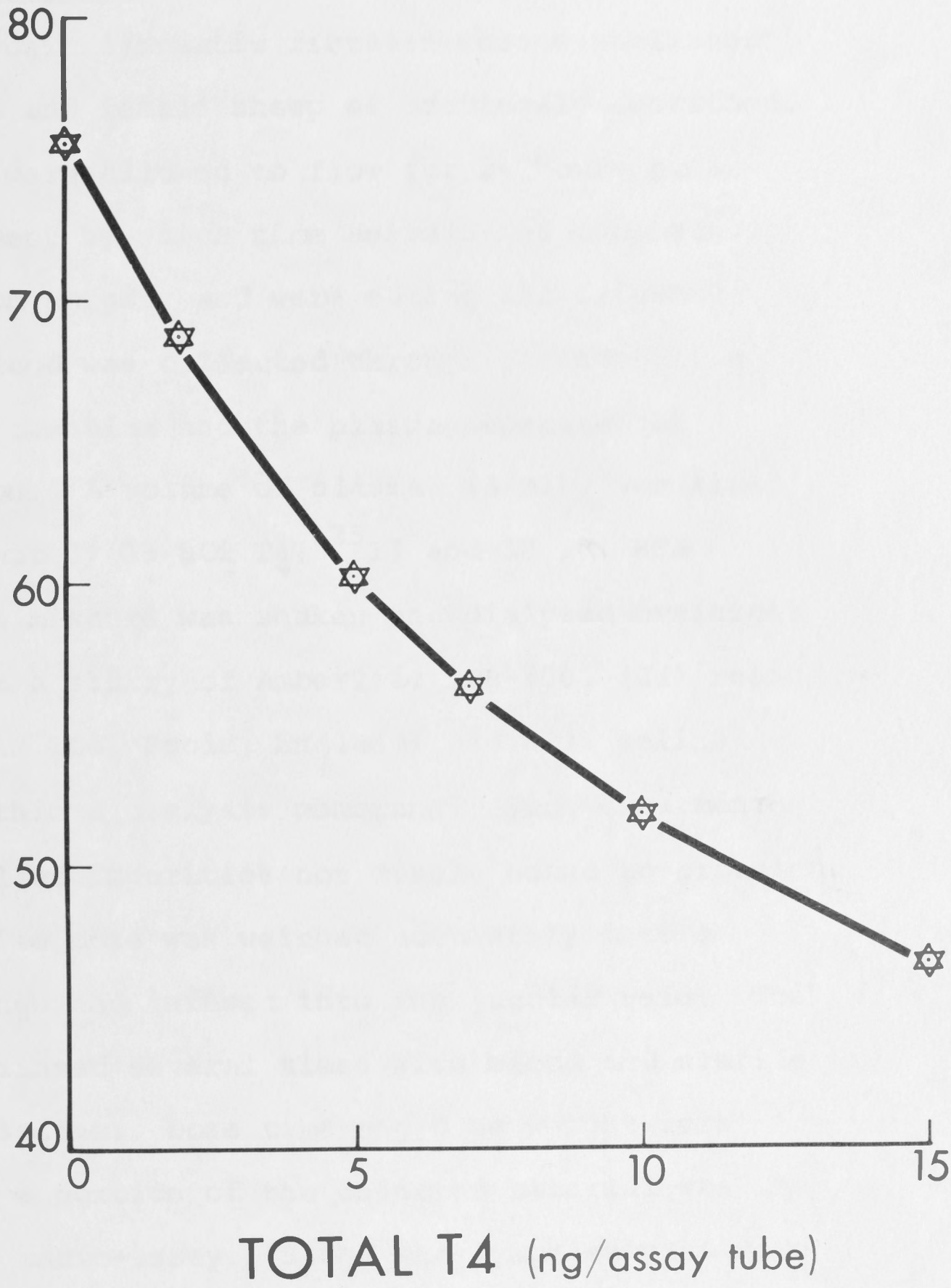


Figure 3.2. A typical standard curve for the estimation of total thyroxine in blood plasma and lymph.

Transcapillary transfer of thyroxine-binding proteins and thyroid hormones

Chronic lymphatic fistulae were established in adult male and female sheep as previously described. The cannulae were allowed to flow for 24 hours prior to an experiment by which time animals had completely recovered from surgery and were eating and drinking normally. Blood was collected through an indwelling jugular vein cannulae and the plasma separated by centrifugation. A volume of plasma, (3 ml), was mixed with approximately 15 µCi $T_4[^{125}I]$ and 10 µCi HSA $[^{131}I]$. This mixture was shaken and dialysed overnight at 2° against a slurry of Amberlite IRA-400, (Cl) resin (BDH Chemicals Ltd, Poole, England) and 0.9% saline contained within a dialysis membrane. Such treatment removed labelled impurities not firmly bound to proteins. The radioactive dose was weighed accurately into a sterile syringe and infused into the jugular vein. The cannula was rinsed several times with blood and sterile heparinized saline. Dose time and dose weight were recorded and a portion of the injected material was retained for radio-assay. Blood and lymph samples were collected at timed intervals after injection and plasma was separated in the usual manner. Lymph flow rates were recorded. Where volumes permitted 3.0 ml of plasma or lymph were counted in a gamma scintillation spectrometer system before and after protein precipitation with two volumes of cold 10% trichloroacetic acid (TCA). The precipitate was deposited by centrifugation and the

supernatant poured off. Results were expressed as the percentage of the dose remaining/litre of plasma or lymph at any fixed time after injection.

In some experiments a large dose of T_4 was injected at the same time as, or 72-98 hours after the tracer injection. L-thyroxine sodium (5 mg) was dissolved in 2.0 ml of 0.9% sterile saline containing 2-3 drops of 1 M NaOH to ensure that the T_4 was completely dissolved. Following the administration of this dose, blood and lymph samples were collected at short time intervals over an appropriate time period.

In experiments where $T_3[^{125}I]$ replaced labelled T_4 TCA precipitation was inadequate in separating T_3 from other labelled plasma iodocompounds. To overcome this problem $T_3[^{125}I]$ was separated on strong anion exchange resin columns as described by Sutherland and Irvine (1973). A 50% (v/v) slurry of AG1-X2, 200-400 mesh, chloride form (Biorad Laboratories, Richmond, California) in 0.1 M acetate buffer, pH 5.0, was prepared and 3.0 ml of this slurry was pipetted into glass columns (0.8 x 20 cm) to give 0.8 x 2.5 cm resin columns. The columns were washed with 8 ml of 0.1 M NaOH. Plasma or lymph (2.0 ml) was added to 8.0 ml of 1 M NaOH, shaken, allowed to stand for 15 minutes and added to the columns which were then eluted sequentially with 8.0 ml of 1% acetic acid, 8.0 ml of 18% acetic acid, 0.8 ml of glacial acetic acid and finally with 6.0 ml of 59% acetic acid. The last fraction which eluted 82.5% of the $T_3[^{125}I]$ was retained for gamma counting.

Perfusion of popliteal lymph nodes. An afferent popliteal lymphatic accompanying the recurrent tarsal vein was cannulated in the direction of lymph flow. The surgical procedure has been described in detail by Hay (1970). Sterile Evan's Blue dye (0.2 ml) was slowly infused up the afferent lymphatic and all efferent lymphatics were identified, tied off, and one cannulated as described by Hall and Morris (1962). A 24 hour collection of sterile lymph was made after clearance of the dye. Cells were spun off and the lymph plasma retained. An infusion system was set up whereby the sterile efferent lymph labelled with T_4 [^{125}I] or T_3 [^{125}I] (0.2 µCi/100 ml) and OSA [^{131}I] (0.2 µCi/100 ml) was infused into the afferent lymphatic at the rate of 1 ml/hour. Varying concentrations of T_4 (0 - 100 µg/ml) were added to the infusion mixture to assess the effect of changes in unbound T_4 concentration on the passage of T_4 through a lymph node. Each infusion was continued for 4 hours with a 1 hour sterile saline wash between treatments. Efferent lymph was collected over half hourly intervals and the radioactivity counted before and after precipitation with TCA. Results were expressed as the T_4:albumin activity ratio in efferent lymph/the T_4:albumin activity ratio in the infusion mixture.

CHAPTER 4

FACTORS AFFECTING THYROXINE BINDING TO PLASMA PROTEINS IN VITRO

INTRODUCTION

Most methods previously described for characterizing T_4BPs in biological fluids have involved dilution of these fluids. Electrophoresis has been the most widely used technique for studying thyroid hormone-protein interactions. With electrophoresis, the sample and its constituent proteins necessarily become diluted during separation, often in unphysiological buffers at unphysiological temperature and pH. In the competitive binding technique developed for the studies reported in this thesis, and indeed in most other techniques used to measure unbound T_4 concentrations, it is necessary to dilute the samples so that the proportion of the total T_4 which is unbound is increased and thus measured more accurately. It is not possible to dilute plasma without changing the ionic composition of the solution since the plasma proteins themselves constitute a considerable proportion of the functional anions in undiluted plasma.

The purpose of the experiments described in this chapter was to study some of the effects of ionic strength, ionic composition, pH, and temperature on the binding of T_4 by plasma proteins, so that conditions most likely to reflect the *in vivo* situation could be used routinely *in vitro*. The methods of parameter fitting used in subsequent chapters of this thesis had not been developed when these experiments were carried out. The results were therefore not suitable for that type of analysis and the effects of the various treatments are

described qualitatively. It was hoped, however, that the differential effects of some ions, e.g. barbital (Tata et al., 1961), and temperature (Takemura et al., 1971) on binding by individual proteins might have a use in characterizing the binding system.

Methods of graphical presentation of binding data

When specific binding proteins are present as homogenous preparations, the binding parameters for a particular ligand can be readily calculated from measured bound and unbound ligand concentrations using simple linear regression techniques. In the case of blood plasma and lymph, non-specific binding proteins are present concomitantly with the specific binding proteins and the above methods are invalid. Human plasma is known to have three T_4BPs and thus the relationship between bound and unbound T_4 is the sum of 3 hyperbolic functions and no linearization is theoretically possible. Linearization can however be attained over narrow concentration ranges but when the range is extended the slope of the initial straight line must be modified to try and fit all points. Therefore the values for the binding parameters are determined rather arbitrarily and inaccurately.

Despite these limitations one such graphical technique, the Scatchard plot, has been used widely to obtain quantitative estimates of binding parameters in mixtures of binding proteins. This type of analysis plots the [B]/[U] ratio on the ordinate against [B] on

the abscissa. A typical Scatchard plot is shown in Figure 4.1.

Rearrangement of the mass action equation shows that if there is only one set of independent binding sites then

$$[B]/[U] = k(C - [B])$$

where k = the association constant

and C = the binding capacity for the ligand under study. The plot of [B]/[U] versus [B] is then a straight line with a slope of -k and intercepts on the ordinate and abscissa of kC and C, respectively (Scatchard, 1949). If there are two or more sets of independent binding sites the Scatchard plot is non-linear, and division of such plots into two or more linear components can at best be only an approximation, since even at the very lowest concentration of ligand some of the ligand is bound to proteins other than the specific binding protein of highest affinity. Quantitative estimates of the binding properties of TBG in plasma using extrapolation of the initial slope of the Scatchard plot can only yield a minimal estimate of the association constant and a maximal estimate of the binding capacity.

In most binding experiments it is necessary to saturate the specific binding proteins to gain maximal information about the binding system. Logarithmic presentations are essential to cover the entire concentration range studied. One such method is the proportional graph of Baulieu and Raynaud (1970). In

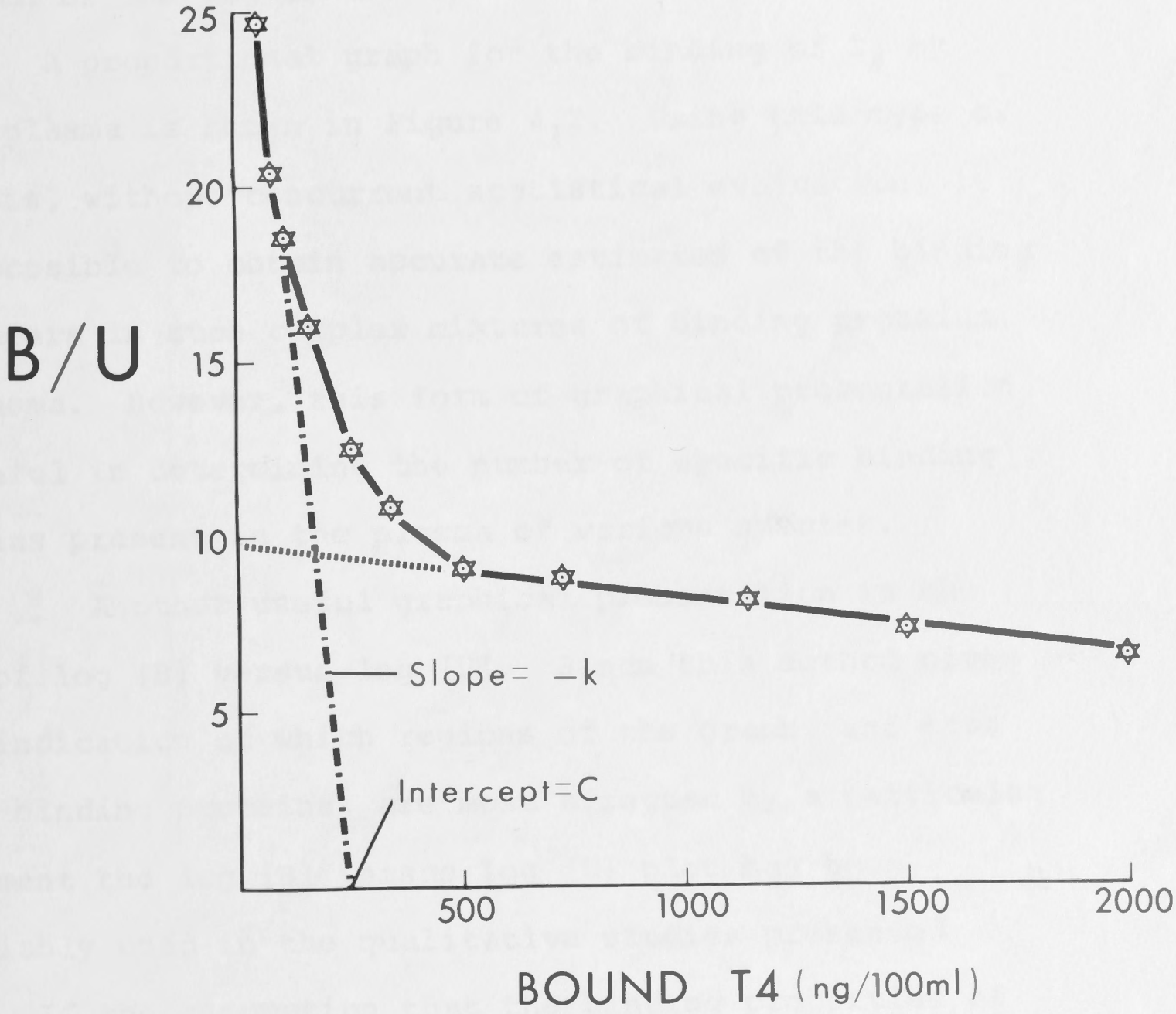


Figure 4.1. A Scatchard plot of data for the binding of T_4 by human plasma diluted 1:111.6 in the excluded volume. The extrapolation technique for the estimation of the T_4 binding parameters is demonstrated. The slope, -k, and the intercept, C, are graphical estimates of the association constant and binding capacity respectively of human TBG.

this presentation the log of the proportion of bound ([B]/[T]) or unbound ([U]/[T]) ligand is plotted as a function of the log of the total ligand concentration ([T]). A proportional graph for the binding of T_4 by human plasma is shown in Figure 4.2. Using this type of analysis, without concurrent statistical evaluation, it is impossible to obtain accurate estimates of the binding parameters in such complex mixtures of binding proteins as plasma. However, this form of graphical presentation is useful in determining the number of specific binding proteins present in the plasma of various species.

Another useful graphical presentation is the plot of log [B] versus log [U]. Since this method gives some indication of which regions of the graph, and thus which binding proteins, are most affected by a particular treatment the log [B] versus log [U] plot has been invariably used in the qualitative studies presented here. If the assumption that the binding properties of T_4BPs are not changed by dilution is true, it is possible to correct [B] for dilution, i.e. multiply by 111.6, and estimate the concentration of unbound T_4 in undiluted plasma directly from a log [B] versus log [U] plot as shown in Figure 4.3.

RESULTS

Effect of ionic strength and ionic composition on T_4 binding to plasma proteins

A normal human plasma sample was assayed

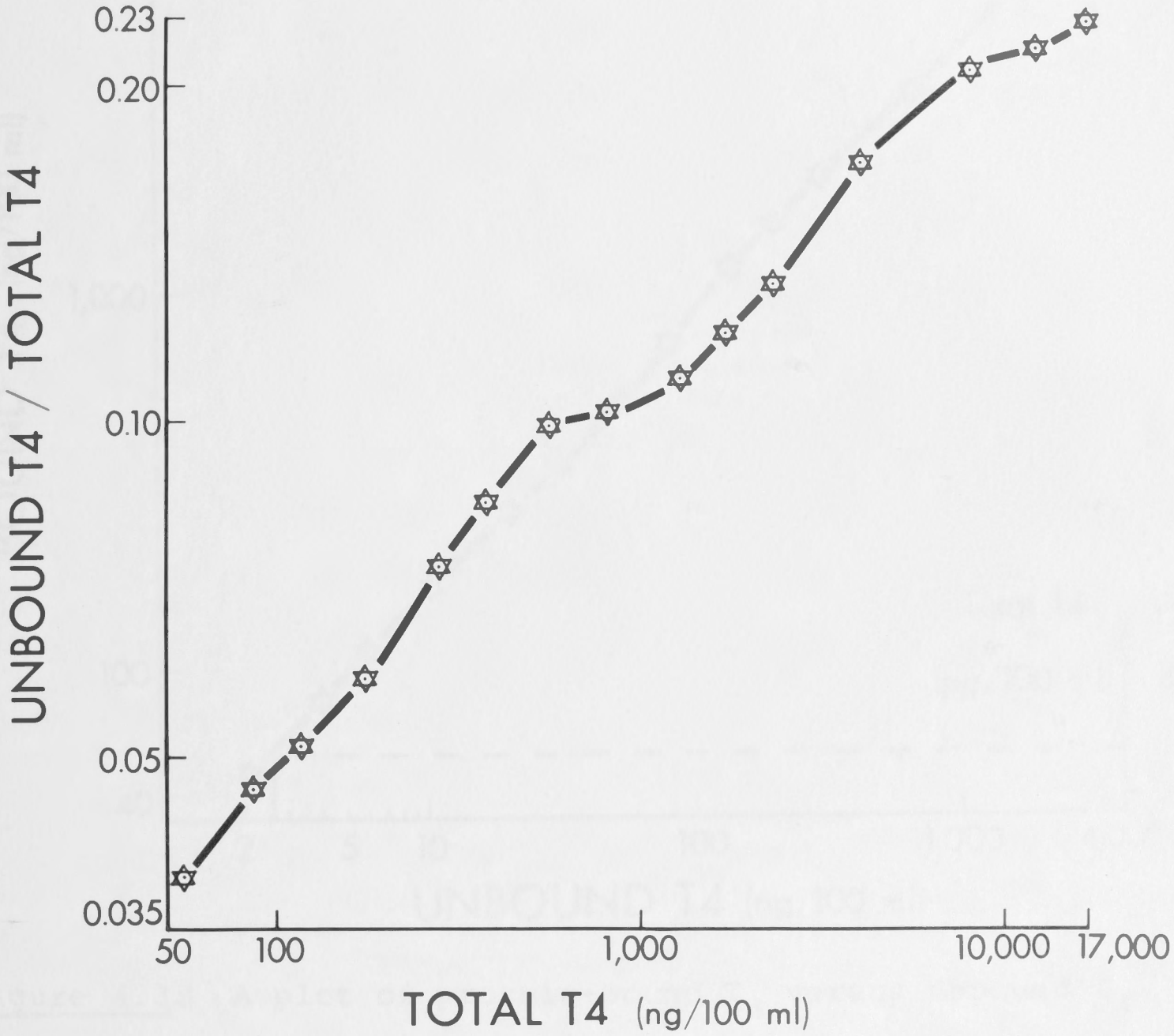


Figure 4.2. A proportional graph (log [U]/[T] versus log [T]) of data for the binding of T_4 to human plasma diluted 1:111.6 in the excluded volume.

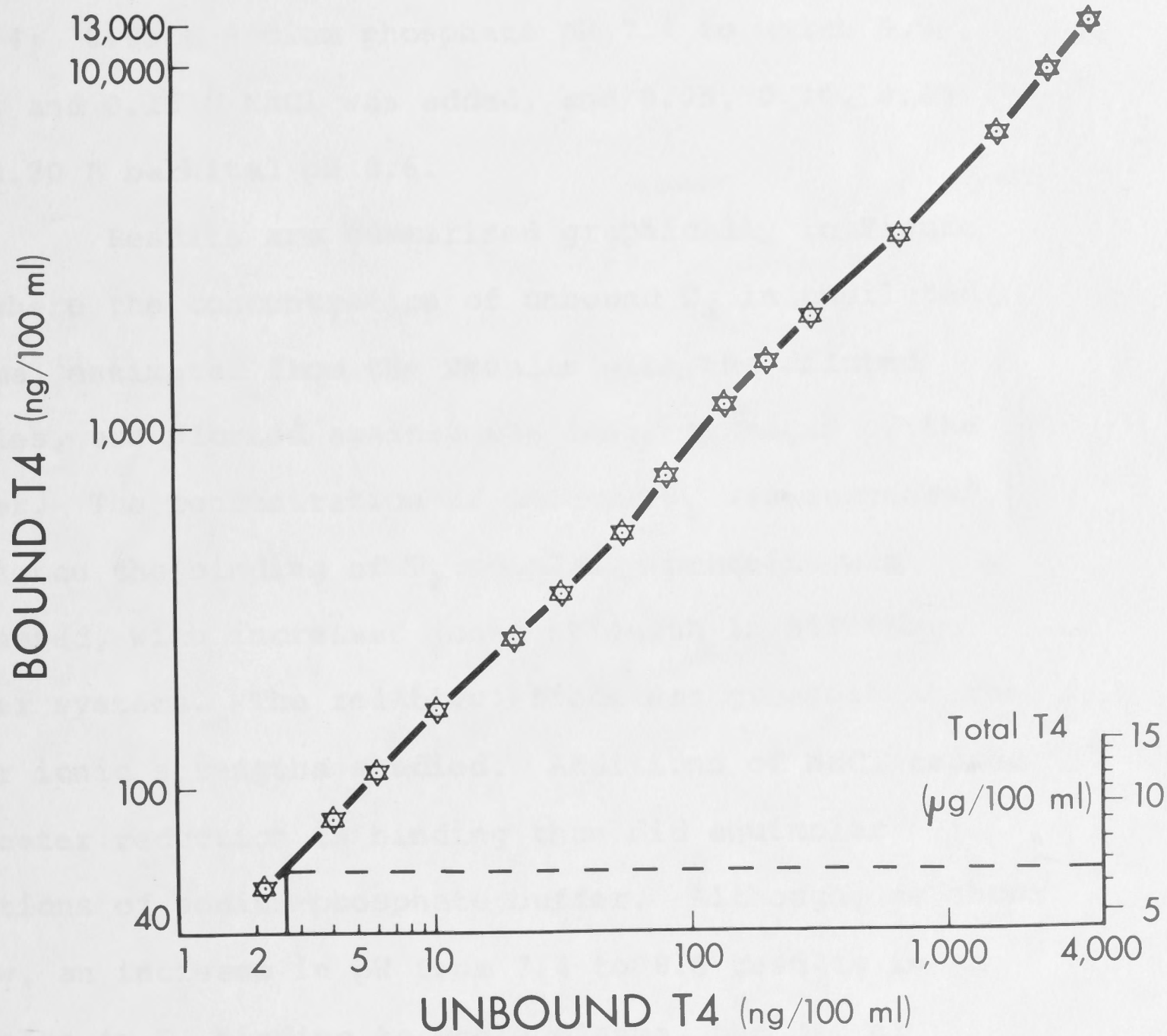


Figure 4.3. A plot of protein-bound T_4 versus unbound T_4 concentration (log [B] versus log [U]) for a human plasma sample diluted 1:111.6 in the excluded volume. The right hand ordinate scale has been corrected for a dilution of 111.6 which allows a direct graphical estimate of the unbound T_4 concentration in undiluted plasma.

following dilution (1:150) in three different buffer systems at different concentrations. The systems used were: 0.05, 0.10, and 0.15 M sodium phosphate buffer pH 7.4; 0.05 M sodium phosphate pH 7.4 to which 0.05, 0.10, and 0.15 M NaCl was added, and 0.05, 0.10, 0.15 and 0.20 M barbital pH 8.6.

Results are summarized graphically in Figure 4.4 where the concentration of unbound T_4 in undiluted plasma, estimated from the results with the diluted samples, are plotted against the ionic strength of the buffer. The concentration of unbound T_4 was increased, and hence the binding of T_4 to plasma proteins was decreased, with increased ionic strength in all three buffer systems. The relative effect was greatest at the lower ionic strengths studied. Additions of NaCl caused a greater reduction in binding than did equimolar additions of sodium phosphate buffer. Although, as shown below, an increase in pH from 7.4 to 8.6 results in an increase in T_4 binding to whole plasma, the use of barbital buffer over a similar ionic strength range at pH 8.6 resulted in greatly diminished binding compared with the other two buffer systems.

Effect of temperature on T_4 binding

A human plasma sample, diluted 1:150 with 0.05 M sodium phosphate, 0.10 M NaCl buffer, pH 7.4, was analysed at 25° and 37°. The results are shown in Figure 4.5 and clearly indicate that the degree of

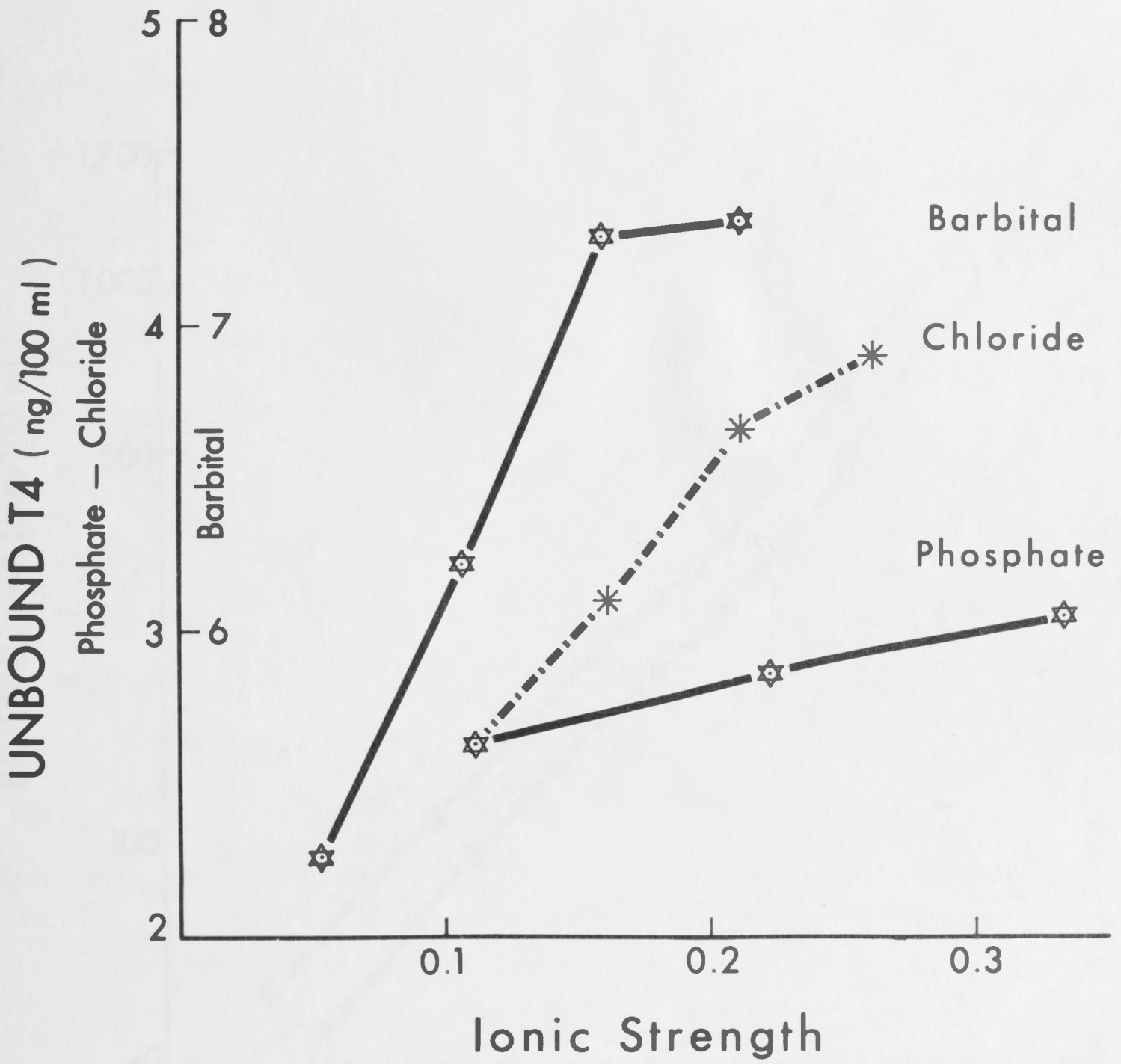


Figure 4.4. The effect of ionic strength and ionic composition of the assay buffer on the unbound T_4 concentration of a human plasma sample. The composition of the buffer systems is described in the text. Unbound T_4 concentrations in barbital buffer are presented on a different ordinate scale than results obtained with the other two buffer systems.

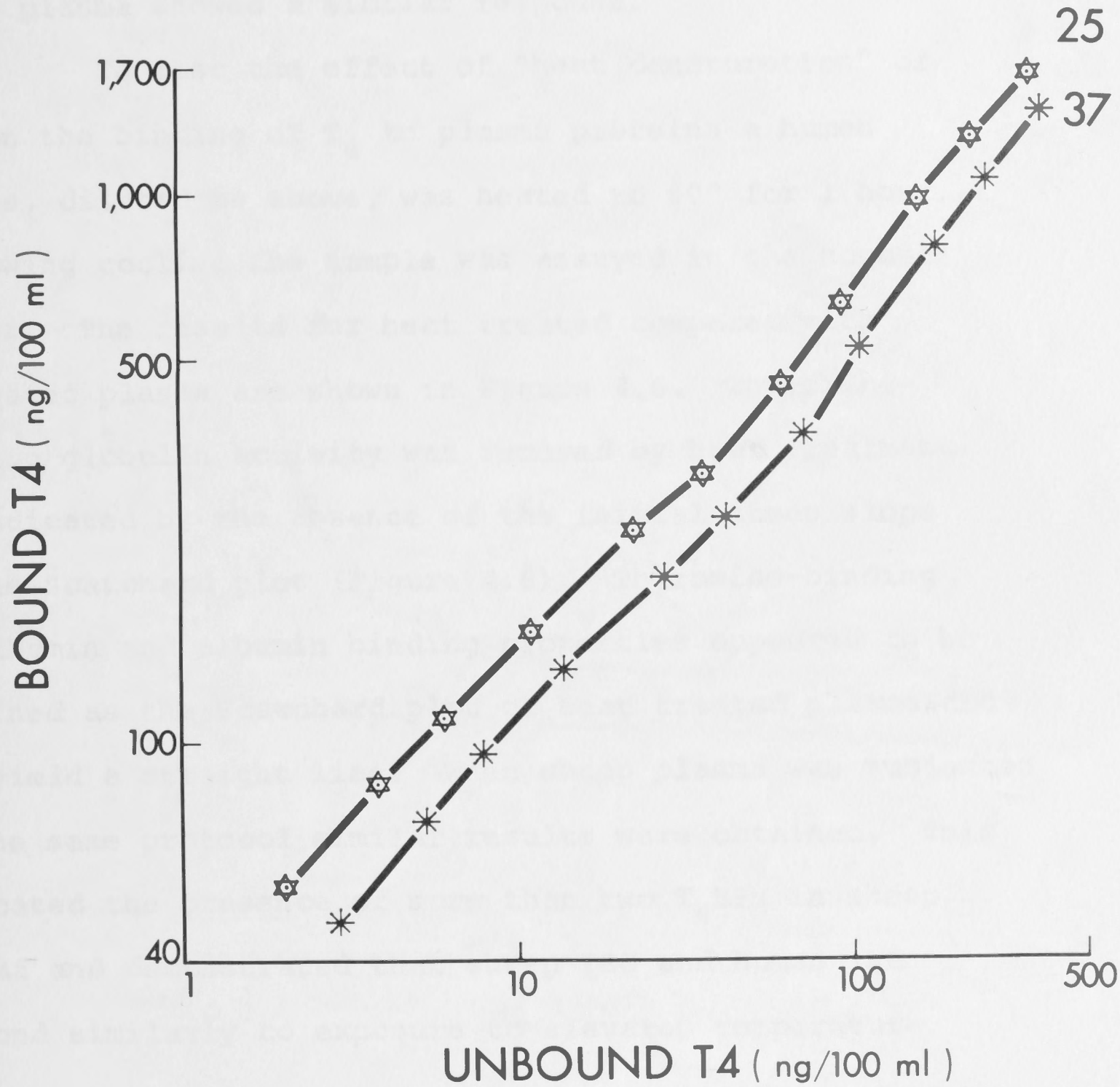


Figure 4.5. The effect of assay temperature on the binding of T_4 to plasma proteins. A normal human plasma sample was assayed at 37° and 25°.

protein-binding was greatly affected by temperature. At any given unbound T_4 concentration the sample assayed at 25° bound considerably more T_4 than when analysed at 37°. Sheep plasma showed a similar response.

To test the effect of "heat denaturation" of TBG on the binding of T_4 to plasma proteins a human sample, diluted as above, was heated to 60° for 1 hour. Following cooling the sample was assayed in the normal manner. The results for heat treated compared with untreated plasma are shown in Figure 4.6. Thyroxine-binding globulin activity was removed by heat treatment as indicated by the absence of the initial steep slope in the Scatchard plot (Figure 4.6). Thyroxine-binding prealbumin and albumin binding properties appeared to be retained as the Scatchard plot of heat treated plasma did not yield a straight line. When sheep plasma was subjected to the same protocol similar results were obtained. This indicated the presence of more than two T_4BPs in sheep plasma and demonstrated that sheep TBG and human TBG respond similarly to exposure to elevated temperature.

Effect of pH on T_4 binding

Since a large number of T_4 binding studies have employed alkaline buffers, especially at pH 8.6, it was important to compare binding at that pH with binding at physiological pH. A human and a sheep plasma sample were analysed in 0.05 M Tris, 0.10 M NaCl buffer at pH 7.4 and pH 8.6. Both samples showed qualitatively similar responses to this change in pH. Results for the

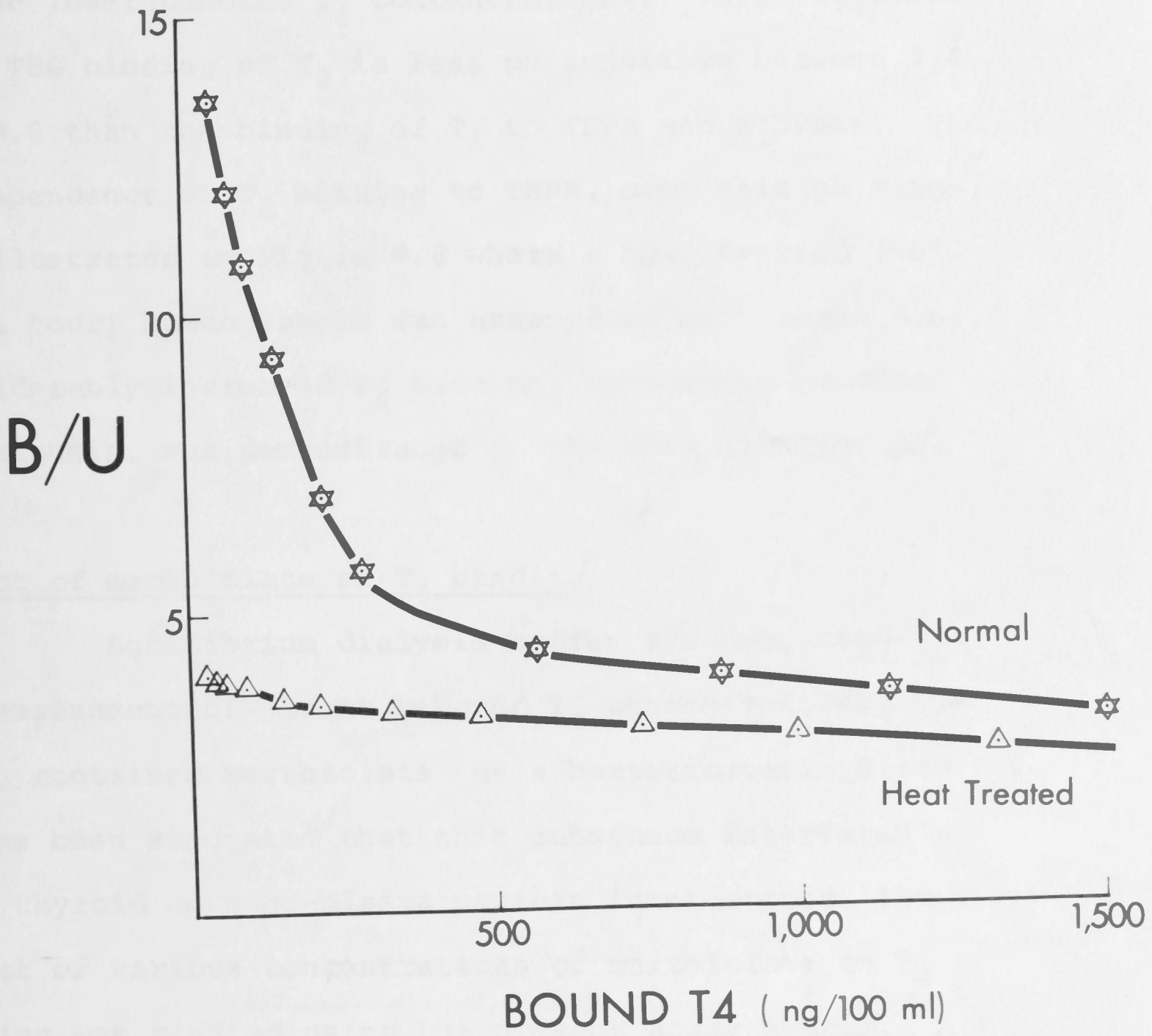


Figure 4.6. A Scatchard plot of T_4 binding to a human plasma sample before (normal) and after (heat treated) exposure to 60° for 1 hour.

human sample are shown in Figure 4.7. At pH 8.6 plasma bound more T_4 at all levels of unbound T_4 than when assayed at pH 7.4, however, the differences were smallest at the lower unbound T_4 concentrations. This suggested that TBG binding of T_4 is less pH sensitive between 7.4 and 8.6 than the binding of T_4 to TBPA and albumin. The pH dependence of T_4 binding to TBPA, over this pH range, is illustrated in Figure 4.8 where a heat treated (60° for 1 hour) human sample was assayed at pH 7.4 and 8.6. Considerably increased T_4 binding, presumably to TBPA and albumin, was demonstrated at the more alkaline pH.

Effect of merthiolate on T_4 binding

Equilibrium dialysis buffer systems, used for the assessment of plasma unbound T_4 concentrations, have often contained merthiolate as a bacteriostat. Since it has been suggested that this substance interferes with thyroid hormone-plasma protein interactions, the effect of various concentrations of merthiolate on T_4 binding was studied using the present assay system. A human plasma sample was diluted 1:150 in 0.05 M phosphate, 0.10 M NaCl buffer, pH 7.4, and increasing concentrations of merthiolate were added over the range 10^{-5} to 10^{-3} gm/ml of buffer. The concentrations of unbound T_4 at each merthiolate concentration are shown in Table 4.1.

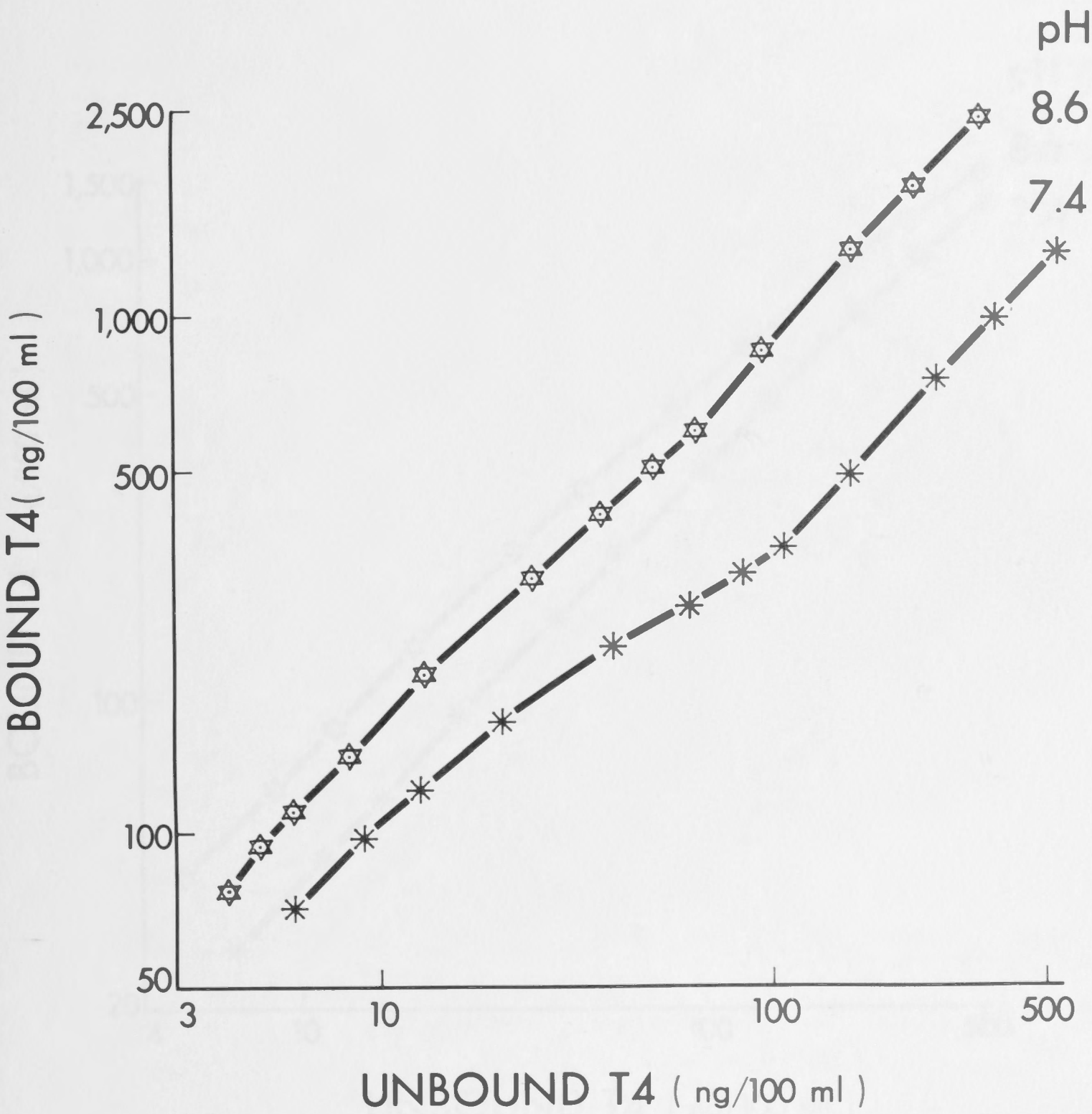


Figure 4.7. The effect of assay pH on the T_4 binding properties of plasma proteins. A human sample was assayed at pH 7.4 and 8.6.

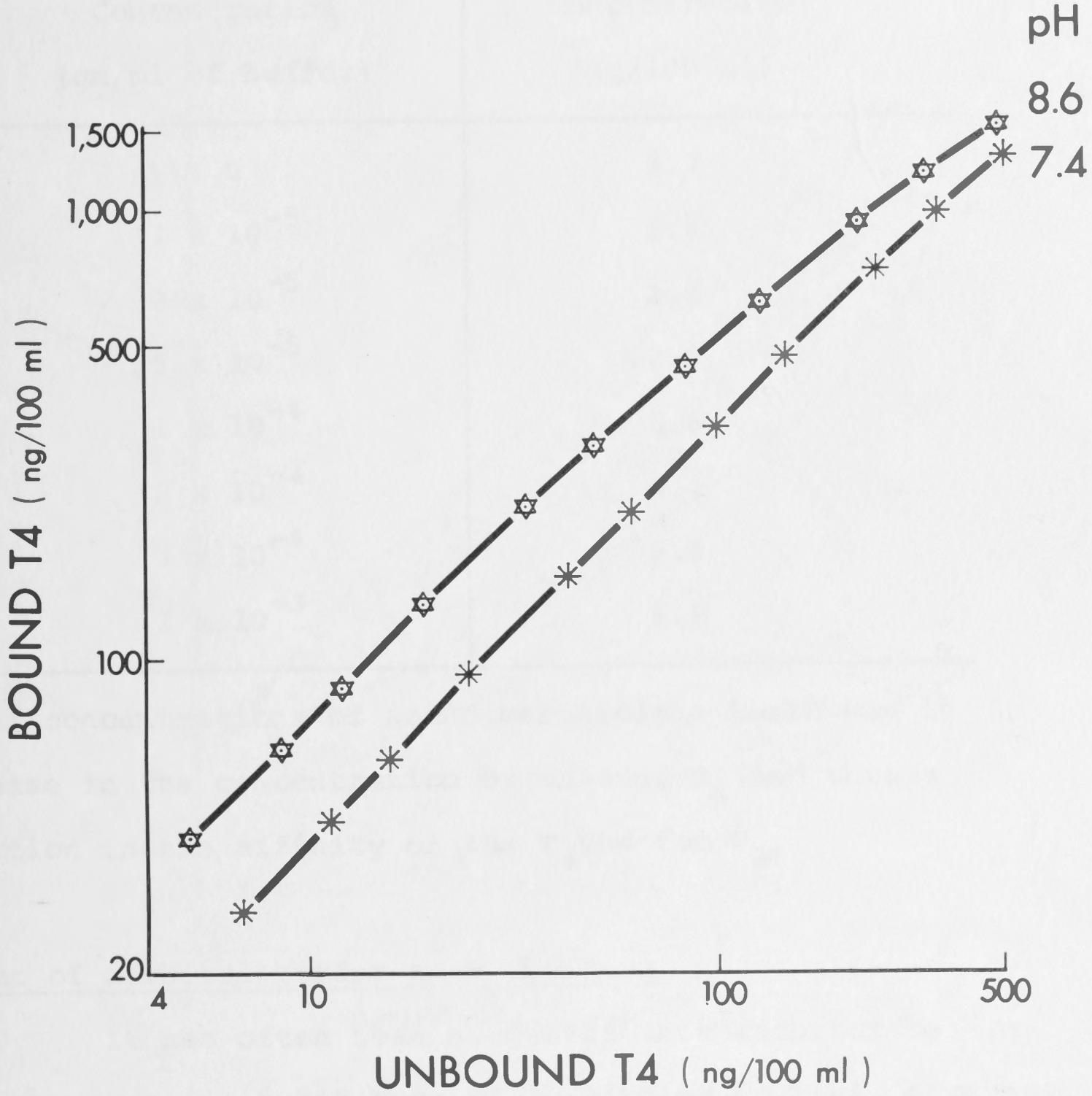


Figure 4.8. The effect of assay pH on T_4 binding to heat treated (60° for 1 hour) human plasma.

Table 4.1. Effect of merthiolate concentration on the estimation of unbound T_4 concentration

Merthiolate Concentration (gm/ml of buffer)	Unbound T_4 Concentration (ng/100 ml)
0	1.7
1×10^{-5}	1.9
2×10^{-5}	2.4
5×10^{-5}	2.9
1×10^{-4}	3.6
3×10^{-4}	5.1
5×10^{-4}	6.0
1×10^{-3}	6.8

At all concentrations of added merthiolate there was an increase in the concentration of unbound T_4 and thus a reduction in the affinity of the T_4BPs for T_4.

Effect of barbital buffer on T_4 binding

It has often been suggested that barbiturate ions are specific inhibitors of T_4 binding to TBPA, however, the elevated unbound T_4 levels found using barbital buffer (Figure 4.4) appeared to be too great to be due entirely to the inhibition of TBPA binding. To study this effect in more detail a human plasma sample was assayed in 0.05 M phosphate, 0.10 M NaCl buffer, pH 7.4 and 0.15 M barbital buffer, pH 8.6. The relationship between bound and unbound T_4 in each buffer system is shown in Figure 4.9.

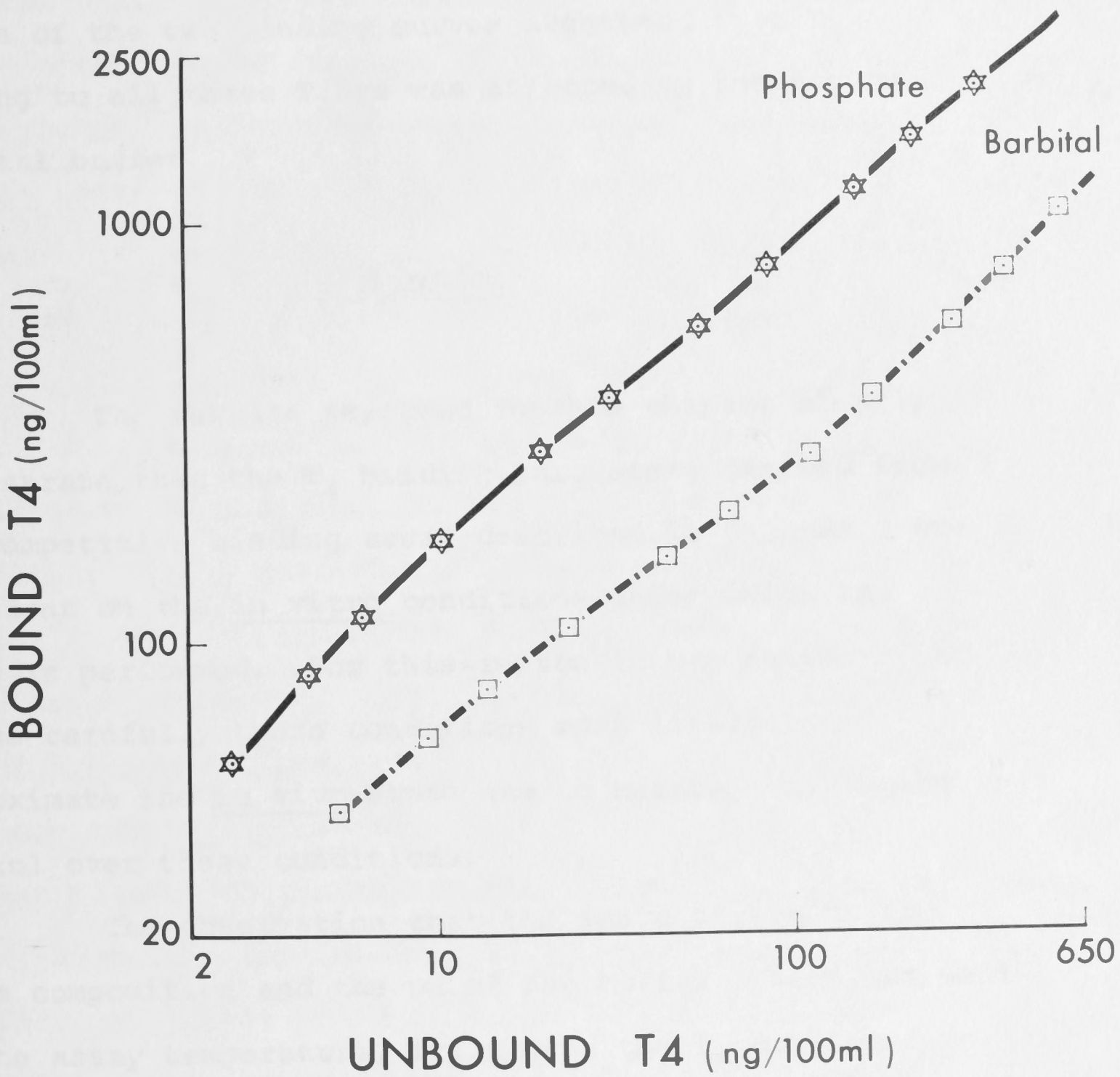


Figure 4.9. The effect of dilution in barbital buffer on the binding of T_4 by human plasma. The composition of the two buffer systems is described in the text.

At all unbound T_4 concentrations studied the plasma sample assayed in phosphate buffer bound considerably more T_4 than when assayed in barbital. The parallel nature of the two binding curves suggested that T_4 binding to all three T_4BPs was affected by the use of barbital buffer.

DISCUSSION

The results reported in this chapter clearly demonstrate that the T_4 binding parameters derived from the competitive binding assay described in Chapter 3 are dependent on the *in vitro* conditions under which the assay is performed. For this reason it was necessary to choose carefully those conditions most likely to approximate the *in vivo* state and to maintain stringent control over these conditions.

The observation that the ionic strength, the ionic composition and the pH of the buffer system, as well as the assay temperature, influenced the T_4 binding properties of plasma proteins is in general agreement with a number of studies in the literature (Gordon and Coutsoftides, 1969; Coutsoftides and Gordon, 1970; Davis and Gregerman, 1971; Spaulding and Gregerman, 1972).

In designing the routine T_4BP assay system use was made of the present qualitative information. While control of assay temperature and pH at 37° and 7.4 was easily attained, choice of an appropriate buffering system

posed some problems. Chloride ions at a concentration of 0.10 M were included because of their profound effect on binding and their presence in normal plasma at approximately that concentration. The buffering ions were supplied by sodium phosphate buffer at a concentration of 0.05 M since changes in phosphate concentration had minimal effects on the binding of T_4 to plasma proteins. Phosphate is not present at this concentration in plasma which may lead to an artefactual situation since Spaulding and Gregerman (1972) have shown that T_4 binding by plasma proteins is increased with decreases in the molarity of phosphate buffer below 0.05 M. Woebar and Ingbar (1968) demonstrated that unbound T_4 concentration was 20% lower in Krebs Ringer bicarbonate buffer than in Krebs Ringer phosphate buffer which suggests that the use of bicarbonate buffer would be more likely to approach the in vivo state. This buffer was not used however because of the technical difficulty of keeping the assay system continuously supplied with carbon dioxide. Whilst it would be naive to postulate that T_4 binding in a simple buffer system, like 0.05 M sodium phosphate, 0.10 M NaCl, pH 7.4, is identical to binding in whole plasma it appears likely from the results presented here that T_4 binding in this buffer system bears a close approximation to T_4 binding in vivo.

Merthiolate should be excluded from the buffer system because of its profound effect on T_4 binding. Such an observation is at variance with published data

which have shown that merthiolate, when present at concentrations of 10^{-4} gm/ml of buffer and at 0.5×10^{-4} gm/ml, had no effect on the concentrations of unbound T_4 and T_3 respectively (Sterling and Brenner, 1966; Nauman et al., 1967). This apparent anomaly in results might be explained by the use of diluted plasma in the current assay system.

The presence of more than two binding species makes graphical interpretation of binding data difficult. Since it has been well documented that human plasma contains three T_4BPs attempts were made to specifically remove one of these binding proteins in the hope of simplifying the binding system to make graphical interpretation more simple. Two approaches, based on phenomena which have been described in the literature, were investigated, viz. the specific denaturation of TBG by exposure to elevated temperature, and the inhibition of T_4 binding to TBPA by barbiturate ions.

Scatchard plots of binding data obtained with both human and sheep plasma which had been heat treated, were very different from those obtained with unheated plasma. With both species, heat treatment reduced markedly the initial steep slope of the Scatchard plots, but the plots were still not linear and indicated the presence of at least 2 binding proteins in the heated plasma. In the case of human plasma, these 2 proteins were probably TBPA and albumin since Takemura et al. (1971) have shown that T_4 binding to TBG is eliminated by

such treatment, while the binding to TBPA is not affected. In the case of sheep plasma, the situation might not be so simple since only 2 binding proteins have been described. If heat treatment affects sheep TBG the same as human TBG, then such a result indicates the presence of more than 2 binding proteins which are not separated by electrophoresis. Alternatively heat might affect sheep TBG by reducing its affinity for T_4.

The attempt to specifically remove the binding of T_4 to TBPA using barbital buffer was clearly unsuccesful due to the fact that barbiturate ions reduced T_4 binding to all the plasma T_4BPs. While such an observation is at variance with most published data it can be readily explained in the light of the present results and some published data. A change in pH, in Tris-NaCl buffer, from 7.4 to 8.6 was accompanied by an increase in the ability of human plasma proteins to bind T_4. Differences were greatest at the higher unbound T_4 concentrations where TBG was saturated (Figure 4.7). This indicated that the binding of T_4 to TBPA and albumin was more sensitive to changes in pH over this range, than was the binding of T_4 to TBG. Such observations are in agreement with published data (Steiner et al., 1966; Lutz and Gregerman, 1969; Marshall and Pensky, 1971). When the same pH change was accompanied by a change in composition with respect to other ions, i.e. phosphate-NaCl at pH 7.4 and barbital at pH 8.6, the results were considerably different. Binding at the more alkaline pH was severely

reduced and the two graphs were essentially parallel suggesting that each of the binding proteins was influenced to a similar extent (Figure 4.9). However if barbital had an identical effect on all three T_4BPs one would expect graphs of the form shown in Figure 4.7 and not of a parallel nature as shown in Figure 4.9. The latter graphical result indicates that the interaction between T_4 and TBG is less affected by barbiturate ions than is the binding of T_4 to TBPA and albumin. This inhibitory effect of barbital on T_4 binding to plasma proteins is probably due to competitive inhibition at the T_4 binding sites which results in a reduction in the apparent association constant between these sites and T_4. The apparent association constant for TBPA is reduced to a greater extent than that for TBG. Such an hypothesis is supported by the following observations reported in the literature:

1. T_4 is bound to TBPA when barbital buffer concentrations are lower than those normally employed (Ingbar, 1960; Davis and Gregerman, 1970).
2. The use of barbital buffer increases the proportion of T_4 bound to TBG over that seen in other buffer systems (Ingbar, 1963; Davis and Gregerman, 1970).
3. Tetraiodothyroacetic acid, which is bound more firmly to TBPA than is T_4, has been shown to bind to TBPA even in the presence of barbiturate ions at a concentration where no binding of T_4 is evident. The amount bound, however, is considerably less than when other buffer systems are used (Ingbar, 1960, 1963).

4. When a mixture of purified TBPA and HSA was subjected to electrophoresis in barbital buffer both proteins were shown to bind T_4 (Ingbar, 1960).

While the qualitative information reported in this chapter was of considerable value in designing an *in vitro* assay system which approximated T_4 binding *in vivo*, it was virtually valueless in assessing the physiological binding properties of plasma T_4BPs. For this reason attempts were made to attain quantitative estimates of the thyroid hormone binding properties of plasma proteins by using the present technique in conjunction with a mathematical technique for fitting non-linear regressions. The quantitative data obtained using these techniques is presented in the following chapter.

CHAPTER 5

THE THYROXINE-BINDING PROPERTIES OF PLASMA PROTEINS. QUANTITATIVE ESTIMATES.

INTRODUCTION

The major requirement for unambiguous determinations of hormone-protein binding parameters is that measurements of protein-bound and unbound hormone concentration be made when the system is in a state of thermodynamic equilibrium (Westphal, 1971). Since hormone-protein complexes are held together by dissociable non-covalent bonds the need for equilibrium techniques can not be overstressed. Currently available electrophoretic methods, and competitive binding techniques employing ion exchange resins, dextran coated charcoal and Sephadex columns do not fulfil this requirement (Robbins and Rall, 1957; Inada and Sterling, 1967; Keane, Pegg and Johnson, 1969; Roberts and Nikolai, 1969; Refetoff et al., 1972; Elewaut, 1973).

The competitive binding technique described in Chapter 3 allows estimation of the concentration of protein-bound and unbound T_4 at equilibrium under conditions closely approximating those existing in vivo. If sufficient data points are available, mathematical analysis of the binding data allows estimation of the number of specific binding proteins, their individual association constants and binding capacities together with an assessment of the unbound hormone concentration. Quantitative results obtained using this technique are summarized in this chapter.

RESULTS

Thyroxine-binding proteins in human serum

Computer simulation studies demonstrated that an appropriate mathematical analysis of binding data could be attained if values of [B] and [U] covered a range of concentrations, from a point where TBG was less than 50% saturated to a concentration where TBPA was greater than 90% saturated. A standard protocol was adopted to ensure this and a typical set of results are shown in Table 5.1. Using such data it was possible to get a seemingly adequate fit to a model consisting of two specific binding proteins (TBG and TBPA) and one non-specific binding protein (albumin) as described in equation 13 of Chapter 3.

Six replicate analyses of the same human plasma sample were subjected to mathematical analysis and the binding parameters calculated. The results are summarized in Table 5.2. The standard deviation for each of the fitted parameters was calculated and expressed as a percentage of the mean (i.e. % coefficient of variation). The coefficient of variation was 10.8% for K_1 and 10%, 13%, 15.6% and 6.0% for C_1, K_2, C_2 and K_n respectively. Because of the method of fitting, individual estimates of K and C for each of the specific binding proteins were significantly correlated, i.e. an estimate of K_1 or K_2 above (or below) the mean value was accompanied by a C_1 or C_2 value above (or below) the corresponding mean C value. With replicate analyses the mean value of K is

Table 5.1. A typical results table for the analysis of a human plasma sample diluted 1:150 with 0.05 M phosphate, 0.10 M NaCl buffer, pH 7.4.

Background Counts = 2,500/400 sec

Excluded Volume = 22.32 ml

α = 7.362

Endogenous T_4 = 19.24 ng

Total Counts/10 ml dilute plasma = 267,564/400 sec

Total Added T_4 (ng)	Count rate in the excluded volume (Counts/ 400 sec)	Unbound T_4 Concentration [U] (ng/100 ml)	Protein-bound T_4 concentration [B] (ng/100 ml)
0	271,185	2.878	62.131
10	263,874	4.740	91.370
20	256,816	6.834	118.662
40	248,419	11.167	172.037
80	231,070	21.647	263.608
120	219,152	33.208	346.151
200	202,025	58.701	491.404
300	191,713	91.098	668.521
500	180,574	158.050	1,004.730
700	173,407	227.733	1,318.100
1,000	165,114	337.160	1,747.150
2,000	146,535	732.039	2,925.460
4,500	131,188	1,756.840	5,556.760
7,000	126,973	2,779.250	8,208.100
10,000	123,395	4,028.330	11,204.200

Table 5.2. Six replicate estimates of the thyroxine-binding properties of a normal human plasma sample diluted 1:111.6 in the excluded volume. K_1, C_1, K_2, C_2 and K_n, the parameters previously described were not corrected for dilution. C_1, C_2 and K_n values calculated from the mean K_1 and K_2 values are shown in parentheses.

Sample No.	K_1 (ng/100ml)	C_1 (ng/100ml)	K_2 (ng/100ml)	C_2 (ng/100ml)	K_n
1	9.70	193 (176)	475	2609 (3007)	1.44 (1.31)
2	7.97	166 (173)	620	2839 (2346)	1.47 (1.61)
3	8.10	164 (169)	591	2817 (2464)	1.39 (1.49)
4	9.42	196 (182)	511	2103 (2273)	1.63 (1.57)
5	7.26	161 (184)	444	2039 (2211)	1.56 (1.52)
6	8.77	200 (196)	513	2109 (2189)	1.46 (1.43)
Mean	8.54	180 (180)	526	2419 (2416)	1.49 (1.49)
SD	0.93	18 (10)	68	377 (307)	0.09 (0.11)
% C.V.	10.8	10.0 (5.6)	13.0	15.6 (12.7)	6.0 (7.4)

likely to be a more precise estimate of the true value of K than any individual estimate of K. For this reason C_1, C_2 and K_n were recalculated using the mean values of K_1 and K_2. These values are shown in parentheses in Table 5.2. While the mean values of C_1, C_2 and K_n were unaffected by the use of mean K_1 and K_2 values the coefficients of variation for C_1 and C_2 were reduced to 5.6% and 12.7% respectively while that for K_n was slightly increased to 7.4%.

Insofar as it is valid to extrapolate from diluted to undiluted plasma the association constants and binding capacities of the two specific binding proteins and the association constant of the non-specific binding protein were calculated using the mean values in Table 5.2. The association constant of the most avid T_4BP, presumably TBG was 9.4×10^9 M^{-1} and its binding capacity was 20 µg/100 ml of plasma. The association constant of the other specific binding protein (TBPA) was 1.5×10^8 M^{-1} with a binding capacity of 270 µg/100 ml of plasma. The association constant of human albumin calculated from K_n, using the measured albumin concentration of 3.7 g/100 ml, and assuming one binding site per albumin molecule was 3.07×10^5 M^{-1}. From the values of K_1 and K_2, and the values of C_1, C_2 and K_n corrected for dilution, it was possible to calculate the unbound T_4 concentration in undiluted serum. Using the six replicate measurements of these parameters a mean unbound concentration of 2.20 ± 0.07 ng/100 ml was calculated which was identical to the unbound T_4 concentration calculated from the mean

estimates of K_1, C_1, K_2, C_2 and K_n corrected for dilution.

Properties of plasma T_4BPs in subjects of differing clinical thyroid states

Plasma samples from 3 normal, 2 hypothyroid, 2 hyperthyroid and 1 pregnant human subject were analysed as described in Chapter 3. The results extrapolated to undiluted plasma are summarized in Table 5.3.

The variance in the estimates of the mean association constants for TBG and TBPA was not significantly different from the variance obtained with replicate estimates on the one plasma sample. It is conceivable that the association constants of the T_4BPs vary between individuals and for this reason it was not valid to use the mean association constants for the calculation of individual binding capacities. The mean values for the association constants of TBG, TBPA and albumin in these samples were 9.5×10^9, 1.6×10^8 and 3.06×10^5 M^{-1} respectively. The TBG binding capacity in the one pregnant subject was 44.5 µg/100 ml which was significantly higher than the mean value of 21.3 ± 3.2 µg/100 ml for the other seven subjects. The mean value for the plasma TBPA binding capacity was 307 ± 78 µg/100 ml.

Using the estimated values of K_1, C_1, K_2, C_2 and K_n and correcting for dilution the concentration of unbound T_4 and the distribution of bound T_4 between its three binding proteins in undiluted plasma was calculated. These are shown in Table 5.3. The unbound T_4 concentrations agreed well with the clinical status, the mean unbound T_4

Table 5.3. The thyroxine-binding properties of human plasma proteins from subjects of differing clinical thyroid status.

Thyroid Status	Total T_4 (µg/100ml)	TBG		TBPA		Albumin			% Distribution of Total T_4				Unbound T_4 Conc. (ng/100ml)
		K_{assoc} ($M^{-1} \times 10^9$)	B.C.* (µg/100ml)	K_{assoc} ($M^{-1} \times 10^8$)	B.C. (µg/100ml)	K_n	Conc. (g/100ml)	K_{assoc} ($M^{-1} \times 10^5$)	TBG	TBPA	Albumin	Unbound	
Normal	5.6	9.4	20.0	1.52	270	166.2	3.7	3.07	73.3	20.1	6.5	0.039	2.2
	8.0	8.5	24.8	1.72	202	256.8	4.2	4.22	74.4	16.1	9.6	0.037	3.0
	4.7	10.7	19.0	1.58	302	215.6	4.3	3.44	72.1	20.4	7.4	0.034	1.6
Hypo-	3.5	11.3	17.3	1.65	457	212.8	5.6	2.62	64.7	28.8	6.5	0.031	1.1
thyroid	2.8	9.8	25.7	1.58	355	233.6	6.0	2.36	75.5	18.4	6.1	0.026	0.7
Hyper-	9.2	8.8	22.9	1.02	304	170.0	4.3	2.74	76.0	16.6	7.3	0.043	4.0
thyroid	8.6	9.8	19.4	1.86	240	218.4	4.3	3.50	68.2	22.8	9.0	0.041	3.5
Pregnant	7.3	7.9	44.5	1.92	326	163.2	4.5	2.52	80.1	16.4	3.4	0.021	1.5
Mean		9.5	21.3**	1.61	307	204.6	4.6	3.06	73.0	20.0	6.97		
SD		1.1	3.2	0.28	78	34.5	0.8	0.63	4.8	4.3	1.90		

* B.C. - binding capacity

** Pregnant sample excluded from mean estimate.

concentration in normal human plasma was 2.3 ng/100 ml while that in the plasma of hypothyroid and hyperthyroid subjects was 0.9 and 3.8 ng/100 ml respectively. The latter level is lower than reported values for the concentration of unbound T_4 in hyperthyroid patients. This may be due to the small number of patients studied, the degree of hyperthyroidism or it may be accounted for by an incorrect diagnosis. The sample from the pregnant subject had an unbound T_4 concentration of 1.5 ng/100 ml which was at the lower end of the range seen in the 3 normal subjects. The mean percentage distribution of total T_4 between TBG-bound, TBPA-bound, albumin-bound and the unbound state was 73.0:20.0:6.97:0.03.

Thyroxine-binding proteins in sheep serum

Sera collected from 16 male and non-pregnant female sheep of various ages were subjected to the same protocol as the human samples. Although data in the literature suggests that there are only two T_4BPs in sheep serum (TBG and albumin) fitting a model with one specific and one non-specific binding protein (S_1 + NS) gave very poor fits over the concentration range used. However, the model used for analysis of human sera, i.e. two specific and one non-specific binding protein (S_1 + S_2 + NS) gave a good fit to the data as is demonstrated in Figure 5.1. The binding parameters extrapoloted to undiluted serum are shown in Table 5.4. In all samples studied there appeared to be at least three T_4BPs. The most avid T_4BP which probably corresponds to TBG of electrophoresis, had a mean association constant of

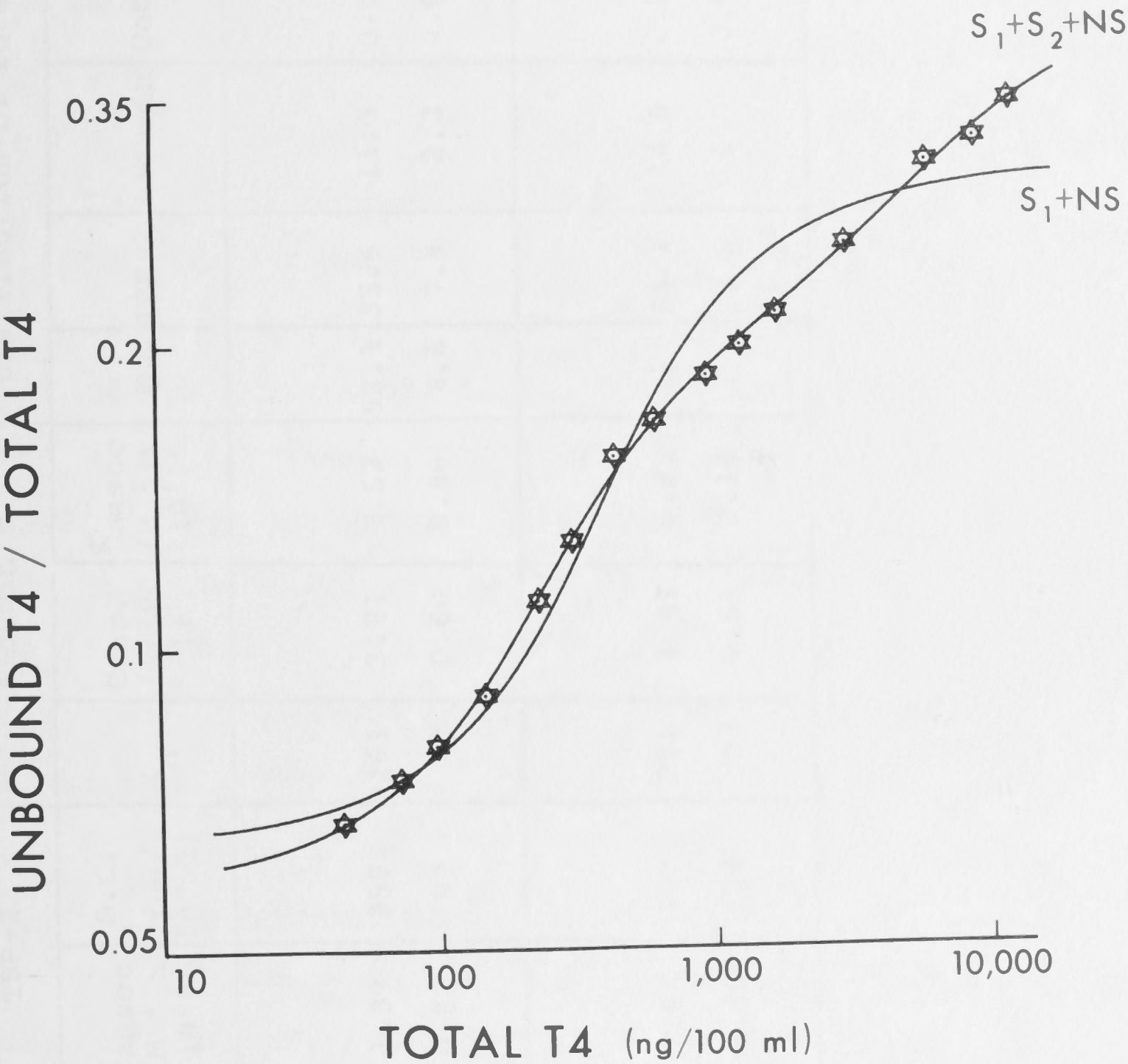


Figure 5.1. Thyroxine-binding by sheep serum diluted 1:111.6 in the excluded volume. The experimental data points were fitted to a two (S_1 + NS) and three (S_1 + S_2 + NS) binding protein model.

Table 5.4. The thyroxine-binding properties of sheep and rat serum proteins

	Total T_4 (µg/100ml)	TBG		TBP-2		Albumin			% Distribution of Total T_4				Unbound T_4 Conc. (ng/100ml)
		K_{assoc} (M^{-1} x 10^9)	B.C.* (µg/100ml)	K_{assoc} (M^{-1} x 10^8)	B.C. (µg/100ml)	K_n	Conc. (g/100ml)	K_{assoc} (M^{-1} x 10^5)	TBG	TBP-2	Albumin	Unbound	
Sheep (n = 16)													
Mean	6.2	8.9	12.8	1.39	359	161	2.81	3.52	53.1	35.6	11.3	0.056	3.54
SD	2.7	2.9	4.8	0.62	99	49	0.60	0.86	8.6	7.5	2.3	0.019	2.04
Rat (n = 7)													
Mean	4.0			3.64	317	228	2.41	6.25		85.1	14.8	0.066	2.42
SD	2.3			0.69	114	33	0.55	2.11		4.9	4.9	0.023	1.08

* B.C. - binding capacity.

8.9×10^9 M^{-1} which was not significantly different from that of human TBG, however the mean binding capacity of this protein was significantly lower than that of human TBG. The association constant and binding capacity of sheep T_4BP-2 were not significantly different from those of human TBPA, nor was the association constant of sheep albumin significantly different from that of human albumin. The mean estimated concentration of unbound T_4 in sheep plasma was 3.5 ± 2.0 ng/100 ml, and many of the sheep had unbound T_4 concentrations greater than those estimated in two hyperthyroid humans. The distribution of total T_4 between the various binding proteins was different from the human, due to the lower binding capacity of sheep TBG. The mean percentage ratio of TBG-bound:T_4BP-2-bound:albumin-bound:unbound in the sheep was 53.1:35.6:11.3:0.06.

Thyroxine-binding proteins in rat serum

The binding of T_4 by rat serum was considerably different from that seen with human and sheep serum (Figure 5.2). Data was best fitted by a model consisting of one specific and one non-specific binding protein. The specific binding protein had a mean association constant of 3.64×10^8 M^{-1} and a mean capacity of 317 µg/100 ml (Table 5.4). Non-specific binding was probably due to albumin. The association constant of rat albumin for T_4 was $6.25 \pm 2.11 \times 10^5$ M^{-1} which is twice the corresponding value found for sheep and human albumin.

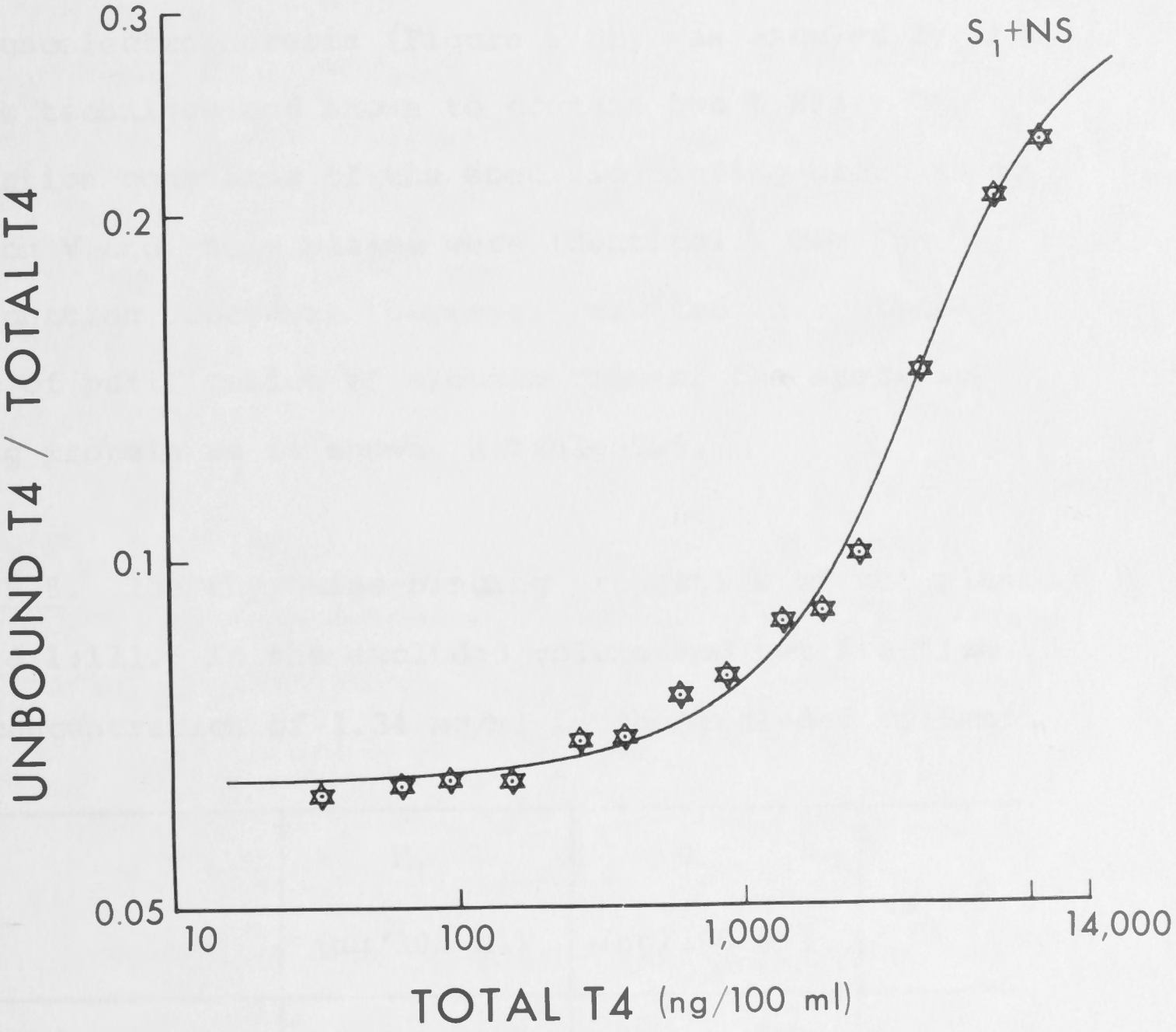


Figure 5.2. Thyroxine-binding by rat serum diluted 1:111.6 in the excluded volume.

Since the results obtained with rat plasma were at variance with those in the literature attempts were made to confirm the present findings using normal biochemical separation techniques.

Rat Cohn fraction V (1 mg/ml), which was pure on immunoelectrophoresis (Figure 5.4), was assayed by the present technique and shown to contain two T_4BPs. The association constants of the specific binding proteins in fraction V and whole plasma were identical. The Cohn fractionation procedure, however, resulted in a greater degree of purification of albumin than of the specific binding protein as is shown in Table 5.5.

Table 5.5. The thyroxine-binding properties of rat plasma diluted 1:111.6 in the excluded volume and rat fraction V at a concentration of 1.34 mg/ml in the excluded volume.

	K_1 (ng/100 ml)	C_1 (ng/100 ml)	K_n
Rat Plasma (n = 7)	219.3	2839	2.04
Rat Fraction V (n = 2)	205.5	7686	10.74
Fraction V/Plasma		2.71	5.26

From the data presented in Table 5.5 it was possible to calculate the molar ratios of the two T_4BPs in the rat fraction V. The specific binding protein was present at less than 1% of the albumin concentration. Assuming that

rat albumin has one binding site per molecule the association constant of purified rat albumin for T_4 was calculated to be $5.54 \times 10^5\ M^{-1}$.

Radio-thyroxine [^{125}I] and rat fraction V [^{131}I] were added to 1 ml of pooled rat plasma and a 50 mg/ml solution of rat fraction V. Each sample was separated in duplicate on the same Sephadex G-200 column. The distribution of protein and radioactivity in the column effluent is shown in Figure 5.3. The optical density peaks for albumin in whole plasma and fraction V coincided with the rat fraction V [^{131}I] radioactivity peak at tube 39. Thyroxine [^{125}I] radioactivity peaked three tubes later indicating the presence of a protein of smaller molecular size than albumin which was present in low concentration but had the ability to bind considerable T_4 presumably due to its high association constant. Tubes from the ascending (34-39) and the descending (40-45) sides of the optical density peak of the rat fraction V separation were pooled and concentrated. A portion of each sample was electrophoresed and reacted against rabbit anti-whole rat plasma. One protein band corresponding to albumin was found in the ascending fraction and two protein bands were found in the descending fraction (Figure 5.4). The latter fraction contained both albumin and a β-globulin. Autoradiographic studies of these immunoelectrophoretograms were unable to demonstrate T_4 [^{125}I] binding to any plasma protein other than albumin.

Attempts were then made to separate these two

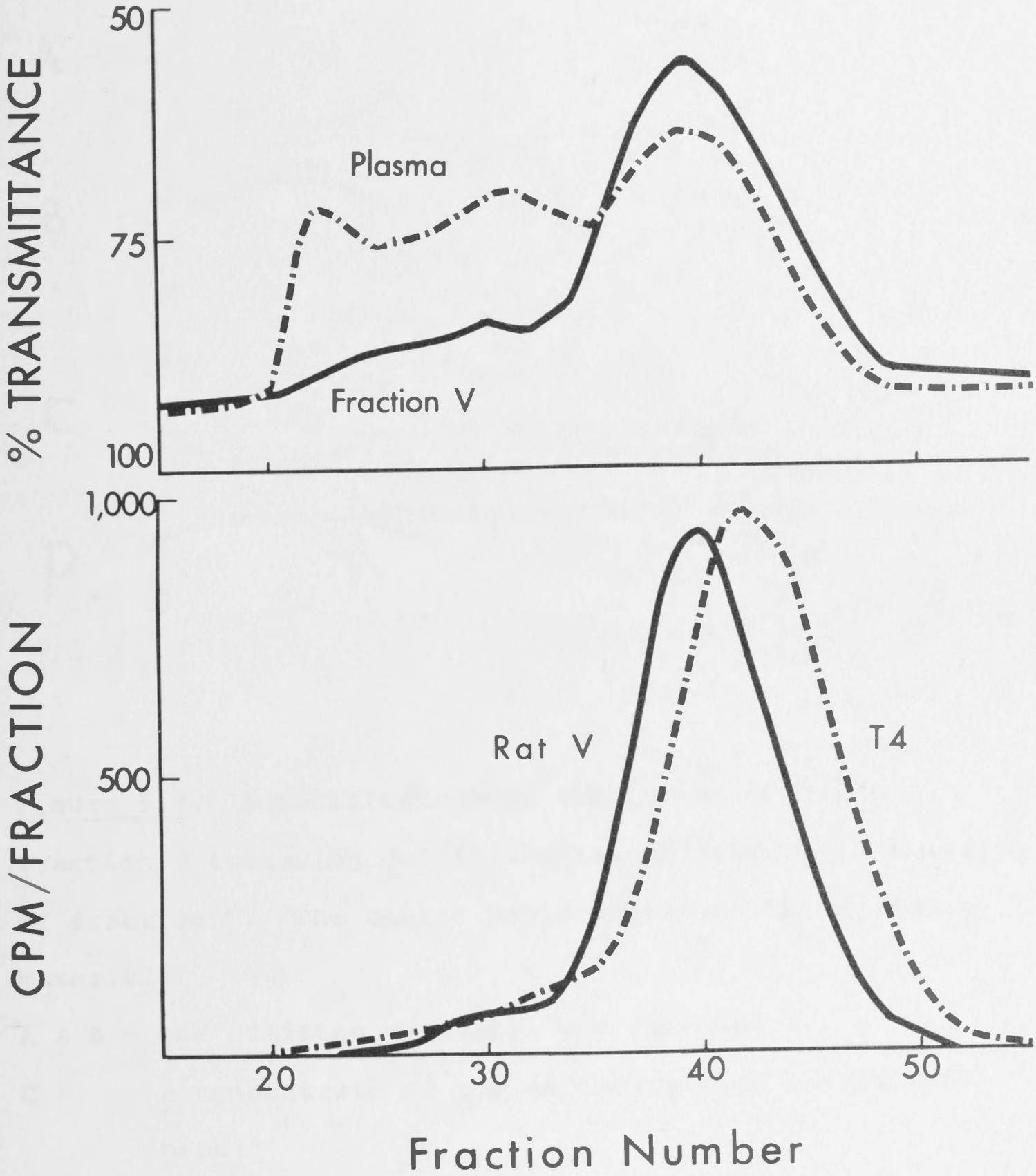


Figure 5.3. Separation of rat T_4BPs by gel filtration on Sephadex G-200 columns. Rat plasma and rat fraction V were labelled with tracer T_4 [^{125}I] and rat fraction V [^{131}I]. The protein elution profiles are shown in the upper diagram.

Figure 5.4. Identification of two proteins in rat fraction V following gel filtration of a 50 mg/ml solution of fraction V. The centre wells contained the following material:

A & B - the starting material, rat fraction V

C - a concentrate of the ascending side of the O.D. peak

D - a concentrate of the descending side of the O.D. peak.

The precipitin arcs were developed using rabbit anti-rat serum. D shows two precipitin lines while A, B and C have only an albumin line.

T_4BPs by polyacrylamide gel electrophoresis. Rat serum, rat fraction V and a concentrate of tubes 40-45 above were labelled with T_4 [^{125}I] and subjected to electrophoretic separation in polyacrylamide gel. A number of protein bands were identified in all samples. Autoradiographic studies using these electrophoretograms demonstrated strong T_4 [^{125}I] binding in the albumin region as shown in Figure 5.5. Occasionally small amounts of radioactivity were present in the immediate postalbumin region. Since the majority of T_4 [^{125}I] would be associated with the specific T_4BP it was concluded that this protein demonstrates electrophoretic mobility similar to that of serum albumin. For this reason it could not be separated from albumin by the electrophoretic techniques employed.

Effect of the added T_4 concentration range on estimates of the T_4 binding parameters

Preliminary results indicated that good solutions could be obtained when sheep binding data were fitted to a two binding protein model (S_1 + NS). This situation arises when the concentration of added T_4 is insufficient to saturate the specific binding proteins. To test the optimum additions of T_4 a human plasma was assayed using 18 additions of T_4 over a 10,000 fold concentration range of added T_4. Identical estimates of the binding parameters were obtained when 14 to 18 data points were used. A 1,000 fold unbound T_4 concentration range was therefore adequate to saturate the specific binding proteins and obtain a solution. When the concentration

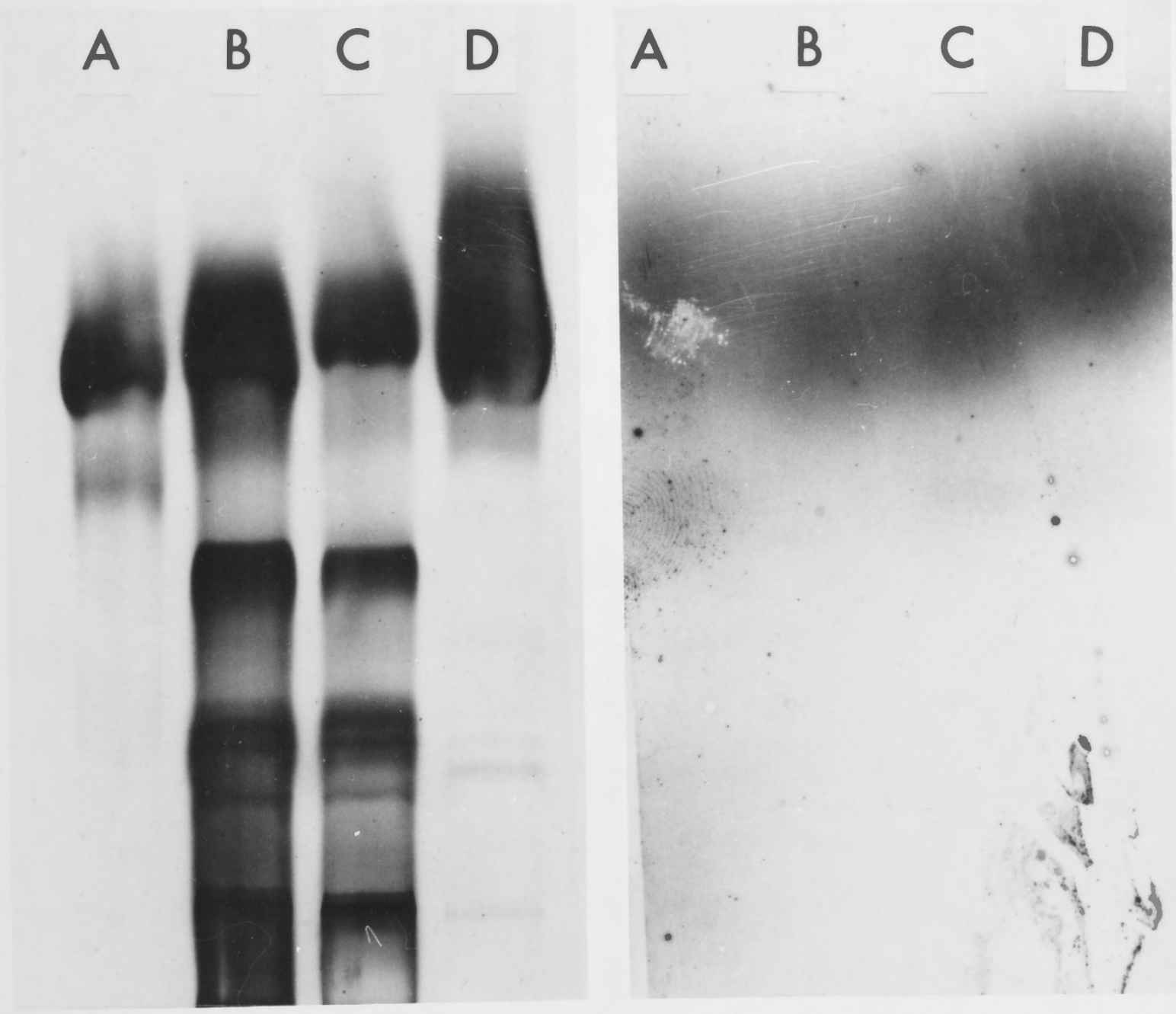


Figure 5.5. Polyacrylamide gel electrophoretograms of rat serum and rat fraction V labelled with T_4 [^{125}I]. Electrophoretograms stained for protein and autoradiographs are shown. The samples studied were 2% protein solutions of the following:

A - rat fraction V

B - rat serum (4% solution)

C - rat serum

D - a concentrate of the descending side (tubes 40-45) of the OD peak for a Sephadex G-200 separation of rat fraction V.

range of added T_4 was further reduced a solution with three T_4BPs (S_1 + S_2 + NS) could not be attained, however, a two binding protein model could be used with the adequacy of fit increasing as the number of data points diminished. This is illustrated in Table 5.6. When the first 10 data points were fitted to a two protein model (S_1 + NS) a seemingly good fit was attained over the concentration range fitted. However the solution deviated considerably from the experimental points when extrapolated beyond that range as is demonstrated in Figure 5.6.

It was of interest to note that when the first 5 data points were fitted, the binding parameters for TBG binding were close to the correct result. At these low unbound T_4 concentrations TBPA probably acts as a non-specific binder, i.e. the relationship between [B] and [U] is linear which would explain the accuracy of the result.

DISCUSSION

Conventional methods of measuring the T_4 binding properties of proteins in whole serum have mainly involved electrophoretic separation techniques. The distribution of T_4 in such electrophoretograms has been measured using radioactively labelled T_4 and the binding capacities of the saturable binding proteins (TBG and TBPA) estimated from the maximum amounts of added T_4 that they can bind. Gordon and Coutsoftides (1969) have shown that the distribution of labelled T_4 is a function of the

Table 5.6. The effect of the added T_4 concentration range on estimates of the T_4 binding parameters of human plasma

Model	No. of Data Points	Maximum [U] (ng/100 ml)	S_1		S_2		NS	Residual Variance
			$K_{assoc.}$ $M^{-1} \times 10^{10}$	Binding Capacity (µg/100 ml)	$K_{assoc.}$ $M^{-1} \times 10^{8}$	Binding Capacity (µg/100 ml)	K_n	
$S_1 + S_2 + NS$	18	40,445	1.77	11.7	1.84	274	303	0.00025
	15	3,827	1.76	11.8	1.78	283	298	0.00032
$S_1 + NS$	18	40,445	0.28	59.2	-	-	383	0.00553
	15	3,827	0.38	44.9	-	-	457	0.00499
	10	204	1.21	16.5	-	-	772	0.00029
	5	21	1.72	11.9	-	-	938	0.00037

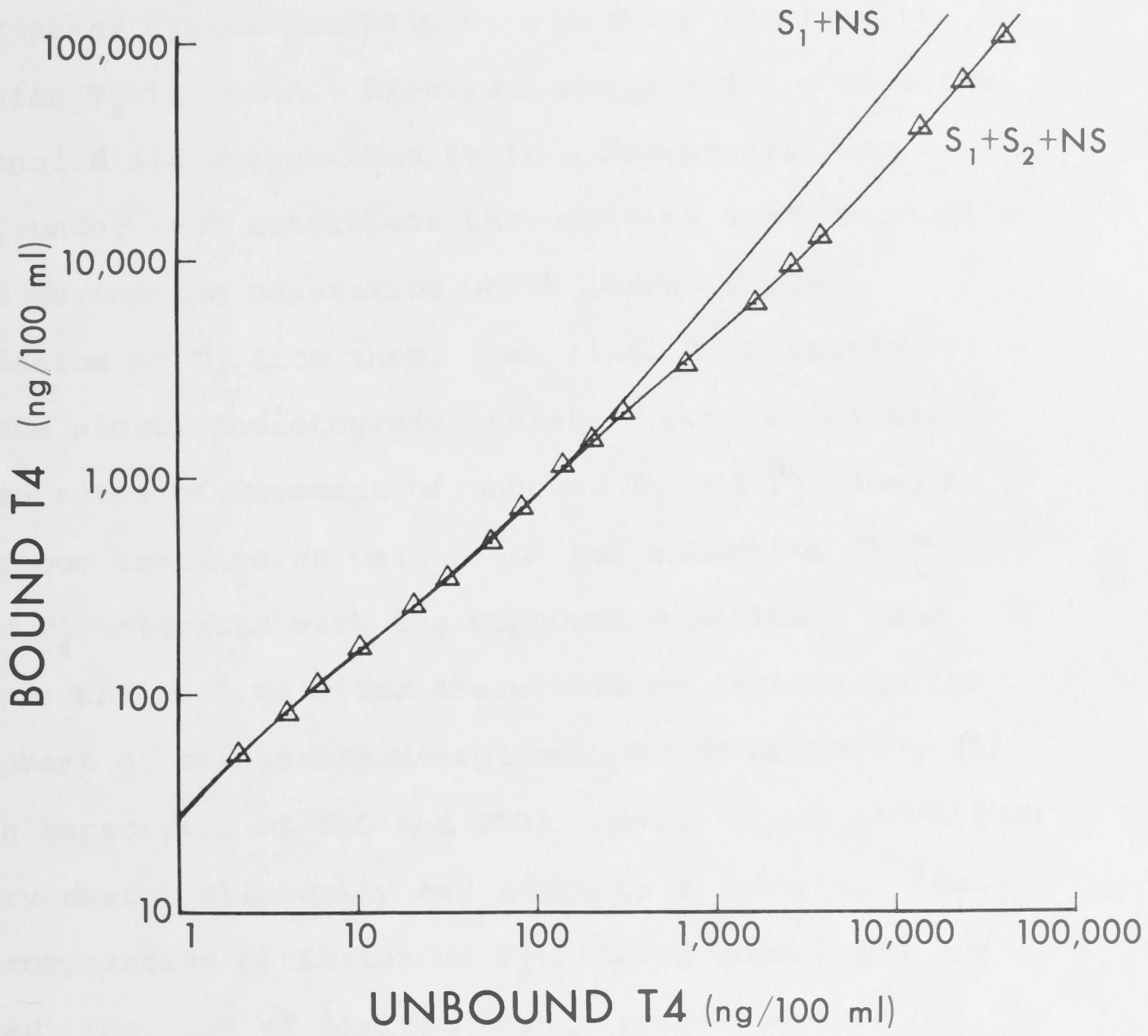


Figure 5.6. The effect of the added T_4 concentration range on estimates of the thyroxine-binding parameters of a human plasma sample diluted 1:111.6 in the excluded volume. A three binding protein model (S_1 + S_2 + NS) gave an excellent fit over the entire concentration range.

ionic strength, composition, pH and temperature of the system used for the electrophoretic separation. These factors have also been shown to influence binding in the present assay system probably by affecting the avidity with which T_4 is bound. Electrophoresis suffers from the additional disadvantage that it is a non-equilibrium system, under such conditions the proteins are continually diluted during the separation which leads to the dissociation of T_4 from them. The final distribution of T_4 in the electrophoretograms therefore depends on the relative rates of movement of unbound T_4 and T_4 bound to the various proteins as well as on the extent to which unbound T_4 interacts with the supporting medium. Some allowance has been made for these various factors in the development of clinically useful methods of measuring the binding capacities of TBG and TBPA. While these techniques are very useful clinically and adequate as research tools where comparative estimates of T_4 binding capacities are required, they are of limited use in assessing the relative importance of the T_4BPs in the transport of thyroid hormones *in vivo*. Estimates of the association constants for TBG and TBPA, necessary to do this, could only be derived from electrophoretic data when values for the association constant of albumin were derived by other means, e.g. equilibrium dialysis studies (Robbins and Rall, 1967; Hamada *et al.*, 1970). In order to measure association constants relevant to the *in vivo* situation it is necessary to use equilibrium techniques under conditions which closely mimic the normal

physiological state. It was found in the present studies that plasma diluted in 0.05 M phosphate, 0.10 M NaCl buffer, pH 7.4 and assayed at 37° was a convenient system and appeared likely to closely approximate T_4 binding in vivo.

It has long been realised that if the concentration of protein-bound and unbound ligand can be measured over a wide range of added ligand concentration then considerable information can be obtained about the binding properties of the proteins involved (Scatchard, 1949). A large number of methods have been applied to that end in the study of steroid hormone-plasma protein interactions. These methods, which have been evaluated in a recent review by Westphal (1971), tend to be more sophisticated than methods currently used for the study of T_4 binding properties in that greater efforts have been made to maintain equilibrium. The method described in this thesis is similar in principle to one previously used for the study of testosterone-plasma protein binding in that Sephadex was used as a competitive binding agent (Pearlman and Crépy, 1967; Pearlman, 1970). With this system, equilibrium concentrations of protein-bound and unbound T_4 could be measured over a 1,000 fold range, enabling accurate statistical estimation of the T_4 binding parameters.

Previously, analysis of binding data relied on linear transformations and graphical techniques (Lineweaver and Burk, 1934; Eadie, 1942; Scatchard, 1949). These methods are liable to systematic errors and are not

adequately suited to situations involving a number of binding species (Baulieu et al., 1970; Westphal, 1971). With the availability of modern computer techniques it is now possible to fit non-linear functions over a wide concentration range. Such a technique has been described in detail by Baulieu and Raynaud (1970). While the present method of analysis is similar to that of Baulieu and Raynaud (1970) the proportional graph was not used for initial estimates of binding affinities; these were found by trial and error using values in the literature as a basis. The proportional graph however has been useful on occasions for assessing the number of saturable binding proteins present and to indicate whether the range of concentrations was sufficient to permit an adequate solution. The plot of log B vs log U has also proven useful for these purposes and has the added advantage of allowing graphical estimates of the concentration of unbound T_4 in undiluted plasma.

The parameter fitting technique described in Chapter 3 relies on the assumption that the relationship between albumin-bound T_4 and unbound T_4 concentrations is linear over the range of unbound T_4 concentration used in the assay. When the estimated association constants for albumin are used, albumin can be shown to be less than 2% saturated at the highest unbound T_4 concentration found in the assay. If there is more than one binding site per molecule of albumin, it would be proportionately less saturated. Thus the approximation

derived in equation (12) in Chapter 3 for non-specific (albumin) binding, and used in fitting the model used in this chapter is an accurate one.

The results presented here demonstrate the applicability of this technique to sera of different species and to individuals of different clinical states. Because of the small numbers in each group it is unlikely that the data is truly representative of the group as a whole however certain trends are evident. The fitting of a three binding protein model to the human data is consistent with recent published information using electrophoretic techniques (Hamada et al., 1970). The values derived for the association constants of human TBG, TBPA and albumin are close to published figures using purified protein preparations when it is realised that the various estimates were made under varying conditions of pH, temperature and buffer composition (Tabachnick, 1964a, 1967; Green et al., 1972a; Pages et al., 1973). Present observations agree with previous publications which showed that the binding capacity of TBG is substantially increased during pregnancy (Refetoff et al., 1972) but is little affected by hypothyroidism (Inada and Sterling, 1967), and that the unbound T_4 concentration is at the lower end of the normal range during pregnancy, is lowered in hypothyroidism and elevated in hyperthyroidism (Sterling and Brenner, 1966; Thorson et al., 1972). The present estimates of the distribution of T_4 between its binding proteins do not differ greatly from those of Woebar and Ingbar (1968) and Hamada et al.

(1970) but are considerably different from earlier estimates (Oppenheimer and Surks, 1964; Robbins and Rall, 1967).

The observation that sheep serum contains three functionally distinguishable T_4BPs is at variance with electrophoretic data which has demonstrated only two T_4BPs (Farer et al., 1962; Refetoff et al., 1970). Statistical analysis of the data presented in the preceding chapter for heat treated sheep and human plasma, showed that heat treatment removed TBG binding in both species, and that sheep T_4BP-2, like human TBPA, was unaffected by the heat. This illustrates the ability of the present technique to detect functionally different T_4BPs and measure their affinities and capacities for T_4. Electrophoresis can only distinguish classes of binding proteins with different electrophoretic mobilities and only the binding capacities of these classes of proteins can be determined. It is conceivable that proteins of widely differing T_4 binding properties could have similar electrophoretic mobility. Similarly the present method has shown two functionally different T_4BPs in the rat which disagrees with findings using a number of electrophoretic media and buffer systems (Farer et al., 1962; Refetoff et al., 1970). However, these are minimal estimates of the number of specific binding proteins since this technique is not capable of distinguishing between different proteins with similar T_4 binding properties.

Further support for the existence of two T_4BPs

in rat serum was obtained with gel filtration studies (Figure 5.3). The existence of two such proteins, however, could not be substantiated using agar or polyacrylamide gel electrophoresis. Davis, Spaulding and Gregerman (1970) have demonstrated that the major T_4BP of rat serum migrates slightly anodal to albumin on polyacrylamide gel electrophoresis and has T_4 binding properties similar to those of human TBPA. The present estimates of the association constant and binding capacity of the most avid T_4BP in rat serum are similar to those of human TBPA (Tables 5.3 and 5.4). It appeared likely that the major T_4BP observed in both studies was the same protein. The inability to separate this protein from albumin in the present study was attributed to the use of a different polyacrylamide gel and buffer system than that employed by Davis et al. (1970). The latter authors also demonstrated T_4 binding to a protein in the immediate postalbumin region. While they suggested that such binding was due to a rat "TBG-like" protein the competitive binding technique was unable to detect a protein with T_4 binding properties similar to those of human or sheep TBG. It appeared more likely that this protein, which has also been isolated in the present polyacrylamide gel separations (Figure 5.5), is a second non-specific T_4BP which binds only tracer quantities of T_4.

The competitive binding technique described here is essentially analogous to the widely used technique of equilibrium dialysis. In both methods binding systems

can be studied at equilibrium under closely controlled conditions but the present technique has the added advantages of reaching equilibrium more rapidly, of being less troubled by contaminating radio-iodide and of being technically more simple. It could be used for all those purposes for which equilibrium dialysis has been used traditionally such as measuring concentrations of unbound ligand or studying the binding of purified proteins. With the methods of data analysis described, this technique can be used for the study of binding in complex mixtures of binding proteins. Since the controlled conditions can be readily varied these can be selected to mimic the situation pertaining *in vivo* or altered to yield information about mechanisms underlying the binding reactions. Seemingly similar competitive binding methods which have been described previously have employed ion exchange resins, Dextran coated charcoal, and Sephadex columns (Keane *et al.*, 1969; Roberts and Nikolai, 1969; Refetoff *et al.*, 1972; Elewaut, 1973). Ion exchange resins have been found to take too long to reach equilibrium, bind T_4 too strongly and bind T_4BPs themselves.

Sephadex columns yield data under non-equilibrium conditions due to the dissociation of protein-bound hormone during its passage through the column. To illustrate the erroneous results obtained from such a technique and to demonstrate the inaccuracy of graphical analysis a recent paper by Elewaut (1973) was studied in

detail. This author used Sephadex G25 columns for separating two forms of T_4; "protein-bound" T_4 passed directly through the columns, while "unbound" T_4 was bound tightly to the Sephadex and could only be eluted with an excess of plasma. The binding parameters for the various proteins were determined from the data obtained, by a graphical method.

Elewaut claimed the unbound T_4 values were too high, the error being due to T_4 which was bound to albumin in the plasma being stripped off the albumin during passage through the column. He estimated the unbound values were 34 times too high. Nevertheless he estimated the binding parameters using these uncorrected values, but corrected the estimated binding parameters subsequently. It seemed unlikely that Elewaut's explanation for his erroneously high estimates of unbound T_4 in his non-equilibrium system could be as simple as he claimed.

Firstly, it seemed unlikely that the amount of T_4 bound to albumin in plasma diluted 1:10 would be 33 times the amount unbound. Second, the speed with which T_4 equilibrates between plasma and Sephadex in the equilibrium technique described in this thesis suggests it likely that T_4 would be stripped off proteins other than albumin during passage through Sephadex columns. Third, in the example shown in Figure 1 of Elewaut's publication (Figure 5.7), the binding curve obtained from the estimated binding parameters fitted the data points very badly, suggesting that either the model fitted, or the method of parameter estimation was incorrect. Fourth,

in the same publication it was suggested that binding of T_4 to TBPA as well as to TBG, is destroyed by heating plasma to 60° and this is at variance with data presented in Chapter 4 and other published observations (Takemura et al., 1971).

To test the adequacy of the model fitted by Elewaut, i.e. 2 specific binding proteins ($S_1 + S_2$) supposedly corresponding to TBG and TBPA, and to evaluate the validity of the graphical method of parameter fitting used, the data was reanalysed using the computer least squares routine of non-linear parameter estimation described in Chapter 3.

When an ($S_1 + S_2$) model was used with the uncorrected bound and unbound T_4 values plotted in Figure 1 of Elewaut's paper, an almost perfect fit was obtained which is in marked contrast with that obtained by Elewaut (models 1 and 2, Figure 5.7). The TBPA binding parameters were quite similar to those estimated by Elewaut, but those for TBG were very different. These estimates, together with those of Elewaut and for fits described below, are shown in Table 5.7. When a model which also allowed for albumin binding ($S_1 + S_2 + NS$) was fitted to the same data, an unreal solution was obtained (a negative value for K_n indicating either a negative association constant or binding capacity for albumin) even though the fit to the actual data points was improved (model 3, Figure 5.7).

Since the use of the erroneously high unbound T_4 values weights the data points incorrectly, the two

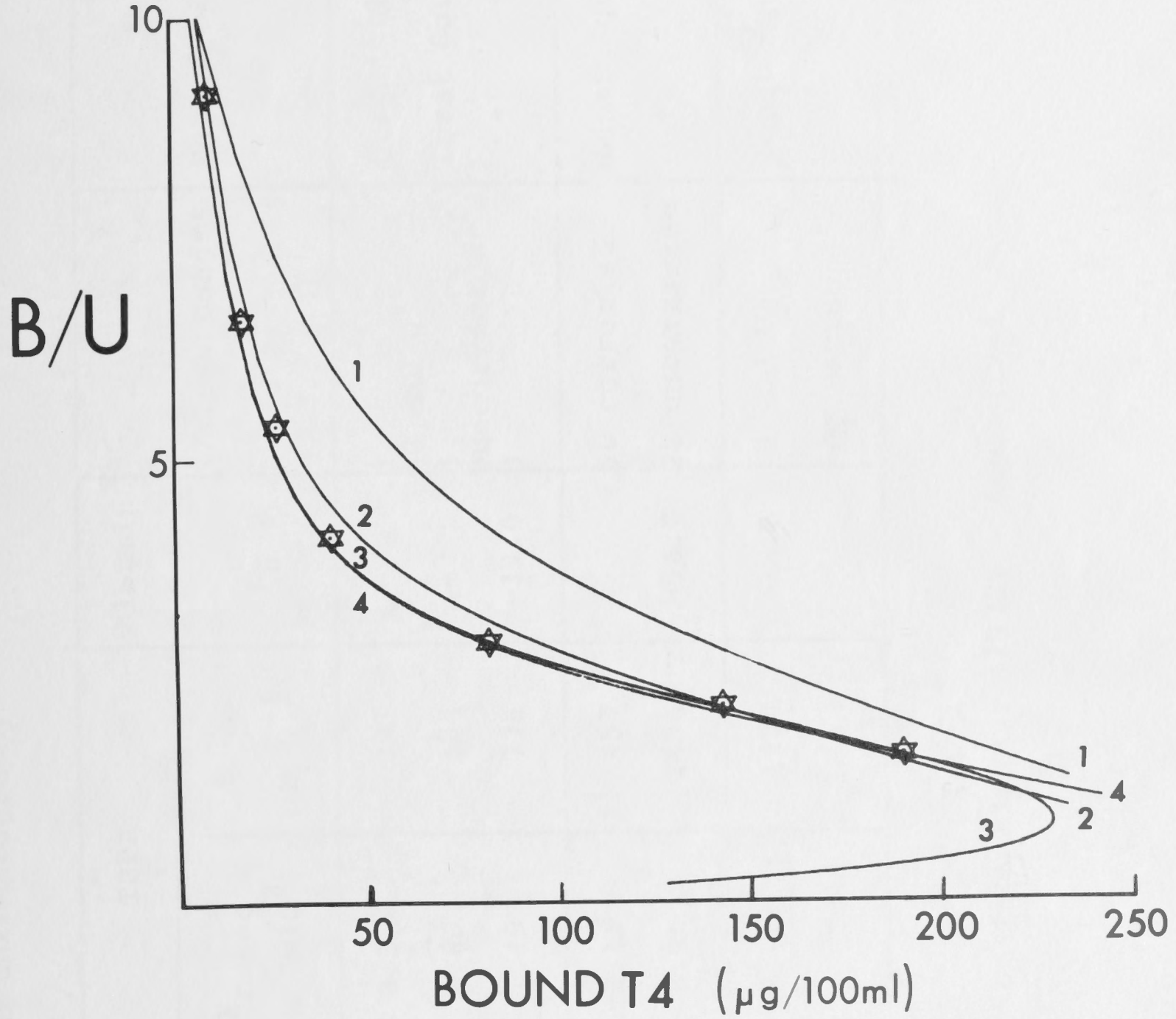


Figure 5.7. A Scatchard plot of T_4 binding to human serum as assessed by the Sephadex column chromatographic procedure of Elewaut (1973). The data points were taken from Elewaut (1973) - see Appendix 1. The four models fitted to the data are described in Table 5.7.

Table 5.7. Estimates of the thyroxine-binding properties of human serum proteins using binding data obtained from Sephadex G25 column chromatography.

Model	No.	TBG		TBPA		Albumin	Values Used**	Method of Fit
		K_{assoc} ($M^{-1}x10^{9}$)	B.C.* ($Mx10^{-8}$)	K_{assoc} ($M^{-1}x10^{8}$)	B.C. ($Mx10^{-8}$)	K_n		
S_1+S_2	1	7.5	35.78	3.3	391	-	) B and U	Graphical
S_1+S_2	2	16.2	21.6	2.86	394	-	) uncorrected	Least Squares
S_1+S_2+NS	3	15.7	22.1	1.49	776	-19.0	)	" "
S_1+S_2	4	16.7	21.0	2.18	457		) U corrected	Least Squares
S_1+S_2+NS	5	14.7	23.6	0.90	1630	-54.8	) B uncorrected	" "
S_1+S_2+NS	6	14.6	23.6	0.90	1450	-14.6	B and U corrected	Least Squares

* B.C. - binding capacity

** Uncorrected B and U values were taken from Elewaut (1973) - Appendix 1.

models fitted above were fitted again using unbound T_4 values corrected as suggested by Elewaut. Fitting the model (No. 4, Table 5.7) for 2 binding proteins (S_1 + S_2) resulted in appreciably different parameters for TBPA, but those for TBG were very similar. The model (No. 5, Table 5.7) which also allows for albumin binding (S_1 + S_2 + NS) still could not be fitted.

If the excess T_4 taken up by the Sephadex (excess over the true unbound) is T_4 originally bound to albumin, then correcting the bound T_4 concentrations by adding to them the amount of T_4 which was subtracted from the unbound concentrations should reproduce the distribution of T_4 present in the diluted plasma before chromatography. It should be possible therefore to fit the model allowing for albumin binding (S_1 + S_2 + NS) to such corrected bound and unbound values. However, it can be seen from model 6, Table 5.7 that such a fit was still not possible.

Sephadex chromatography carried out under the conditions employed by Elewaut disturbs the equilibrium between T_4 and its binding proteins. Not only is T_4 removed from albumin by the Sephadex, as claimed by Elewaut, but almost certainly from other binding proteins. The proportions removed from the individual species would depend on the distribution of T_4 between the various species of binding proteins. Since this would vary with the amount of added T_4, no simple correction, such as that suggested by Elewaut, can be applied to data so obtained. Similarly, if T_4 dissociates from proteins

during chromatography, no simple correction for dilution can be applied to the calculated binding parameters. This is because the plasma is progressively diluted during chromatography. It should also be pointed out that the actual dilution of plasma applied to the Sephadex columns by Elewaut was 1/14 not 1/10. This was brought about by the addition of 200 µl of various T_4 solutions to 500 µl of the plasma diluted 1/10.

Thus, the analyses presented in Table 5.7 and Figure 5.7 indicate that the data obtained by the procedure of Elewaut is not suitable for kinetic analysis. Without prior knowledge of the binding parameters which are to be measured, this would only be possible if the procedure is carried out at such a low temperature that dissociation of protein-bound T_4 during chromatography is negligible. However, association constants calculated from such data would have little relevance to the situation in vivo.

The deficiencies of graphical methods for estimating binding parameters are clearly shown. Unless the binding parameters so calculated are progressively altered by trial and error to obtain a good fit to the data, as suggested by Berson and Yalow (1959), they are of little value. A simpler and more accurate procedure is to use the least squares method presented in this study.

CHAPTER 6

THE THYROID HORMONE BINDING PROPERTIES OF HUMAN AND OVINE ALBUMIN

INTRODUCTION

The competitive binding technique described in this thesis has supplied the first estimates of the T_4 binding properties of human and ovine albumin in samples of dilute plasma. It was therefore important to compare such estimates with those derived from studies using purified human and sheep serum albumin.

The ready availability of human and bovine serum albumin as Cohn fraction V made albumin, until recently, the most extensively studied pure T_4BP. Most workers used human Cohn fraction V and there was general agreement that it was not contaminated, to any large extent, with other T_4BPs (Sterling, 1964; Steiner et al., 1966). While results in this laboratory confirm that observation, we have found that sheep, rat and rabbit Cohn fraction V preparations are contaminated with T_4 binding moieties other than albumin (Sutherland and Brandon, unpublished observations). For this reason sheep fraction V was purified on DEAE Sephadex prior to experimentation.

With the availability of pure human and ovine serum albumin (OSA) experiments were conducted to assess the T_4 binding properties of these proteins under varying conditions of ionic strength, ionic composition, pH and temperature.

Experimental procedure

The assays were performed as described in Chapter 3 with the following modifications. The relationship between albumin-bound T_4 and unbound T_4 was established by making serial additions of T_4 (0 - 500 ng) to albumin diluted 1 mg/ml in an appropriate buffer at 37°. Data was plotted as albumin-bound T_4, [B], against unbound T_4 concentration, [U], and the relationship was linear over the concentration range studied ($4.4 \times 10^{-6} - 7.1 \times 10^{-4}$ M T_4 bound per mole of albumin). It should be pointed out however, that the relationship between [B] and [U] maintains linearity over a much greater concentration range than this. If normal human plasma has a mean total T_4 concentration of 8.0 µg/100 ml and 7% of this is bound to albumin present at 4 g/100 ml, then human albumin in normal whole plasma would bind T_4 at the rate of approximately 1.2×10^{-5} M T_4 per mole of albumin. Therefore over a range of albumin bound T_4 concentrations likely to be encountered *in vivo* the relationship between [B] and [U] is linear and can be expressed by the following equation:

$$[B]_{alb} = C_{alb}\,[U]/K_{alb}$$ See Equation (12) Chapter 3

This formulation assumes 1 strong binding site per albumin molecule. It is generally agreed however, that albumin has more than one type of T_4 binding site (Tritsch *et al.*, 1961; Tabachnick, 1964a) and if this is the case equation (12) becomes

$$[B]_{alb} = \Sigma\, n_i k_i\, [C_{alb}]\, [U]$$

where n_i is the number of each type of binding site/molecule

and k_i is the association constant of the i th site. For this to be dimensionally correct [B], [U] and [C_{alb}] must be expressed in molar concentrations and k_i in reciprocal molar concentrations. Since [C_{alb}] is a constant, changes in $\Sigma\ n_i k_i$ will reflect changes in the ability of albumin to bind hormone. Because of the limited concentration range of added T_4 used in the present study it was not possible to detect whether changes in $\Sigma\ n_i k_i$ were due to changes in n_i or to changes in k_i.

Estimates of $\Sigma\ n_i k_i$ were made under various experimental conditions by calculating the linear regression of [B] on [U], using least squares methods, under those conditions. The regression coefficient was equal to $\Sigma\ n_i k_i$ [C_{alb}].

RESULTS

Effect of ionic strength and ionic composition on the T_4 binding properties of serum albumin

Increasing the buffer concentration from 0.05 M to 0.20 M in both phosphate at pH 7.4 and barbital at pH 8.6 resulted in a reduction in the affinity of albumin for T_4, as assessed by changes in $\Sigma\ n_i k_i$ (Table 6.1). The rate of decline in binding affinity was greatest between 0.05 M and 0.10 M, and changed little between 0.15 M and 0.20 M. A similar reduction in binding affinity was observed when increasing concentrations of NaCl (0.05 - 0.15 M) were added to 0.05 M phosphate buffer, pH 7.4. When 0.05 M NaCl,

0.05 M sodium phosphate buffer, pH 7.4 was used the binding affinity was 4.76×10^5 M^{-1} which is significantly ($P < 0.01$) lower than the value of 5.45×10^5 M^{-1} found using 0.10 M phosphate pH 7.4 (Figure 6.1). Similarly 0.05 M phosphate, pH 7.4, containing 0.10 M (3.90×10^5 M^{-1}) and 0.15 M (3.28×10^5 M^{-1}) NaCl resulted in significantly ($P < 0.01$) lower binding affinities than when 0.15 M (5.02×10^5 M^{-1}) and 0.20 M (4.96×10^5 M^{-1}) phosphate buffer, pH 7.4, was used. These results indicate that the observed reduction in binding affinity with increased concentration of buffer was not due entirely to changes in ionic strength but was also dependent on the ionic species present (Figure 6.2). This is further exemplified in the case of barbital buffer. Assays performed in barbital buffer, pH 8.6, resulted in a diminished binding affinity when compared with phosphate and phosphate-NaCl buffers at pH 7.4 (Table 6.1 and Figure 6.2). Since a change in pH from 7.4 to 8.6 causes an increase in the binding affinity in phosphate buffer (Table 6.2 and Figure 6.3) the reduced binding observed with barbital at pH 8.6 would seem to be a direct effect of the barbiturate ions. Human and ovine serum albumin showed essentially the same responses to changes in buffer ionic strength and ionic composition.

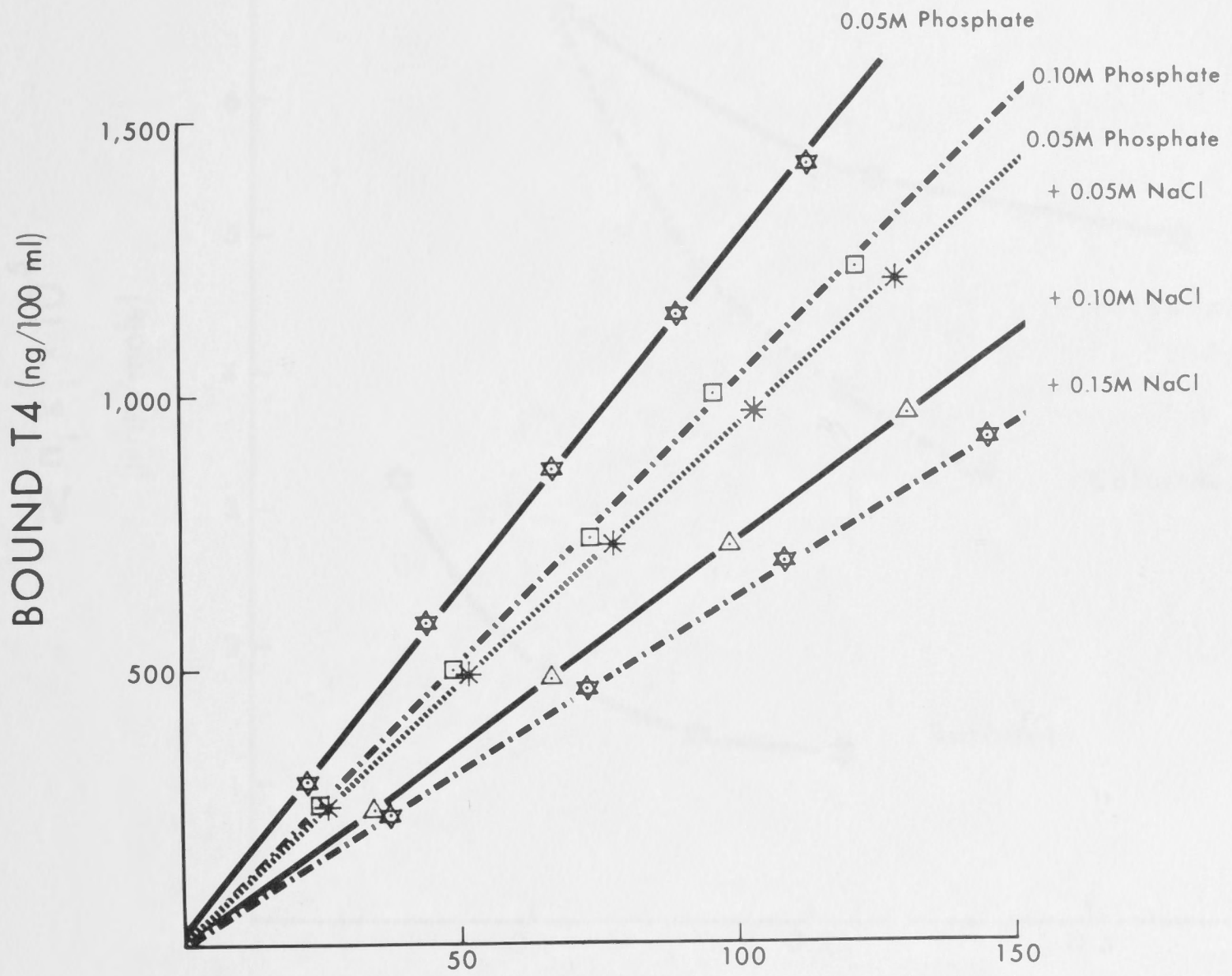


Figure 6.1. The effect of increasing the concentration of sodium phosphate and sodium chloride on the T_4 binding properties of HSA. The buffer systems used are described in the text.

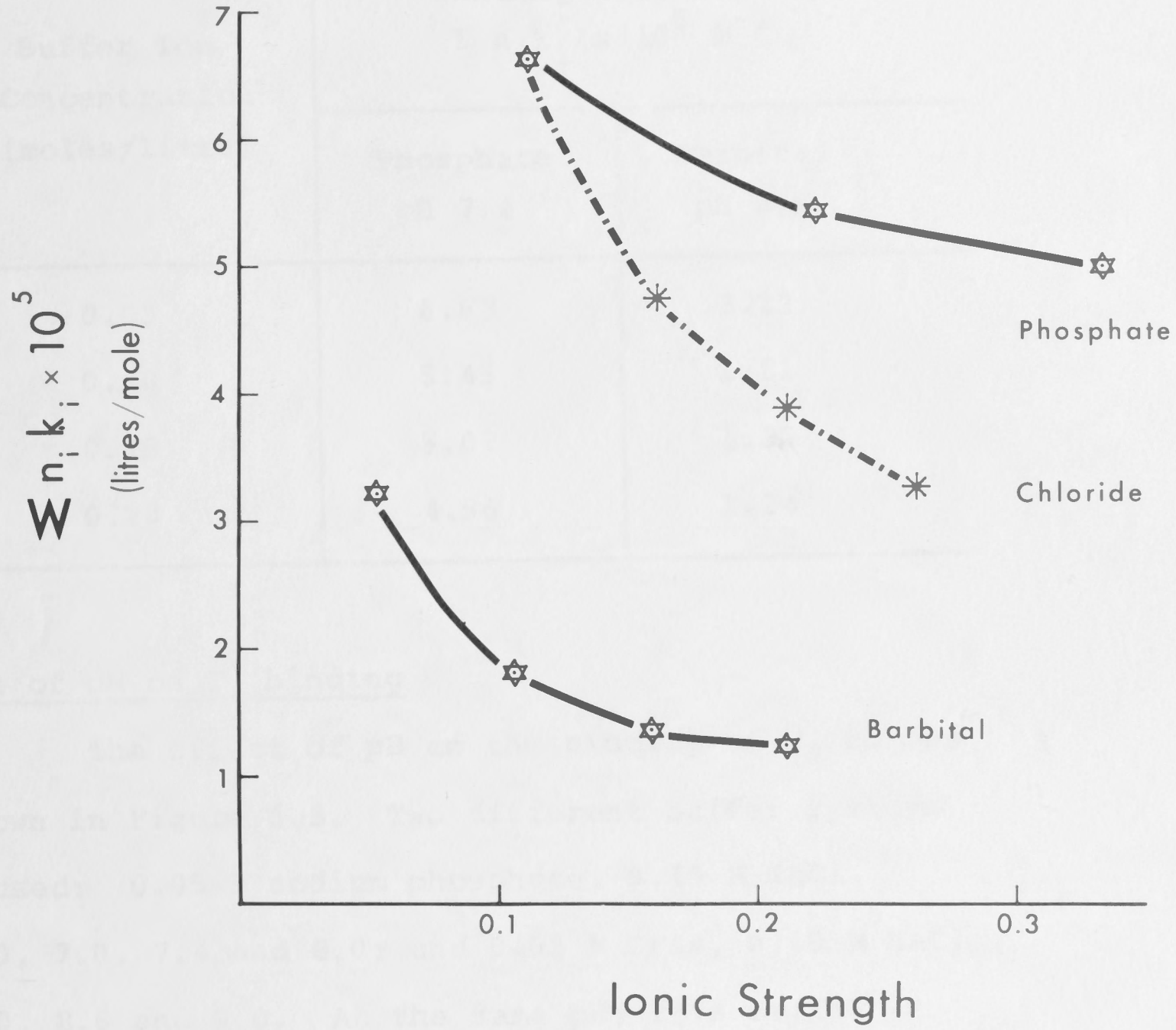


Figure 6.2. The effect of ionic strength and ionic composition of the assay buffer on the binding affinity of HSA for T_4. The composition of the buffer systems is described in the text.

Table 6.1. Effect of buffer concentration on the binding of T_4 to HSA.

Buffer Ion Concentration (moles/litre)	Binding Affinity $\Sigma n_i k_i \times 10^5$ M^{-1}	
	Phosphate pH 7.4	Barbital pH 8.6
0.05	6.63	3.22
0.10	5.45	1.81
0.15	5.02	1.35
0.20	4.96	1.24

Effect of pH on T_4 binding

The effect of pH on the binding of T_4 to HSA is shown in Figure 6.3. Two different buffer systems were used: 0.05 M sodium phosphate, 0.10 M NaCl, pH 6.0, 7.0, 7.4 and 8.0; and 0.05 M Tris, 0.10 M NaCl, pH 8.0, 8.6 and 9.0. At the same pH, Tris buffer resulted in a slightly greater $\Sigma n_i k_i$ value than the corresponding phosphate buffer. This may be due to the lower ionic strength of the Tris-NaCl buffer. Over the pH range studied the affinity of albumin for T_4 increased seven fold from 1.43×10^5 M^{-1} at pH 6.0 to 1.01×10^6 M^{-1} at pH 9.0. Human and sheep albumin behaved similarly (Table 6.2).

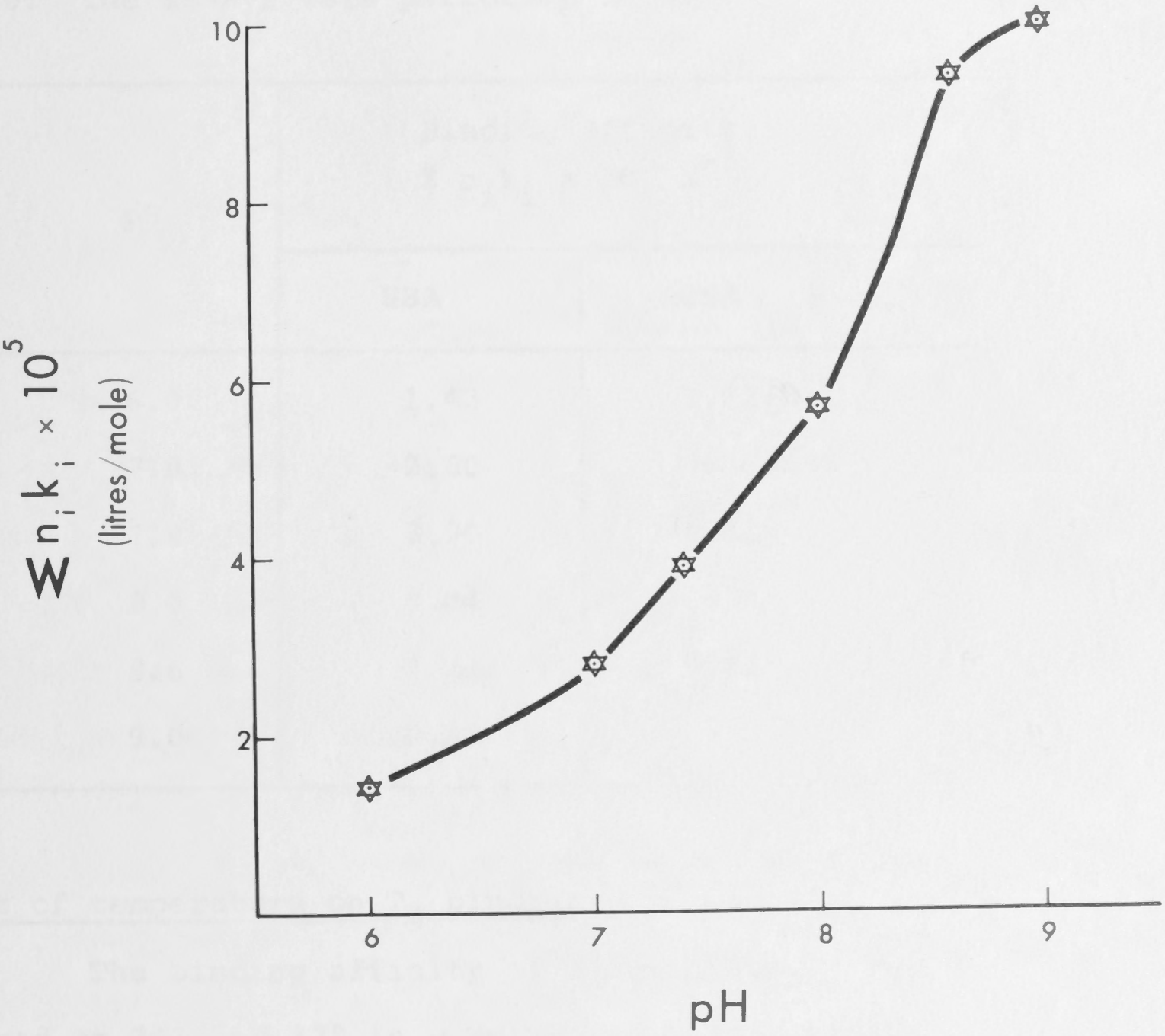


Figure 6.3. The effect of pH on the T_4 binding properties of HSA.

Table 6.2. Effect of pH on the binding of T_4 by HSA and OSA. 0.05 M sodium phosphate buffer in 0.10 M NaCl was used at pH 6.0, 7.0, 7.4 and 8.0 while 0.05 M Tris in 0.10 M NaCl was employed at pH 8.0, 8.6 and 9.0. The assays were performed at 37°.

pH	Binding Affinity $\Sigma n_i k_i \times 10^5$ M^{-1}	
	HSA	OSA
6.0	1.43	1.62
7.0	2.80	-
7.4	3.90	4.11
8.0	5.68	-
8.6	9.41	9.66
9.0	10.08	-

Effect of temperature on T_4 binding

The binding affinity of albumin for T_4 was measured at 25° and 37° in a buffer of 0.05 M sodium phosphate, 0.10 M NaCl, pH 7.4. At these two temperatures a significant difference in the degree of protein binding was observed ($P < 0.01$). A decrease of 12° in temperature resulted in an increase in $\Sigma n_i k_i$ from 3.90×10^5 M^{-1} at 37° to a value of 5.45×10^5 M^{-1} at 25°.

Binding of T_4 and T_3 to serum albumin under "physiological conditions"

The routine assay system used for the study of

the thyroid hormone binding properties of plasma proteins employs a 0.05 M sodium phosphate, 0.10 M NaCl buffer, pH 7.4 at 37°. When T_4 binding to 1 mg/ml solutions of HSA and OSA was studied under such conditions the $\Sigma n_i k_i$ values were 3.90×10^5 and 4.11×10^5 M^{-1} respectively. Triiodothyronine was bound with lower affinity than T_4. These $\Sigma n_i k_i$ values were 1.15×10^5 and 1.26×10^5 M^{-1} for human and sheep albumin respectively.

DISCUSSION

The ready availability of serum albumin in relatively pure form, its good stability, the availability of considerable information on its binding affinity for a wide range of compounds, as well as the relative scarcity of other purified T_4BPs probably explains the wide use of albumin as a model for the study of thyroid hormone-plasma protein interactions. Most studies have investigated the nature of T_4 and T_3 binding sites. The number of sites, their amino acid composition, their affinity for T_4 and its analogues, have been the main parameters studied.

Until the development of the present competitive binding assay there were no techniques available which allowed estimation of the thyroid hormone binding properties of albumin in samples of whole plasma. With the advent of such a technique it was important to compare estimates of albumin binding in whole plasma with those derived from experiments using purified serum

albumin. Preliminary results which were reported in Chapter 4, indicated that a number of factors: ionic strength, ionic composition, pH and temperature, had a profound effect on estimates of hormone binding parameters derived from *in vitro* assay systems. The effect of these factors on estimates of T_4 binding to serum albumin was studied, firstly, to determine whether the observed changes in albumin binding seen in the whole plasma assay system were accompanied by similar changes in the binding properties of purified albumin, and secondly, to allow comparison of albumin binding parameters calculated using the present technique with those derived under different experimental conditions.

The effect of changes in ionic strength on the binding activity of serum albumin has received little attention. Tritsch *et al*. (1961) noted that the ability of HSA to bind T_4 was influenced by the ionic strength below values of 0.3 in phosphate buffer at pH 7.35. Increases in ionic strength above 0.3, however, had no significant effect on the binding activity. In contrast Tabachnick (1967) reported that there was no difference in T_4 binding to human albumin when either, a 0.03 M potassium phosphate buffer, pH 7.4., ionic strength 0.064, or a similar phosphate buffer of ionic strength 0.175 was used. Data presented in this chapter clearly demonstate that increases in ionic strength reduce T_4 binding to human and sheep serum albumin in three buffer systems at two pHs. Changes were minimal with increases in ionic strength above 0.2 except in the

case of phosphate-NaCl buffer. A similar phenomenon has been described for the binding of estrone to HSA. Increasing the concentration of phosphate buffer from 0.05 M to 0.10 M resulted in a slight decline in the amount of estrone that was protein-bound (Sandberg, Slaunwhite and Antoniades, 1957).

Inhibition of binding by NaCl is of particular interest in view of its high plasma concentration and its normal omission from buffers used in binding studies. Tabachnick (1967) found a decline in T_4 binding to HSA of 41% and 47% when 0.05 M and 0.10 M NaCl was added to 0.03 M potassium phosphate buffer, pH 7.4. The present experiments demonstrate a similar decline. Thyroxine binding was reduced by 28%, 41% and 51% with addition of 0.05, 0.10 and 0.15 M NaCl respectively to a 0.05 M sodium phosphate buffer at pH 7.4. This decline was considerably greater than that observed with equimolar increases in the concentration of sodium phosphate buffer, despite the greater ionic strength of the latter buffer.

The results obtained with barbital buffer, pH 8.6 are interesting because veronal ions were previously thought to be specific inhibitors of T_4 binding to TBPA (Tata et al., 1961; Ingbar, 1963). Results presented in Table 6.1 show that barbital also inhibits T_4 binding to serum albumin. It now appears likely that barbital buffers inhibit the binding of T_4 to all plasma T_4 binding sites since results presented in Chapter 4 demonstrated inhibition of T_4 binding to TBG

in barbital buffer. Whilst it is only recently that the effects of ionic strength and ionic composition have been discussed with respect to thyroid hormone binding they have long been known to influence the binding of many small anions to HSA. As early as 1953 Klotz stated that phosphate buffers cause minimal interference to anion binding by HSA while veronal buffers produce a significant decrease in binding affinity.

Data presented here show that pH has a profound effect on the affinity of albumin for T_4. The marked increase in binding affinity as pH is increased from 6 - 8.6 is in general agreement with the results of a number of other workers (Tritsch et al., 1961; Tritsch and Tritsch, 1963; Steiner et al., 1966). In view of this agreement, using a number of different techniques, it is difficult to understand why Sterling et al. (1962) and Tabachnick (1964a) were unable to detect changes in the affinity of T_4 binding to HSA between pH 6.8 and 9.8. The pH dependence of thyroid hormone binding to serum albumin has been attributed to changes in the degree of ionization of ionizable groups on both the hormone and the protein (Tritsch and Tritsch, 1963; Steiner et al., 1966). Such changes may be accompanied by conformational changes in albumin which expose new or auxillary binding sites (Klotz, 1953).

Thyroxine binding to serum albumin is known to be temperature dependent although previously reported

results do not show the magnitude of change reported here. Sterling et al. (1962) found no difference in the binding affinity at 30° and 38°, however, binding was stronger at 5°. Similarly, Tabachnick (1967) demonstrated no difference in $\Sigma\ n_i k_i$ between 30° and 37° although this value was increased by 25% with a decline in assay temperature to 6°.

Previously reported estimates of the affinity of HSA for T_4, expressed as $\Sigma\ n_i k_i$ values, are summarized in Table 6.3. The present estimate of $3.90 \times 10^5\ M^{-1}$ for $\Sigma\ n_i k_i$ in 0.05 M phosphate, 0.10 M NaCl, pH 7.4 at 37° is in close agreement with the early estimates of Sterling and Tabachnick (1961b) and Sterling et al. (1962) but is somewhat lower than more recent estimates of $\Sigma\ n_i k_i$ (Tabachnick, 1964a, b, 1967; Tabachnick and Giorgio, 1964; Steiner et al., 1966). These differences can be largely explained by the effects of NaCl concentration and temperature on T_4 binding to albumin currently reported. Thyroxine binding by OSA differed little from the binding activity of HSA. Although the binding of thyroid hormones by OSA has not been reported previously, Tritsch et al. (1961) and Steiner et al. (1966) have shown that HSA and bovine serum albumin have similar T_4 binding properties.

Triiodothyronine was bound with 1/3rd of the affinity of T_4 by both human and ovine serum albumin. Such a binding affinity is greater than

Table 6.3. Reported estimates of $\Sigma n_i k_i$ for the binding of T_4 to HSA.

$\Sigma n_i k_i$ (M^{-1})	Experimental Conditions	Reference
2.8×10^6	0.03 M Phosphate buffer, pH 7.35, 25°	Tritsch *et al.*, 1961
4.4×10^5	Phosphate buffer, ionic strength 0.15, pH 7.4, 38°	Sterling and Tabachnick, 1961b
4.2×10^5	Phosphate buffer, ionic strength 0.15; 0.05 M Tris-0.10 M NaCl; pH 7.4, 38°	Sterling *et al.*, 1962
7.0×10^5	Phosphate buffer, ionic strength 0.15, pH 7.4, 38°	Tabachnick, 1964a
7.0×10^5	0.06 M Phosphate buffer, pH 7.4, 30°	Tabachnick and Giorgio, 1964
7.5×10^5	0.06 M Phosphate buffer, pH 7.4, 30° Fatty acids extracted	Tabachnick, 1964b
1.6×10^6	0.10 M Phosphate buffer, pH 7.4, 24°	Steiner *et al.*, 1966
1.6×10^6	0.06 M Phosphate buffer, pH 7.4, 30°	Tabachnick, 1967

previously reported values which indicated that T_3 was bound to HSA with 1/4 to 1/6th the affinity of T_4 (Tabachnick, 1964a; Steiner et al., 1966) but this might be due to the use of different buffers. This decreased affinity for T_3 has been attributed to the decreased ionization of the phenolic hydroxyl group of this hormone (Sterling and Tabachnick, 1961b; Steiner et al., 1966).

The $\Sigma n_i k_i$ values, 3.90 and 4.11 x 10^5 M^{-1}, for the binding of T_4 to purified human and ovine serum albumin respectively, were greater than the mean K_n values of 3.06 and 3.52 x 10^5 M^{-1}, found for the corresponding binding affinities in whole plasma assayed under identical conditions. However, in both species estimates of K_n varied considerably between individuals and the range of values included the corresponding $\Sigma n_i k_i$ values estimated with purified albumin. Thus the assumption that non-specific binding measured in samples of plasma was due to albumin would seem to be valid.

CHAPTER 7

THE TRANSCAPILLARY EXCHANGE OF THYROID HORMONES AND THYROXINE-BINDING PROTEINS BETWEEN BLOOD AND TISSUE FLUIDS

INTRODUCTION

The first barrier that the thyroid hormones must surmount on leaving the blood to enter the tissue fluids, and hence the tissue cells, is the endothelial lining of the blood capillaries. Data presented in Chapter 5 and that of many other workers (Robbins and Rall, 1967) demonstrate that the majority of plasma T_4 is tightly but reversibly bound to protein, while only a minute proportion, 0.06% in sheep, circulates in the unbound state. Clearly, protein-binding must have a profound effect on the passage of hormone across the endothelium, however, few experiments have been conducted to elucidate this phenomenon.

It has long been thought that unbound T_4 is the freely diffusible, metabolically active component of plasma T_4 (Ingbar and Freinkel, 1960; Ingbar and Woebar, 1968). This interpretation has been based on a number of clinical and experimental observations. Clinical thyroid status is known to be more highly correlated with unbound plasma T_4 concentration than with total plasma T_4 concentration (Ingbar and Woebar, 1968). Recent experimental evidence indicates that unbound T_4 is the form in which T_4 is captured by tissue cells (Hillier, 1968a, b, 1969, 1971). Inherent in this interpretation is the concept that unbound T_4 is freely diffusible across the capillary endothelium while the diffusion of protein-bound T_4 is severely restricted due to the molecular size of its binding proteins. In

contradiction to this "free thyroxine theory", Oppenheimer, Surks and Schwartz (1969) have postulated that the escape of T_4 from the blood vascular system is due entirely to the diffusion of T_4-protein complexes and that the uptake of T_4 by tissues results from direct interaction between protein-bound T_4 and tissue binding sites. This has been called "the collision theory".

Earlier work in this laboratory has provided evidence that unbound T_4 is removed from the circulation at a greater fractional rate than protein-bound T_4. Such an interpretation was based on the observation that radioactively labelled T_4 appeared more rapidly in the lymph of chronically cannulated sheep than did labelled albumin when both isotopes were injected simultaneously into the blood circulation (Simpson-Morgan and Irvine, 1972; Irvine and Simpson-Morgan, 1974).

The aim of the work described in this chapter was to extend that initiated by Irvine and Simpson-Morgan (1974) by sampling lymphatics afferent to a lymph node, and by sampling lymph from a large number of different drainage areas throughout the body of sheep. Two main approaches were adopted. Firstly, the rate of equilibration of radioactively labelled T_4 and albumin was measured in a number of different tissue fluid pools following the simultaneous injection of both labelled compounds into the circulation, and secondly, the steady state concentrations of T_4 and the T_4BPs were measured in plasma and in lymph draining many different regions of the body. In addition, distribution experiments were performed in

animals in which the proportion of unbound thyroid hormone was higher than is normal for T_4. This was achieved either by administering large doses of T_4 or by using tracer doses of labelled T_3. The removal of T_4 from lymph during its passage through a lymph node was also studied.

RESULTS

The rate of movement of tracer T_4 and serum albumin from blood to lymph

Chronic lymphatic fistulae were established in lymphatics efferent to the popliteal, prefemoral and prescapular lymph nodes, the lumbar lymph trunk, the intestinal lymph trunk and the afferent testicular lymphatics. Following simultaneous intravenous injection of T_4 [^{125}I] and HSA [^{131}I] samples of plasma and lymph were collected at appropriate time intervals. Since Irvine and Simpson-Morgan (1974) were unable to detect labelled compounds other than T_4 and iodide in any of the body fluids studied in similar experiments, the TCA precipitated ^{125}I and ^{131}I activities were taken to represent T_4 and HSA respectively. In all animals studied labelled T_4 was removed from the plasma and appeared in the lymph at a greater fractional rate than did labelled albumin. Representative experiments illustrating the equilibration of labelled T_4 and albumin between plasma and lymph in four different tissue fluid pools are shown in Figures 7.1 - 7.4. The rate of transfer of labelled

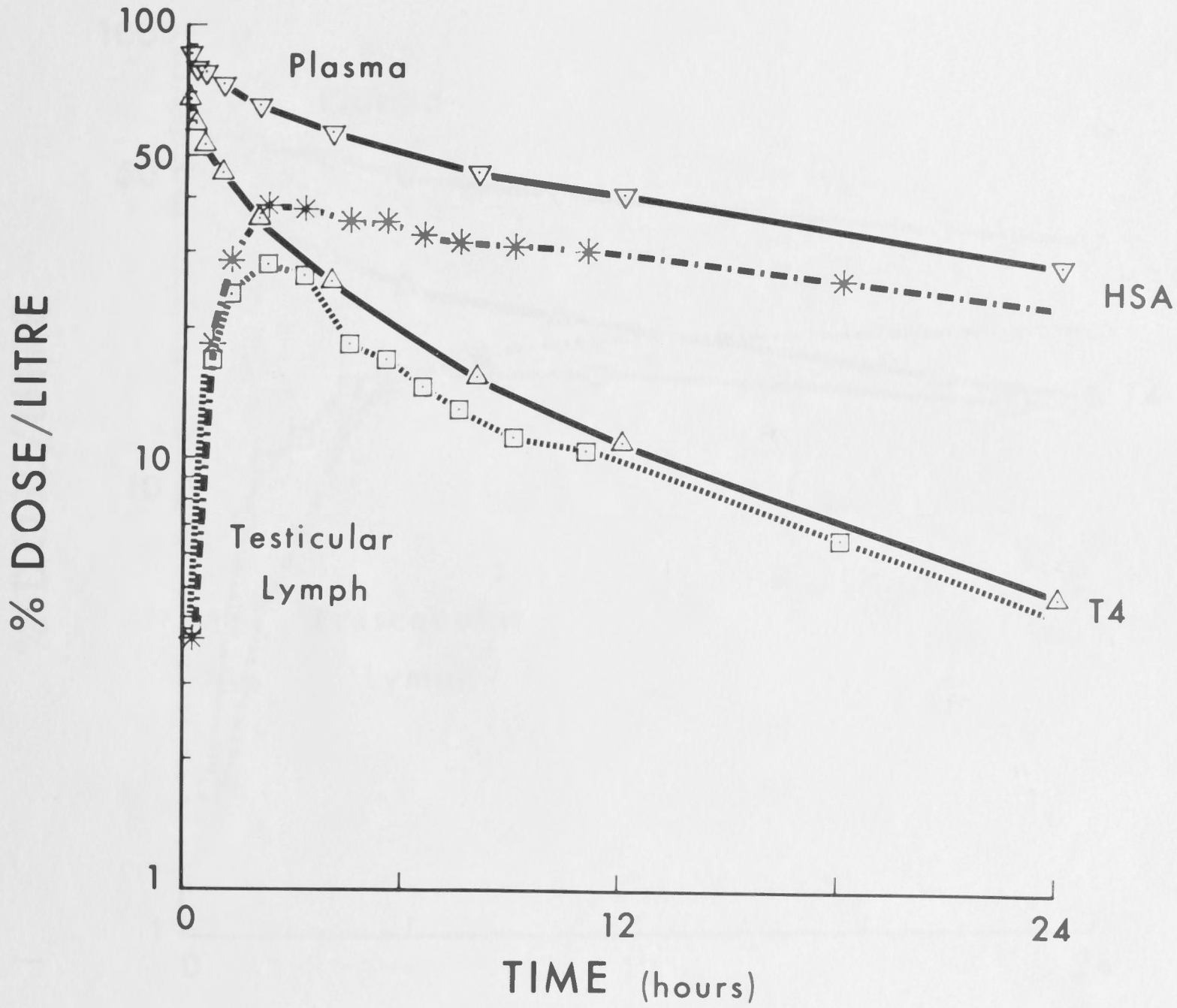


Figure 7.1. The disappearance of T_4 [^{125}I] and HSA [^{131}I] from plasma and their appearance in afferent testicular lymph following simultaneous intravenous injection of the two isotopes.

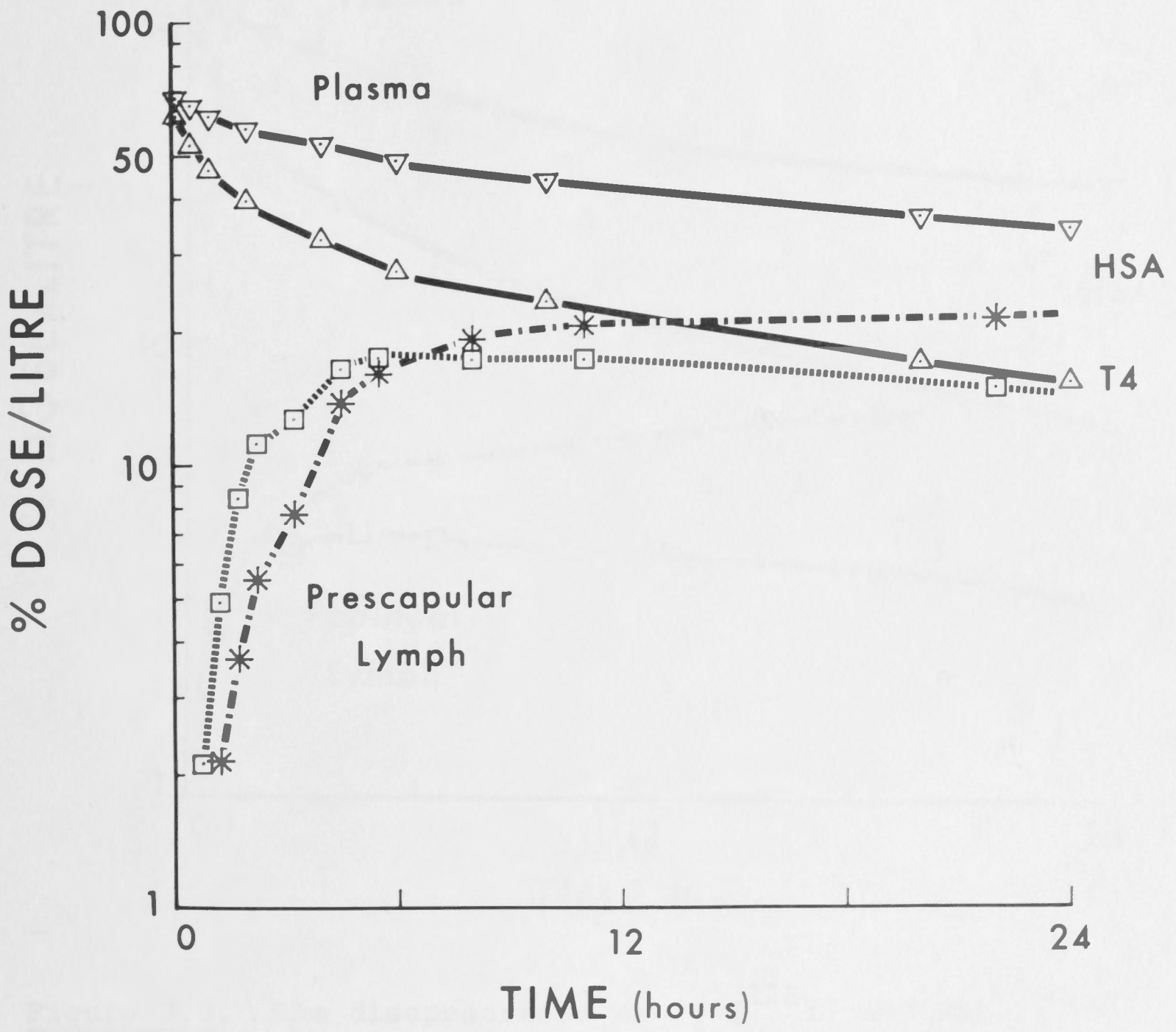


Figure 7.2. The disappearance of T_4 [^{125}I] and HSA [^{131}I] from plasma and their appearance in efferent prescapular lymph following simultaneous intravenous injection of the two isotopes.

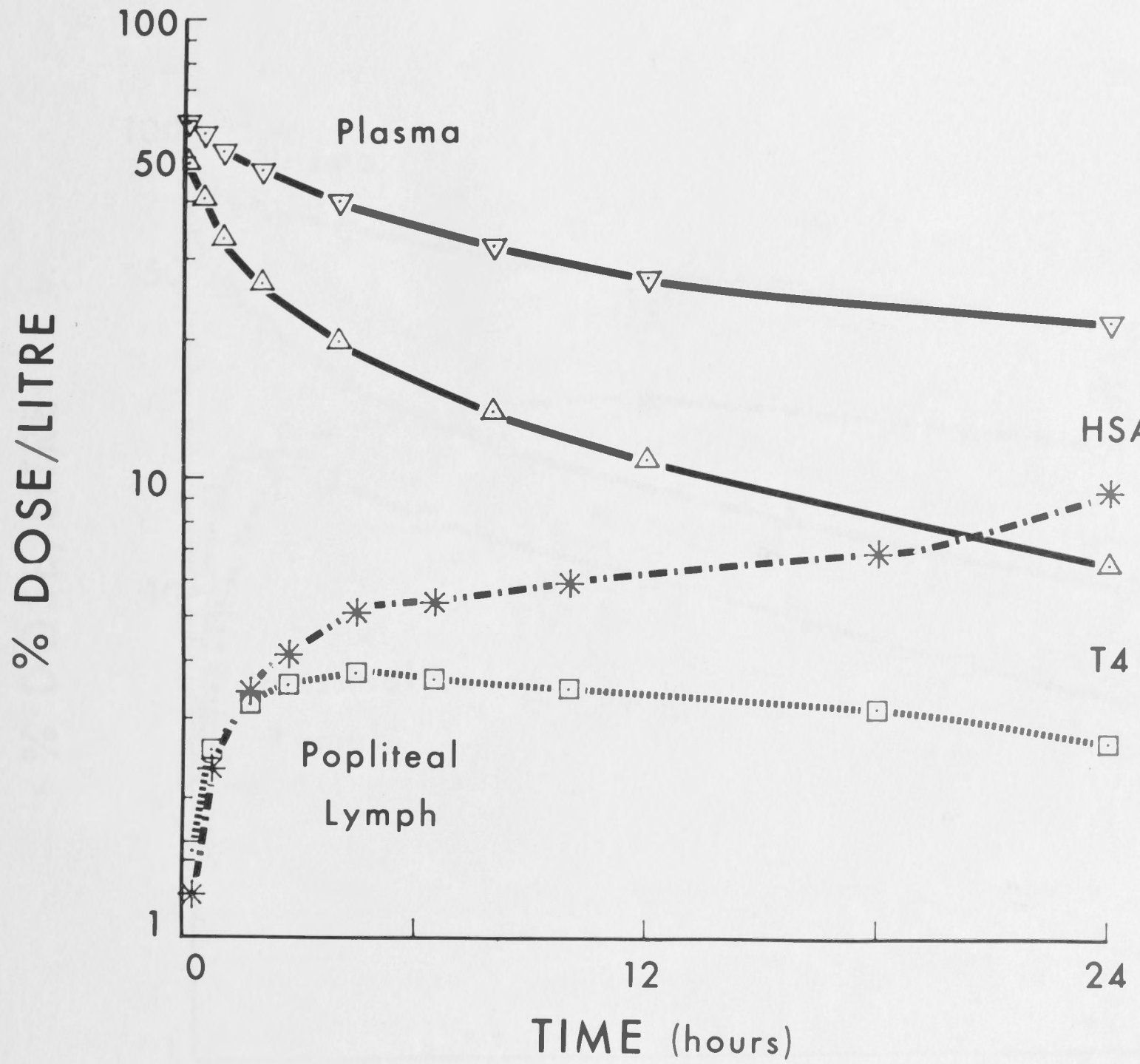


Figure 7.3. The disappearance of T_4 [^{125}I] and HSA [^{131}I] from plasma and their appearance in efferent popliteal lymph following simultaneous intravenous injection of the two isotopes.

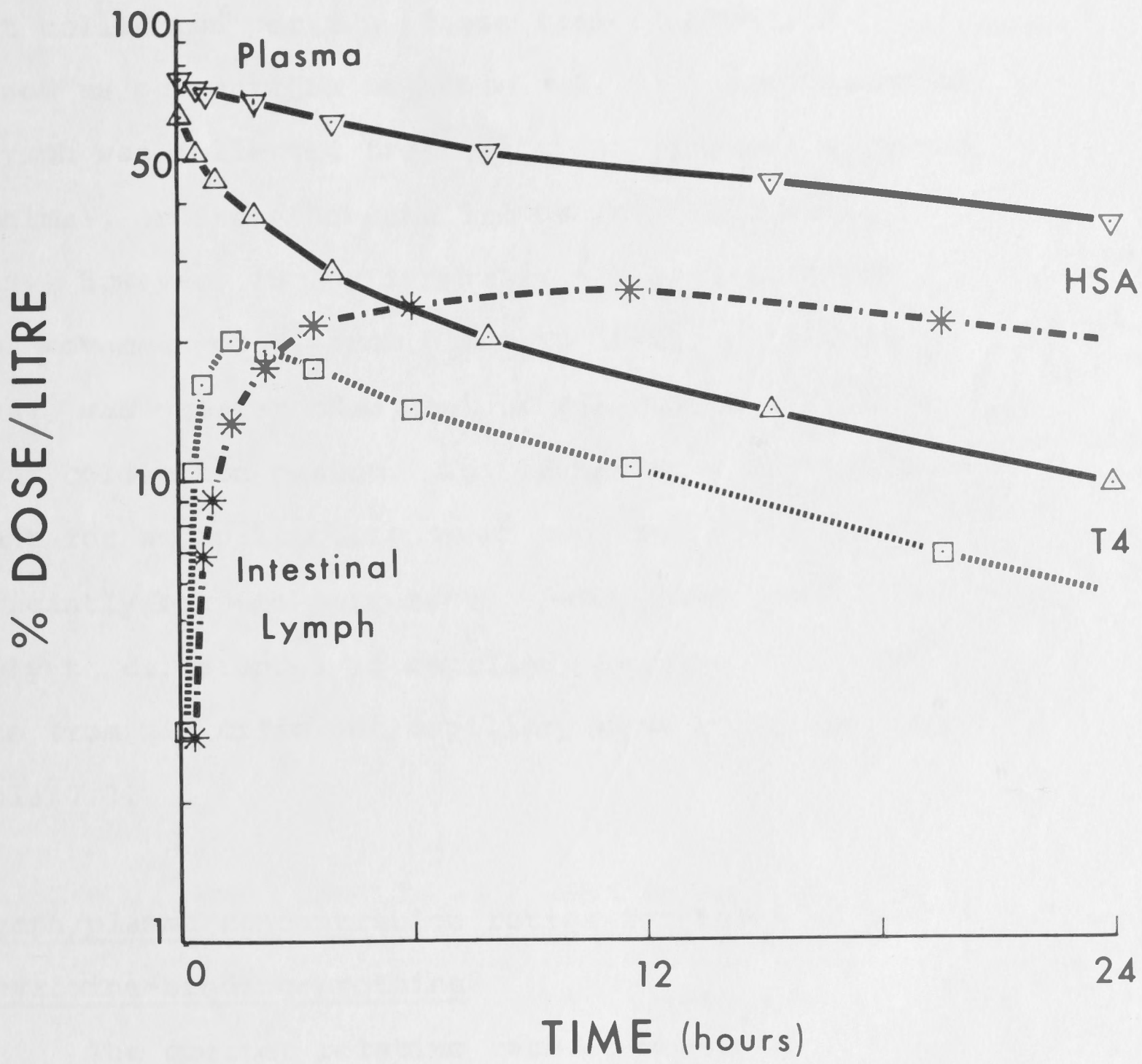


Figure 7.4. The disappearance of T_4 [^{125}I] and HSA [^{131}I] from plasma and their appearance in intestinal lymph following simultaneous intravenous injection of the two isotopes.

T_4 and HSA from blood to lymph during a given collection period were estimated from the ratios of the activities in the lymph to the activities in plasma at the mid-point of that collection period. These ratios which are expressed as percentages in Table 7.1, varied considerably when lymph was collected from different lymphatics in the same animal, or from the same lymphatic in different animals. However, in any lymphatic the nett relative rate of movement of T_4 from blood to lymph, estimated this way, was greater than that of HSA during the first 1/2 hour collection period. This difference was relatively constant for any particular lymph pool but differed significantly between different tissue fluid pools due possibly to differences in capillary permeability. The results from six different capillary beds are summarized in Table 7.1.

The lymph/plasma concentration ratios for total T_4 and the thyroxine-binding proteins

The greater relative rate of movement of labelled T_4 than of HSA from blood to tissue fluids could be explained either by the rapid diffusion of unbound T_4 or by the preferential transfer of T_4 bound to protein. To test the latter possibility, assays were performed to quantitate the T_4 binding properties of proteins in various samples of plasma and lymph. In all samples studied three T_4BPs were identified with association constants corresponding to TBG, TBP-2 and albumin of sheep plasma.

Table 7.1. The relative rates of movement of T_4 [^{125}I] and HSA [^{131}I] from blood to lymph during the first 1/2 hour after injection of the labelled dose.

Lymph Sample	No. of animals	Lymph/Plasma Activity Ratios (%)		Relative Rate of Transfer**
		T_4 [^{125}I]	HSA [^{131}I]	T_4/HSA
Lumbar Trunk	1	8.18	6.61	1.25
Afferent Testicular	4	7.92 ± 6.32*	6.33 ± 5.74	1.31 ± 0.13
Efferent Prescapular	1	1.38	0.416	3.32
Efferent Popliteal	4	2.73 ± 1.92	0.768 ± 0.389	3.42 ± 1.85
Intestinal Trunk	1	4.81	0.91	5.29
Efferent Prefemoral	1	8.35	1.56	5.35

* Mean ± SD

** A ratio greater than 1 indicates more rapid trans-capillary exchange of T_4 relative to HSA.

Plasma and lymph samples from 24 animals, representing 10 different tissue fluid pools were analysed for total T_4, albumin and T_4BP concentrations. The results are summarized in Table 7.2 where the lymph/plasma concentration ratios of those substances are given. In the cases of afferent testicular or efferent popliteal lymph, which were studied in a number of animals, there were no significant differences between

Table 7.2. Lymph/plasma concentration ratios for total thyroxine and thyroxine-binding proteins in samples of lymph from several tissue fluid pools.

Lymph Sample	No. of samples	Total T_4 Conc.	TBG Conc.	TBP-2 Conc.	Albumin Conc. (K_n)	Albumin Conc. (by Debro et al., 1957)
Afferent Hepatic	1	0.780	0.909	0.979	0.820	0.935
Efferent Hepatic	1	0.667	0.858	0.858	0.828	0.880
Afferent Testicular	9	0.757±0.187	0.782±0.133	0.792±0.163	0.841±0.141	0.774±0.139
Efferent Prescapular	1	0.508	0.732	0.781	0.774	0.879
Efferent Prefemoral	1	0.557	0.663	0.683	0.773	0.688
Cervical Lymph Duct	1	0.775	0.598	0.686	0.558	0.655
Lumbar Lymph Trunk	1	0.400	0.662	0.674	0.635	0.716
Afferent Renal	1	0.390	0.418	0.572	0.549	0.730
Efferent Popliteal	8	0.503±0.188	0.418±0.160	0.482±0.226	0.473±0.167	0.483±0.090
Intestinal Lymph Trunk	1	0.250	0.487	0.425	0.415	0.490

the mean lymph/plasma concentration ratios of total T_4, albumin, and the three T_4BPs. These results suggest that the lymph/plasma ratio for total T_4 is governed by the lymph/plasma ratio of its binding proteins, and that the molecular size of the two specific T_4BPs in sheep plasma differ little from that of sheep albumin. The data from the other 8 tissue fluid pools are consistent with such hypotheses. If preferential transport of the specific T_4BPs occurred, either by an active process or due to their smaller molecular size relative to albumin, one would expect a high lymph/plasma concentration ratio for the specific T_4BPs relative to albumin. Since this was not so it is likely that the more rapid movement of T_4 than of albumin from blood to interstitial fluid is due to considerable transcapillary movement of unbound T_4.

Gel filtration studies on sheep thyroxine-binding proteins

Since the measured lymph/plasma concentration ratios for the three T_4BPs of sheep serum indicated that these proteins were of similar molecular size attempts were made to confirm this using gel filtration. Samples of sheep plasma containing tracer amounts of T_4 [^{125}I] and OSA [^{131}I] were fractionated in triplicate on a Sephadex G-200 column. The protein and radioactivity elution profiles of a typical separation are shown in Figure 7.5. Both the optical density and the radioactivity peaks for albumin coincided at tube 32 while the T_4 [^{125}I] peak occurred one tube earlier. Since it has been shown

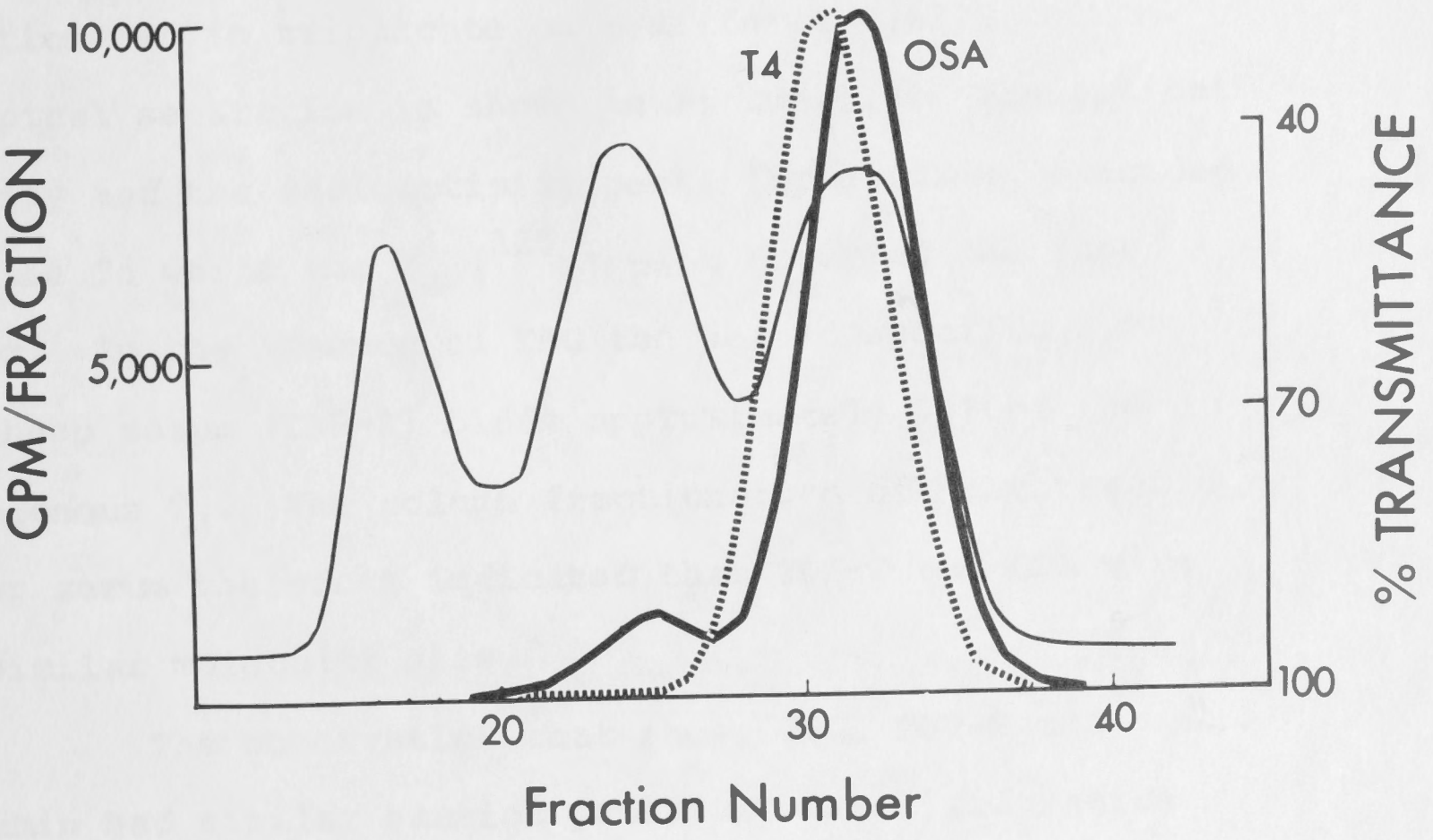


Figure 7.5. Protein and radioactivity elution profiles of a Sephadex G-200 separation of normal sheep serum labelled with tracer T_4 [^{125}I] and HSA [^{131}I].

in Chapter 5 that sheep TBG binds approximately 55% of endogenous T_4 this result was taken to indicate that sheep TBG and OSA were of similar molecular size.

The binding of T_4 to sheep TBG was then destroyed by subjecting sheep serum to 60° for 1 hour. Tracer T_4 [^{125}I] and OSA [^{131}I] were added and the sample fractionated in triplicate on a different G-200 column. A typical separation is shown in Figure 7.6. The optical density and the radioactivity peaks for albumin coincided at tube 36 while the T_4 [^{125}I] peak occurred one tube later. In the absence of TBG the second specific T_4BP of sheep serum (TBP-2) binds approximately 70% of the endogenous T_4. The column fractionation of heat treated sheep serum therefore indicated that TBP-2 and OSA were of similar molecular size.

The observation that sheep TBG, TBP-2 and albumin had similar elution profiles on gel filtration agrees with the lymph/plasma concentration ratio data which indicated that the three T_4BPs of sheep serum were of similar molecular size. It could therefore be expected that these three molecules would have similar distribution kinetics thus validating the use of albumin as a model to study the distribution of the specific T_4BPs in the sheep.

Gel filtration studies also demonstrated that HSA and OSA had identical elution profiles which indicated that the use of tracer HSA [^{131}I] in sheep was a valid marker for endogenous albumin distribution in that species.

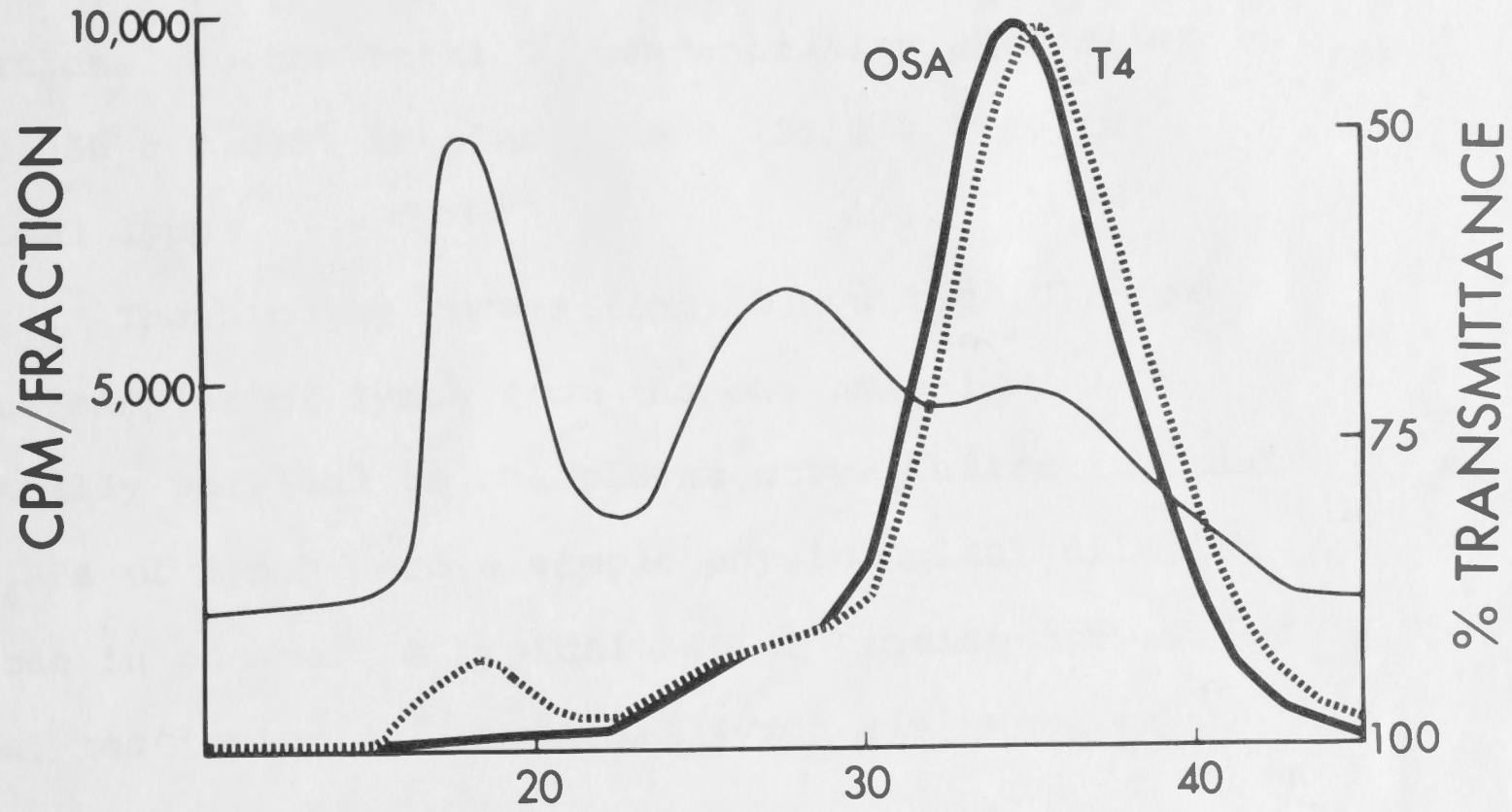


Figure 7.6. Protein and radioactivity elution profiles of a Sephadex G-200 separation of heat treated (60° for 1 hour) sheep serum labelled with tracer T_4 [^{125}I] and HSA [^{131}I].

Unbound T_4 concentrations in plasma and lymph

Although the total T_4 concentration varied appreciably between plasma and various samples of lymph from the one animal the concentration of unbound T_4 was relatively constant from sample to sample. Hence the proportion of total T_4 that was unbound was inversely proportional to the total T_4 concentration and varied from 0.056 ± 0.019% in plasma to 0.125 ± 0.066% in popliteal lymph.

The binding curves (log [B] vs log [U]) for various samples of lymph from the one animal were essentially parallel to the plasma curve indicating that the T_4BPs of lymph were a simple physiological dilution of those in plasma. A typical set of binding curves for plasma, testicular and popliteal lymph are shown in Figure 7.7.

The rate of movement of tracer T_3 and serum albumin from blood to lymph

Triiodothyronine is less firmly bound to human plasma proteins than is T_4. The proportion of unbound T_3 has been estimated to be of the order of 0.35% (Larsen, 1972). Studies using the present competitive binding technique have shown that in the sheep T_3 is also less firmly bound to protein than is T_4 (Figure 7.8). The binding data was best fitted by a two binding protein model (S_1 + NS) presumably TBG and albumin. The association constant of TBG for T_3 was approximately

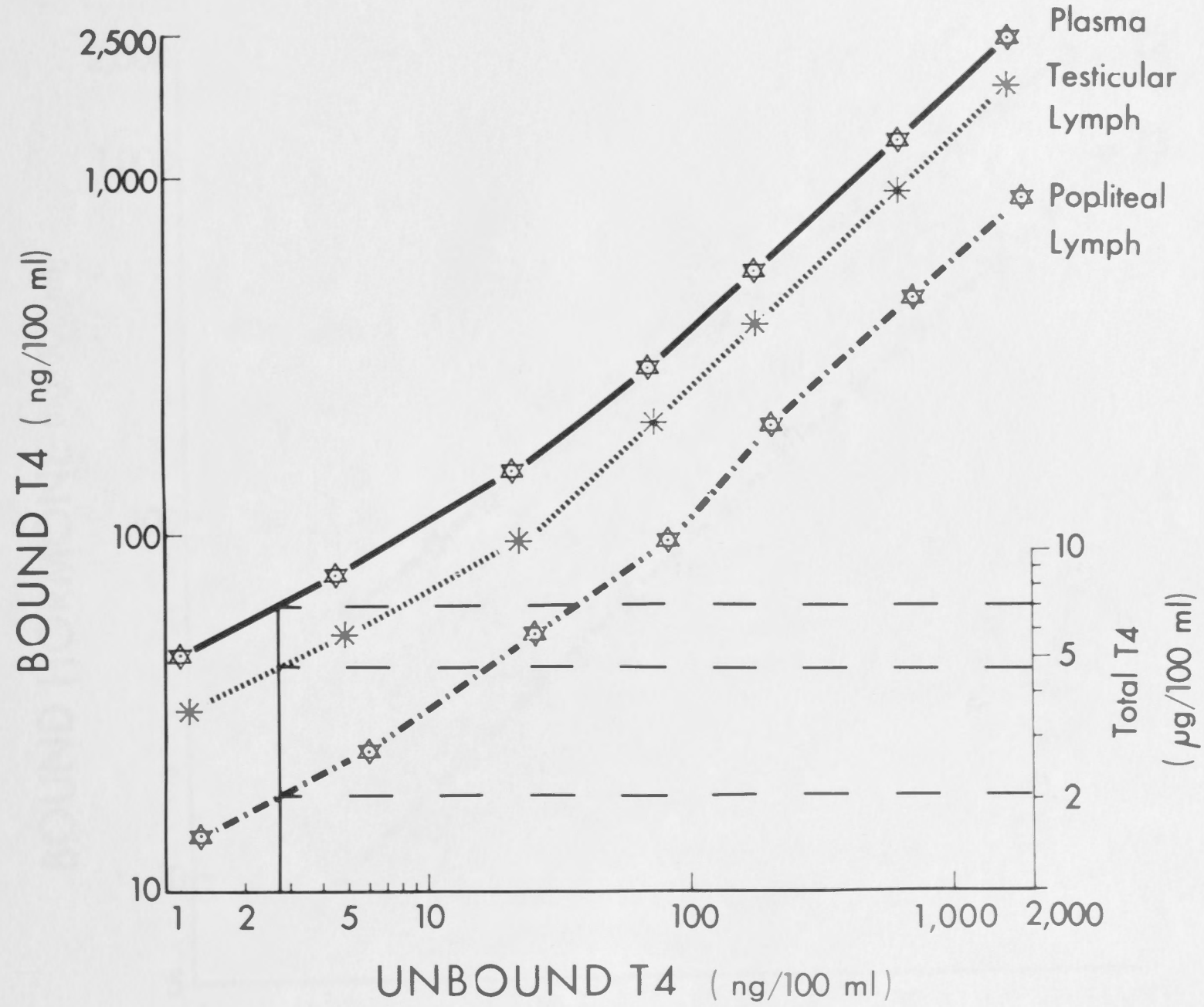


Figure 7.7. The thyroxine binding curves (log [B] vs log [U]) for samples of plasma, afferent testicular lymph, and efferent popliteal lymph collected from the same animal.

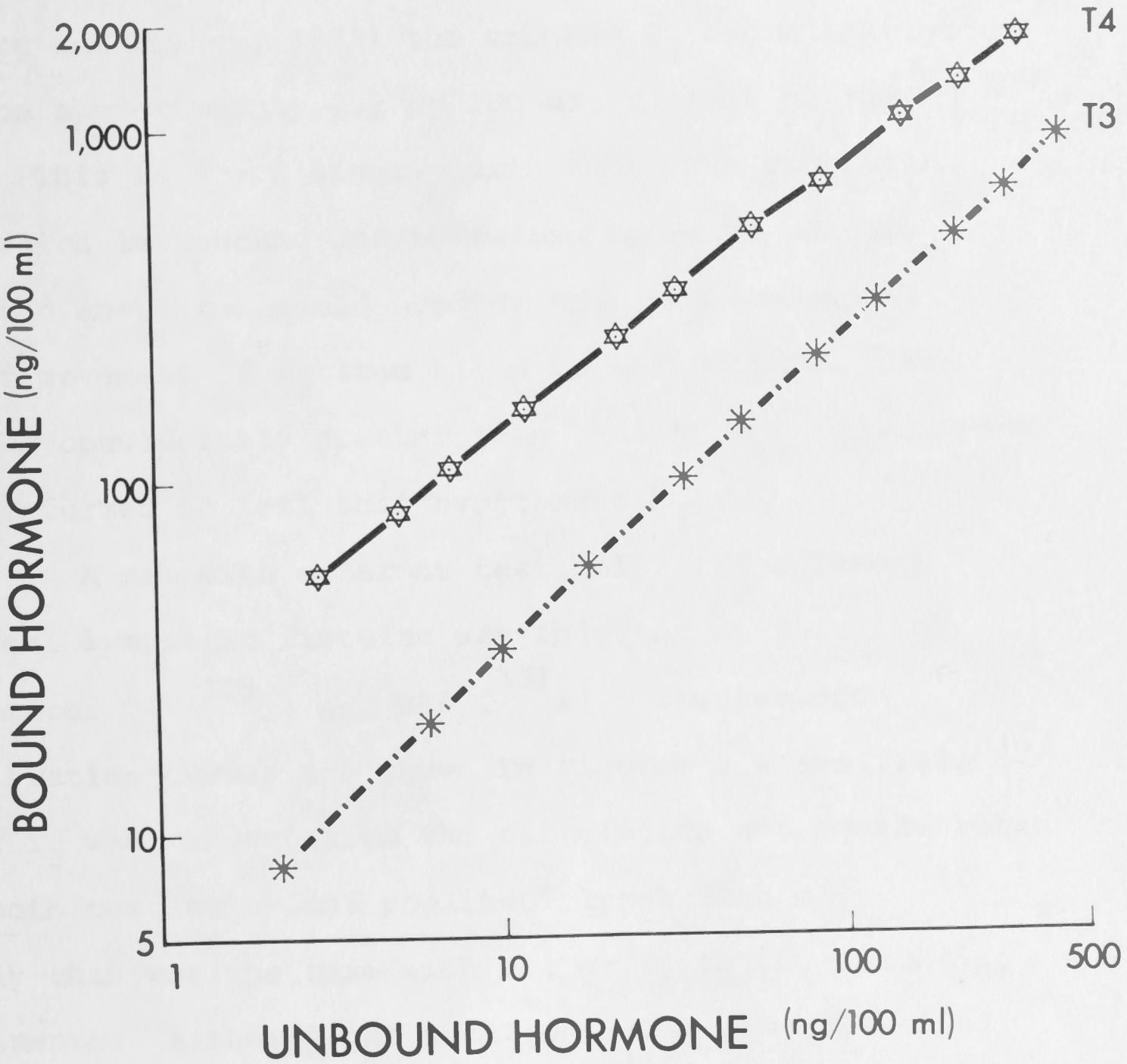


Figure 7.8. The thyroxine and triiodothyronine binding curves (log [B] vs log [U]) for a sample of sheep serum.

1/10th of that for T_4. This may underestimate the true value since no allowance was made for the endogenous T_4 in the assay. If the normal sheep has a total T_3 concentration of 80 ng/100 ml (Dussault, Hobel, DiStefano, Erenberg and Fisher, 1972) the unbound T_3 concentration would be approximately 0.2 ng/100 ml or 0.25% of the total. This is 4 - 5 times greater than the proportion of T_4 which is unbound and therefore from the results presented above one would predict that the fractional rate of movement of T_3 from blood to interstitial fluid would be considerably greater than that of T_4. Experiments were performed to test this hypothesis.

A ram with afferent testicular and efferent popliteal lymphatic fistulae was injected at zero time with tracer T_3 [^{125}I] and HSA [^{131}I]. The isotope equilibration curves are shown in Figures 7.9 and 7.10. Tracer T_3 was removed from the circulation and equilibrated with both testicular and popliteal lymph much more rapidly than was the case with tracer T_4 in the preceding experiments. Although the relative rates of movement of T_3 and HSA from blood to testicular lymph did not differ from those observed with tracer T_4 (the T_3:HSA lymph/plasma activity ratio during the first 1/2 hour was 1.23), the T_3 appeared in popliteal lymph at more than twice the relative rate recorded for tracer T_4. In this latter case the T_3:HSA lymph/plasma activity ratio during the first 1/2 hour after injection was 7.84.

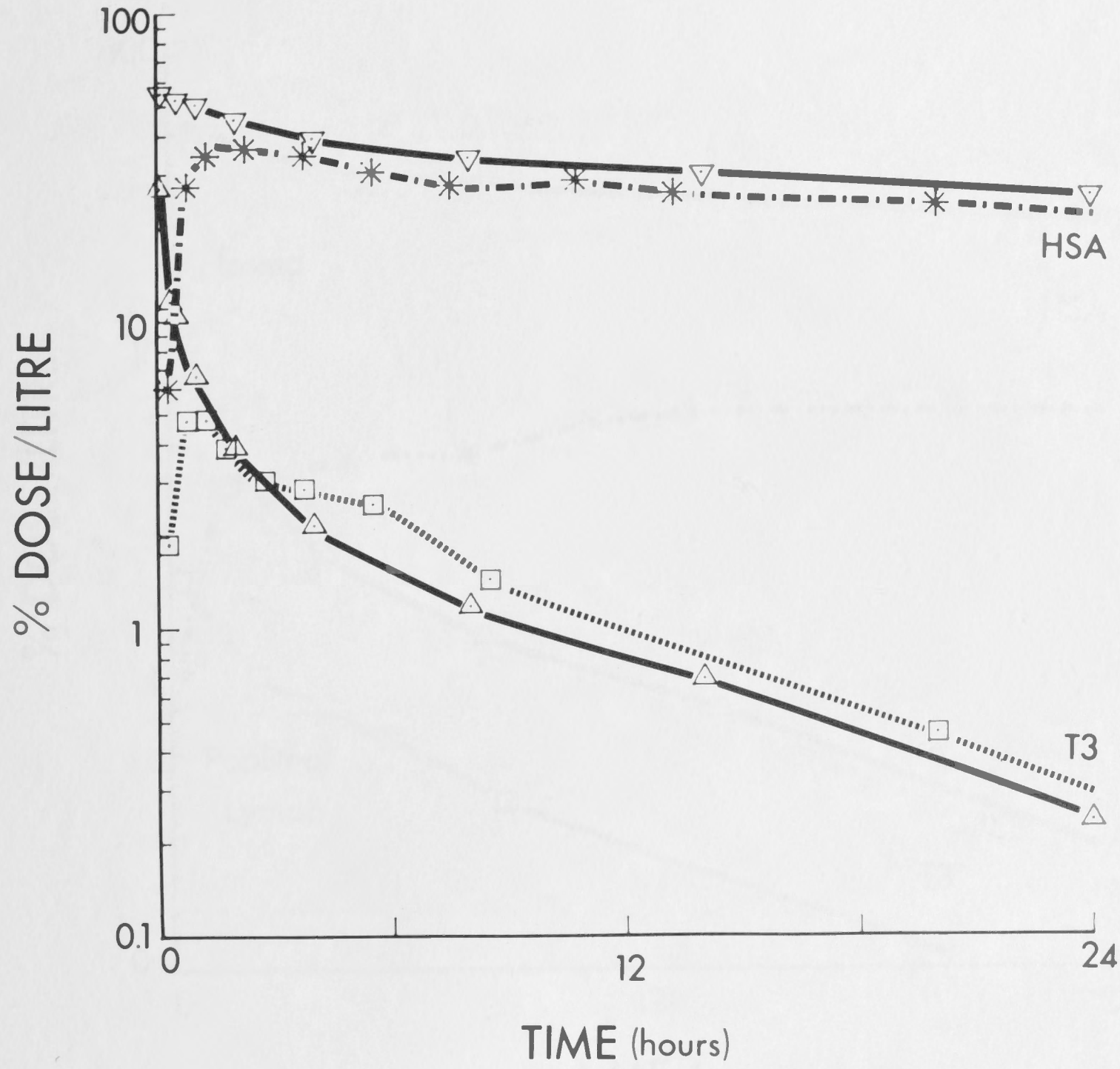


Figure 7.9. The disappearance of T_3 [^{125}I] and HSA [^{131}I] from plasma and their appearance in afferent testicular lymph following simultaneous intravenous injection of the two isotopes.

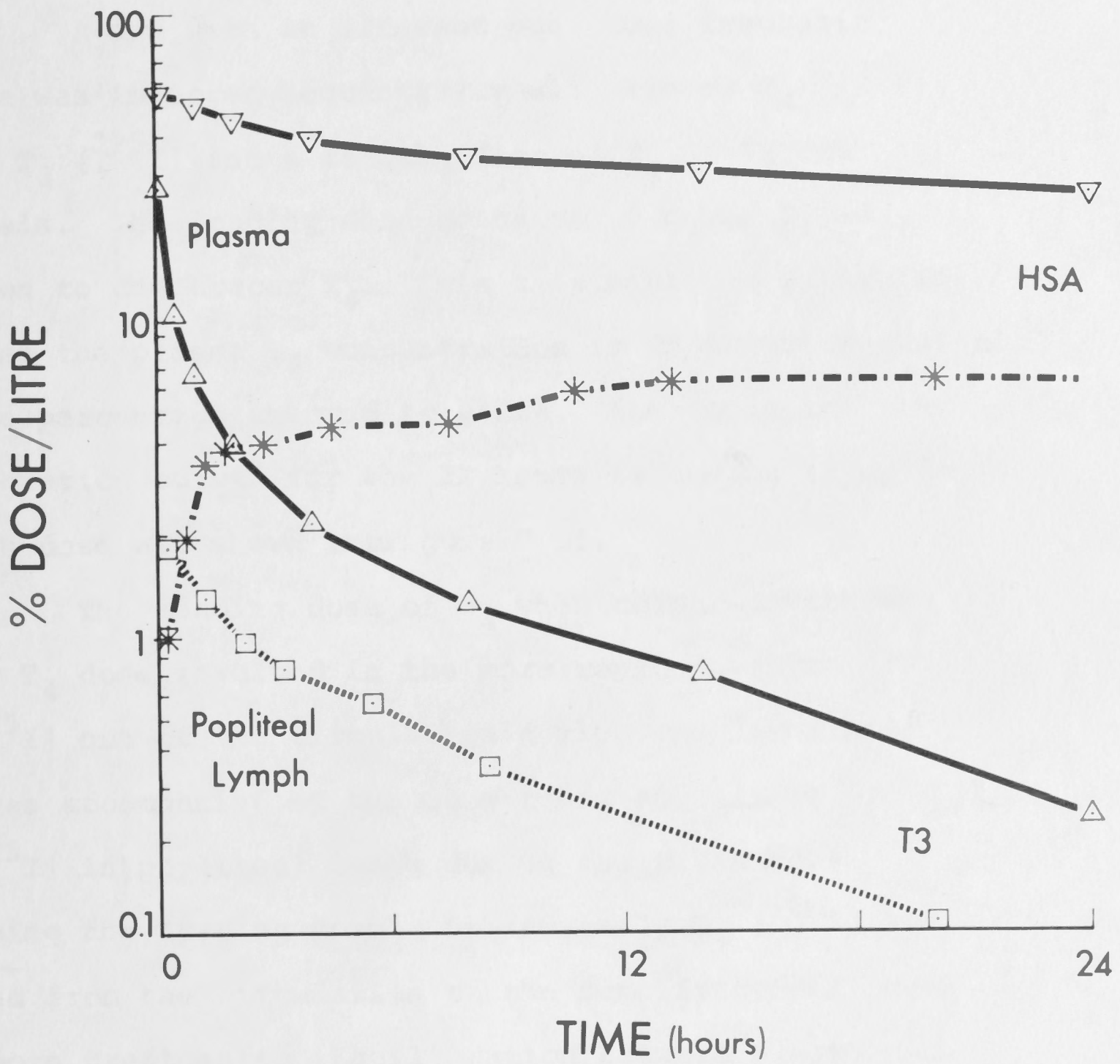


Figure 7.10. The disappearance of T_3 [^{125}I] and HSA [^{131}I] from plasma and their appearance in efferent popliteal lymph following simultaneous intravenous injection of the two isotopes.

The effect of elevated unbound thyroid hormone concentrations on the rate of movement of labelled hormone from blood to lymph

A ewe with an efferent popliteal lymphatic fistula was injected sequentially with tracer T_4 [^{125}I], tracer T_3 [^{125}I] and a loading dose of T_4 at 72 hour intervals. The loading dose contained 5 mg of T_4 in addition to the tracer T_4. This treatment was estimated to raise the plasma T_4 concentration to about 300 µg/100 ml and the percentage unbound to 0.23%. The isotopic equilibration curves for the 12 hours following injection of each dose are shown in Figure 7.11.

The loading dose of T_4 when compared with the tracer T_4 dose resulted in the more rapid movement of T_4 [^{125}I] out of the circulation during the first hour. This was accompanied by the more rapid appearance of T_4 [^{125}I] in popliteal lymph during the first hour following the loading dose. Subsequently T_4 [^{125}I] was removed from the circulation at the same fractional rate with both treatments. Equilibration between plasma and lymph occurred earlier following the loading dose but following equilibration the lymph/plasma ratios for radioactive T_4 were the same in both cases.

Despite the fact that almost identical proportions of hormone in the circulation were unbound following the loading T_4 (0.23%) and tracer T_3 (0.25%) doses the equilibration curves were vastly different. Triiodothyronine [^{125}I] was removed from the circulation

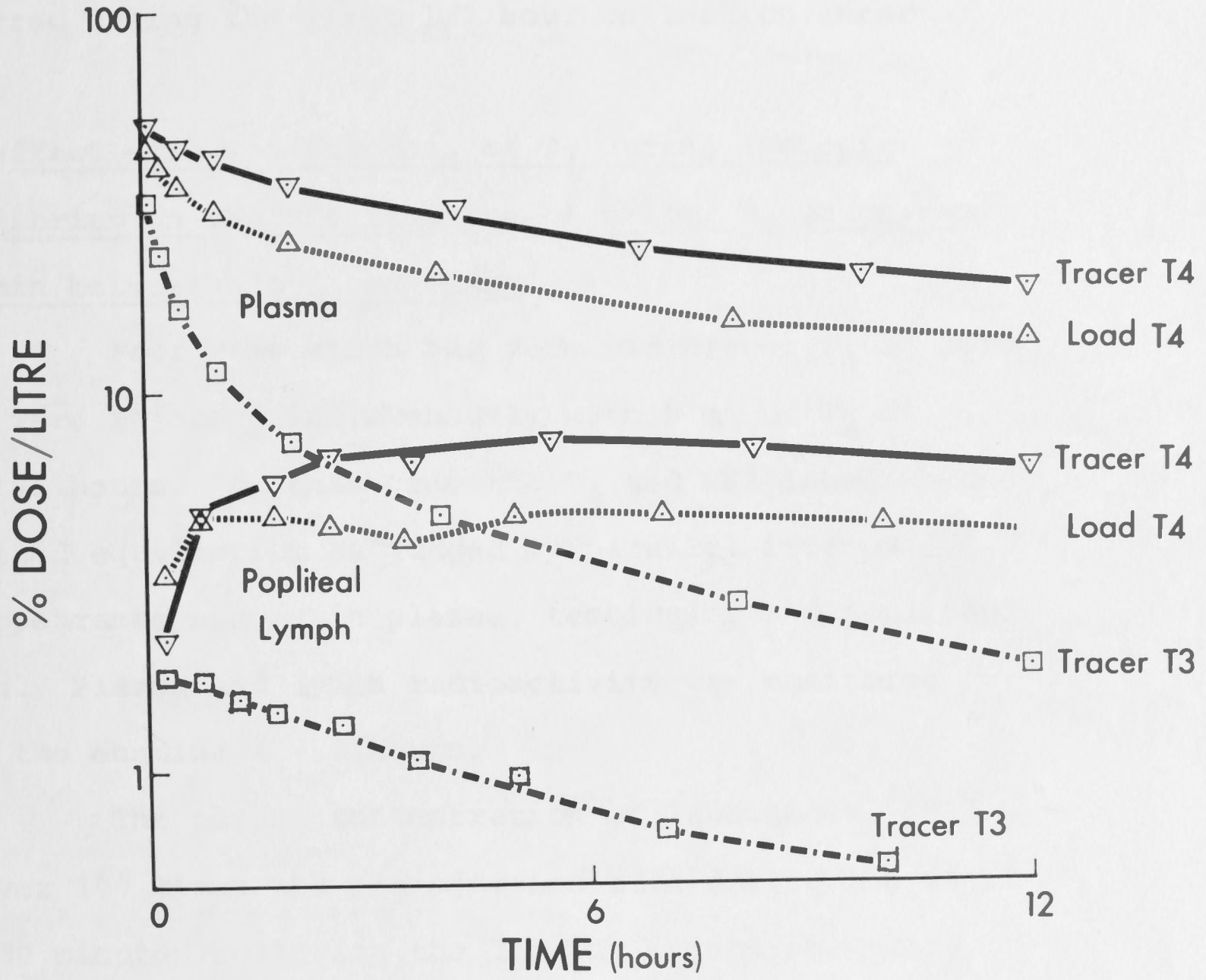


Figure 7.11. The disappearance of radioactively labelled thyroid hormones from plasma and their appearance in popliteal lymph following sequential intravenous injections of tracer T_4 [^{125}I], loading dose T_4, and tracer T_3 [^{125}I] at 72 hour intervals.

at a significantly greater fractional rate than both T_4 treatments over the entire time period studied. Equilibration of T_3 between plasma and popliteal lymph occurred during the first 1/2 hour collection interval.

<u>The effect of a loading dose of T_4 during isotopic equilibrium on the distribution of tracer T_4 and serum albumin between blood and lymph</u>

Four rams which had received tracer T_4 at zero time were injected intravenously with 5 mg of T_4 at 72 - 96 hours. At this time the T_4 and HSA labels had attained equilibrium as judged by parallel isotope disappearance slopes in plasma, testicular and popliteal lymph. Plasma and lymph radioactivity was monitored over the ensuing 4 - 8 hours.

The plasma concentration of labelled T_4 fell at over 100 times the preinjection rate during the first 5 - 30 minutes following the loading injection. This rapid rate of removal was followed by a T_4 [^{125}I] disappearance rate which appeared to differ little from the preinjection removal rate. The rate of removal of labelled T_4 from testicular and popliteal lymph followed that of plasma in 3 of the 4 animals studied. Typical disappearance curves for one such animal are shown in Figure 7.12. In one animal however, the initial rapid removal of T_4 [^{125}I] from plasma during the first 1/2 hour after injection was accompanied by a rapid rise in the level of T_4 [^{125}I] in testicular lymph. This elevated T_4 level fell equally as rapidly during the next 1/2 hour

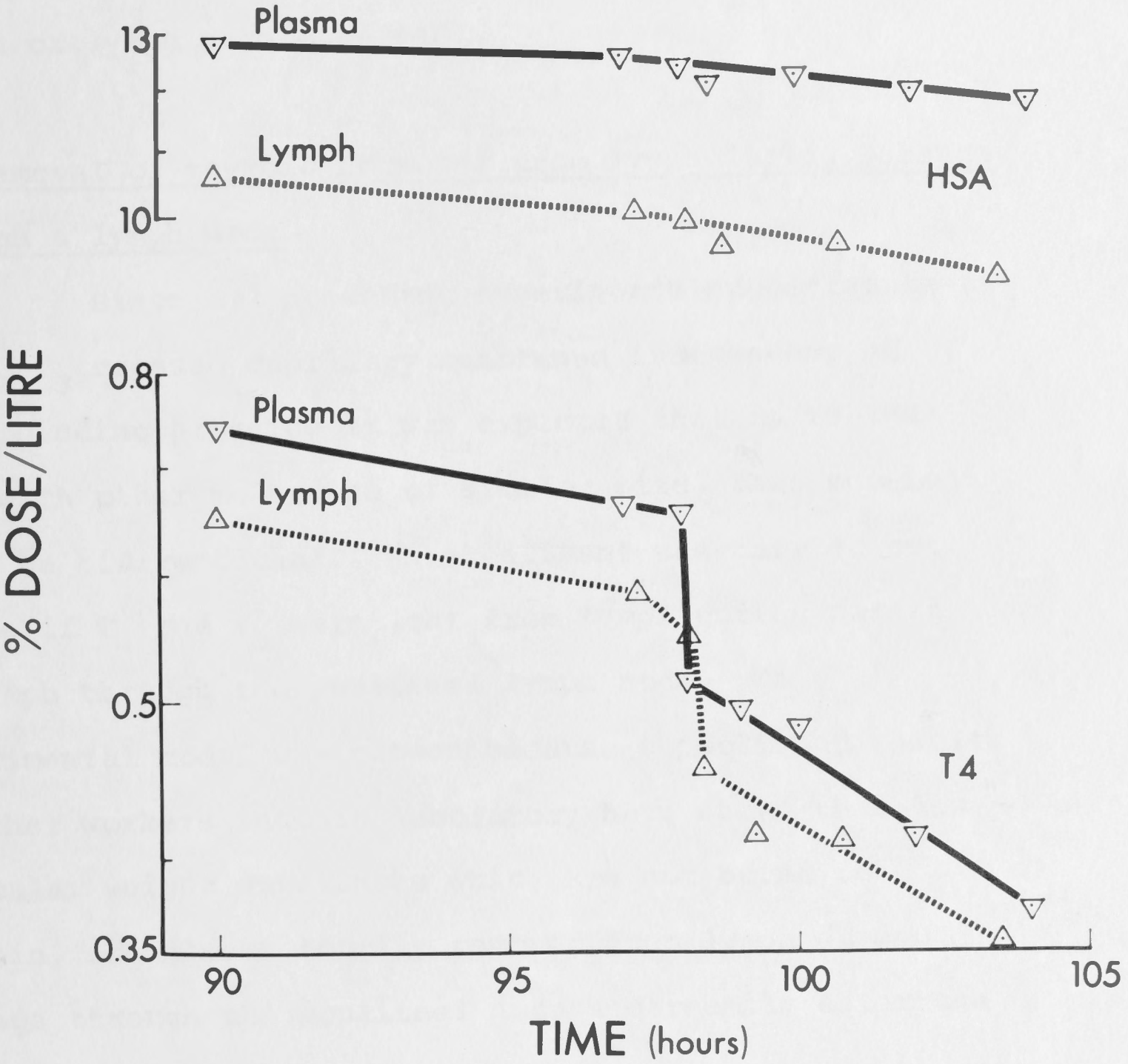


Figure 7.12. The effect of an intravenous loading dose of T_4 at isotopic equilibrium on the distribution of T_4 [^{125}I] and HSA [^{131}I] between plasma and afferent testicular lymph. The loading dose was injected at 98 hours after tracer T_4 [^{125}I] and HSA [^{131}I].

and subsequently showed a parallel decline to the disappearance of T_4 from plasma (Figure 7.13). No comparable changes in the rate of removal of HSA from plasma or lymph were observed.

The removal of thyroid hormones from lymph during passage through a lymph node

Since all preceding experiments suggested that T_4 and T_3 crossed capillary membranes independent of their binding proteins it was expected that as is the case with other molecules of similar size, this movement would be bidirectional. An experiment was carried out to see if T_4 and T_3 were lost from lymph during passage of lymph through the popliteal lymph node. This experimental model was chosen because unpublished results of other workers in this laboratory have shown that low molecular weight substances which are not bound to protein, are almost totally removed from lymph during passage through the popliteal node and readily enter the blood stream.

The recovery of T_4 [^{125}I] during passage through a popliteal node was compared with that of OSA [^{131}I] at four concentrations of T_4 in the lymph. These were compared with the loss of tracer T_3. The results are summarized in Table 7.3 where it can be seen that significant portions of T_4 were lost during passage through the node and these increased with increasing concentrations of T_4. Triiodothyronine loss at tracer concentrations was greater than that at the highest

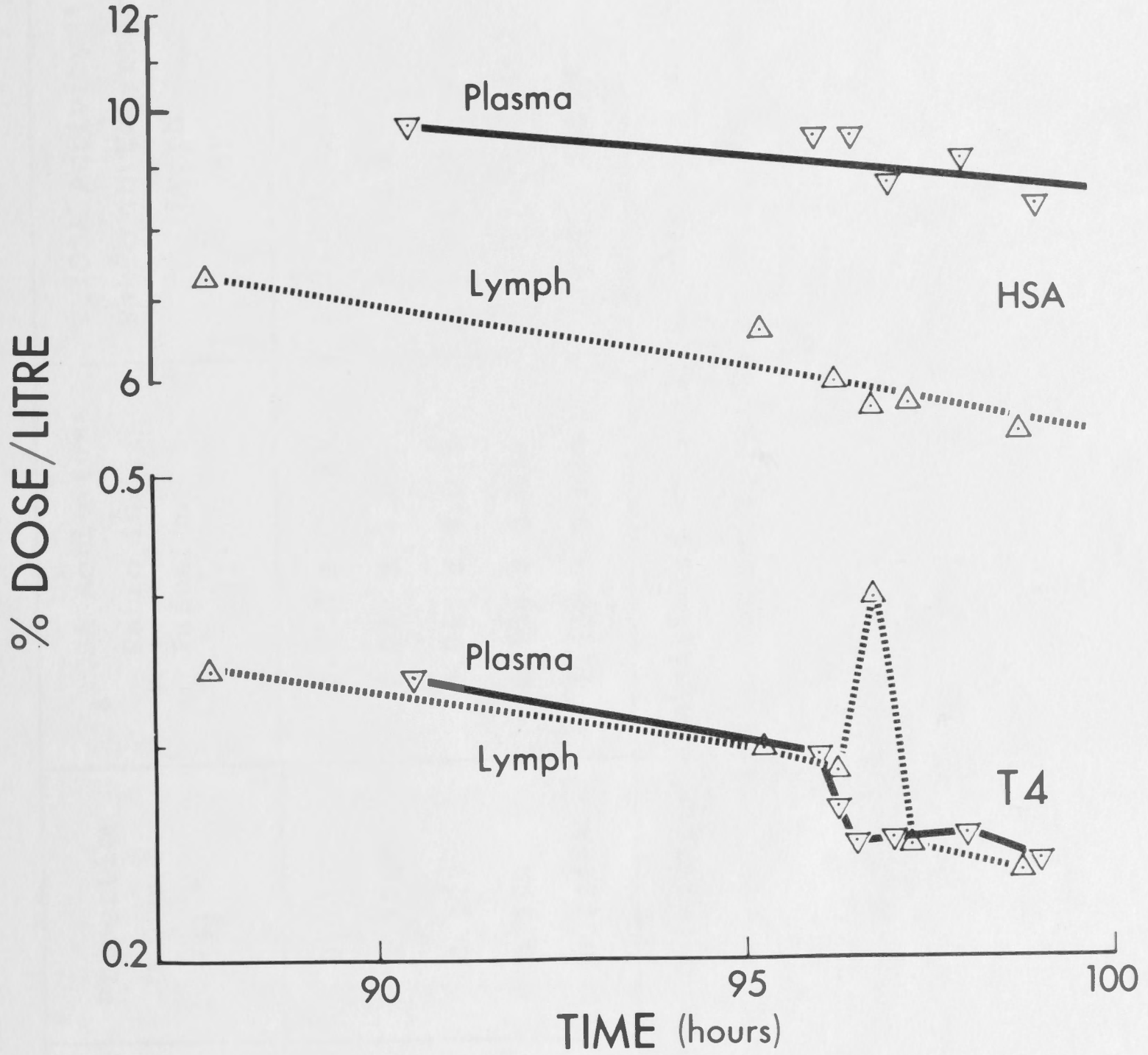


Figure 7.13. The effect of an intravenous loading dose of T_4 at isotopic equilibrium on the distribution of T_4 [^{125}I] and HSA [^{131}I] between plasma and afferent testicular lymph. The loading dose was injected 96 hours after tracer T_4 [^{125}I] and HSA [^{131}I].

Table 7.3. The effect of changes in the proportion of unbound thyroid hormones on their rate of loss from lymph during perfusion of a popliteal lymph node.

Total* Hormone Concentration (µg/100 ml)	Unbound* Hormone Concentration (ng/100 ml)	Proportion Unbound (%)	T_4:OSA Activity** Ratio in Infusion (I)	T_4:OSA Activity*** Ratio in Efferent Lymph (E)	E/I (%)
1.8	2.10	0.117	0.858 ± 0.001	0.851 ± 0.003	99.2
11.8	20.98	0.178	1.022 ± 0.003	0.967 ± 0.004	94.6
101.8	328.92	0.323	0.980 ± 0.013	0.845 ± 0.024	86.2
1,001.8	5,970.35	0.596	0.956 ± 0.014	0.777 ± 0.047	81.3
0.04****	0.196	0.489	1.110 ± 0.008	0.795 ± 0.023	71.6

* Concentrations of hormone in the infusion administered at 1 ml/hr. The mean efferent lymph flow rate was 7.9 ml/hr. Samples containing exogenous T_4 would be diluted almost six fold in the node.

** Mean ± SD values for triplicate analysis on 1 ml of infusion

*** Mean ± SD values for 1/2 hourly collections of efferent lymph during the infusion period.

**** Triiodothyronine.

concentration of T_4. Because of the large amounts of T_4 removed at high T_4 concentrations and since the material removed from the lymph was not recovered during the period of saline perfusion between treatments, these results are unlikely to be explained by tissue uptake in the node. They are more likely to indicate transfer into the blood stream. Unfortunately the amounts of radioactivity used were too low to allow reliable radioassay of the circulating blood.

DISCUSSION

The experiments described in this chapter supply convincing evidence that labelled thyroid hormones are removed from the circulation and appear in interstitial fluid at a greater fractional rate than labelled human serum albumin. This was true for all tissue fluid pools sampled and at all levels of hormone studied. If it is assumed that HSA has identical distribution kinetics to the specific T_4BP of sheep serum, these results indicate that thyroid hormones are removed from the circulation mainly as the unbound moiety.

A systematic study of samples of lymph from various regions of the body demonstrated that the three T_4BPs present in sheep plasma were also present in all samples of lymph. In addition the lymph/plasma concentration ratios for these three proteins were the same in any sample of lymph and similar to the corresponding lymph/plasma ratios for total T_4 and serum albumin.

These results indicate that there is no preferential transcapillary movement of any of the T_4BPs compared with albumin, or each other, and suggest that the T_4BPs of sheep are all of similar molecular size. The latter suggestion is at variance with recent estimates in the literature which indicate that the molecular weights of human TBG and TBPA are significantly lower than that of human serum albumin (Raz and Goodman, 1969; Marshall and Pensky, 1971; Sterling et al., 1971; Branch et al., 1971). Gel filtration chromatography however, confirmed that the T_4BPs of sheep serum had molecular sizes similar to human and sheep albumin; if anything TBG was slightly larger and TBP-2 slightly smaller than serum albumin. Similar chromatographic separations of normal and heat treated human serum have shown that TBG, TBPA and albumin have similar elution profiles to sheep TBG, TBP-2 and albumin respectively (Sutherland and Brandon, unpublished observations). It should be pointed out however, that gel filtration results are markedly affected by molecular shape as well as molecular weight. While such a technique is a good model for assessing comparative transcapillary diffusion, it is difficult to make accurate comment on molecular weights.

The concentration of unbound T_4 was similar in plasma and any sample of lymph from the same animal. Such a situation would arise whether T_4 and its binding proteins moved independently, or if T_4 crossed capillary membranes bound only to proteins. In the former case an unmeasurable transcapillary concentration gradient would

be expected for a substance with molecules as small as those of T_4 even if there was considerable transcapillary transport of that substance (Landis and Pappenheimer, 1963). If lymph is produced just as a simple filtrate of plasma with the proteins being reduced in concentration, then T_4 would dissociate from its binding proteins in the interstitial fluid so that the concentration of unbound T_4 would be very similar to that in the blood (Oppenheimer and Surks, 1964). In either case the concentration of total T_4 in blood or interstitial fluid would be determined by the concentration of the T_4BPs. Such a transcapillary gradient for total T_4 has previously been suggested as providing evidence that T_4 leaves the circulation bound to its binding proteins (Ismail, El-Ridi, Badran, Khalifa, Abel-Hay and Talaat, 1967) but by itself such an observation is insufficient for this purpose.

In experiments where the proportion of unbound T_4 was elevated, equilibration of labelled hormone between plasma and lymph occurred more rapidly, supporting the thesis that T_4 exchanges between these pools as unbound T_4. Following equilibration in the extracellular fluid, the proportion of labelled T_4 in plasma and lymph relative to each other was no different from that with tracer doses. This would be expected from consideration of the binding properties of plasma and lymph as plotted in Figure 7.7. However, the proportion of total labelled T_4 present in these pools was reduced. Since the fractional rate of elimination of T_4 from the body was not increased, lower concentrations in the extracellular fluid most likely

indicate higher concentrations within cells. Thyroxine is known to be bound intracellularly (Oppenheimer et al., 1969) but the relationship between intracellular bound T_4 and unbound T_4 must be different from that for plasma and lymph and would seem to favour cellular uptake when T_4 concentrations are elevated.

Triiodothyronine equilibrated more rapidly between plasma and lymph than did T_4 due most likely to the higher proportion of T_3 which is unbound. The equilibration curves for labelled T_3 were quite different from those for T_4, even when a loading dose of T_4 was used. This most likely represents different fractional rates of destruction, or different steady state distribution between intracellular and extracellular pools.

Experiments in which T_4 loading doses were administered at isotopic equilibrium in general support the above arguments. Plasma levels of labelled T_4 declined abruptly following the loading dose. In three experiments out of four, lymph levels declined similarly. These results suggest that the loading dose was distributed very rapidly. After re-establishment of equilibrium a smaller proportion of T_4 was present in the extracellular fluid and a larger proportion presumably in the intracellular fluid. In only one experiment was the reduction in plasma T_4 followed by an increase in labelled T_4 in the lymph. This was transitory and lymph levels soon fell to parallel the plasma levels as in the other experiments. If this result was not artefactual it could reflect T_4 en route from blood to tissues but this is thought more likely to be due

to experimental error. Similar effects of loading doses of T_4 in the human have been reported (Ingbar and Freinkel, 1960) and the effects similarly interpreted as here. However, since all such loading experiments disturb the steady state profoundly, it is difficult to derive useful quantitative data from them, and they are difficult to interpret critically.

If T_4 does move across capillaries in the unbound form then like other molecules of similar size, it would move in both directions with equal facility. Experiments carried out in a perfused lymph node indicated that T_4 and T_3 move from interstitial fluid to blood and the extent to which this occurs is related to the proportion of the hormones which is unbound.

Thus the experiments reported in this chapter support earlier claims that unbound T_4 is the freely diffusible form of T_4 (Ingbar and Freinkel, 1960; Robbins and Rall, 1967) and that cellular uptake of T_4 is related to the proportion of T_4 in the unbound state (Hillier, 1969). Unbound T_4 captured by the tissue cells is supplied by the circulating unbound T_4 pool and by dissociation of T_4 from the circulating binding proteins during passage through the capillaries (Hillier, 1969, 1971). The present results do not support the claim that T_4 is distributed in the body bound to its binding proteins (Oppenheimer et al., 1969). It should be pointed out that because of movement of T_4 in both directions across capillaries, and possible cellular uptake of T_4, the relative rates of movement of T_4 and protein from plasma

to lymph will tend to under-estimate the rate of trans-capillary movement of T_4 relative to that of its binding proteins.

Even though most T_4 seemed to cross capillaries in most tissues as unbound T_4, in all tissues a proportion of T_4 which crossed the capillaries must have done so bound to protein. Those differences which were observed in the transcapillary exchange of both T_4 and protein between various tissues can be explained in terms of known differences in capillary permeability of the tissues (Yoffey and Courtice, 1970). Certainly no evidence was found to suggest that T_4BPs had a functional role in the facilitated transport of T_4 into any tissue.

CHAPTER 8

THE EFFECT OF THYROIDECTOMY AND ADMINISTRATION OF SEX STEROIDS ON PLASMA THYROXINE-BINDING PROTEIN CONCENTRATIONS IN SHEEP

INTRODUCTION

Despite the vast amount of knowledge that has been accumulated with respect to the thyroid hormone binding properties of plasma proteins, no definite functional role has yet been assigned to the specific T_4BPs. It was hoped that some insight into such a functional role for the T_4BPs might be attained by studying factors which affect their concentrations in the body. Two different experimental treatments, namely, surgical thyroidectomy and administration of sex steroids were employed in an attempt to alter the levels of specific T_4BPs in sheep.

The concentration of circulating plasma T_4 appears to have little effect on the maximal binding capacity of human TBG. Several studies have shown that in humans thyrotoxicosis might slightly lower, and hypothyroidism might slightly elevate TBG binding capacity (Oppenheimer et al., 1963; Inada and Sterling, 1967; Gordon et al., 1971). Preliminary experiments with sheep, however, suggested that surgical thyroidectomy resulted in the reduction of plasma TBG concentrations (Sutherland and Simpson-Morgan, 1974). The binding capacities of two other specific binding proteins; corticosteroid-binding globulin in the rat and sex steroid-binding globulin in the human have also been shown to be affected markedly by the level of circulating T_4 (Labrie, Raynaud and Fortier, 1965; Dray, Mowszowicz, Ledru, Crépy, Delzant and Sebaoun, 1969; De Moor, Steeno, Heyns and Van Baelen, 1969;

Crépy, Dray and Sebaoun, 1972).

The effects of the administration of stilboestrol and testosterone propionate were also studied since these two hormones have previously been shown to cause changes in the plasma concentration of TBG in the human, a species which has functionally similar specific plasma T_4BPs to the sheep (Dowling, Freinkel and Ingbar, 1956; Engbring and Engstrom, 1959; Robbins and Rall, 1967).

RESULTS

The effect of thyroidectomy on the concentration of plasma T_4BPs in the sheep

Four adult female sheep were subjected to surgical thyroidectomy. Plasma T_4 was undetectable 10 days after surgery. The animals tolerated the operation well and were eating and drinking normally within a few hours. Despite complete absence of thyroid hormones, these animals remained in good health over the ensuing 3 months and showed no obvious behavioural differences to normal control animals housed under identical conditions. Ten weeks after surgery the four thyroidectomized animals and two normal controls were injected with 100 µCi of NaI [^{125}I] and the subsequent disappearance of total (^{125}I) and protein precipitable (^{125}I) was monitored over the following 3 weeks. Typical disappearance curves for a thyroidectomized and a normal control animal are shown in Figure 8.1. Plasma proteins were precipitated with 2 volumes of 10% TCA, the precipitate spun down and

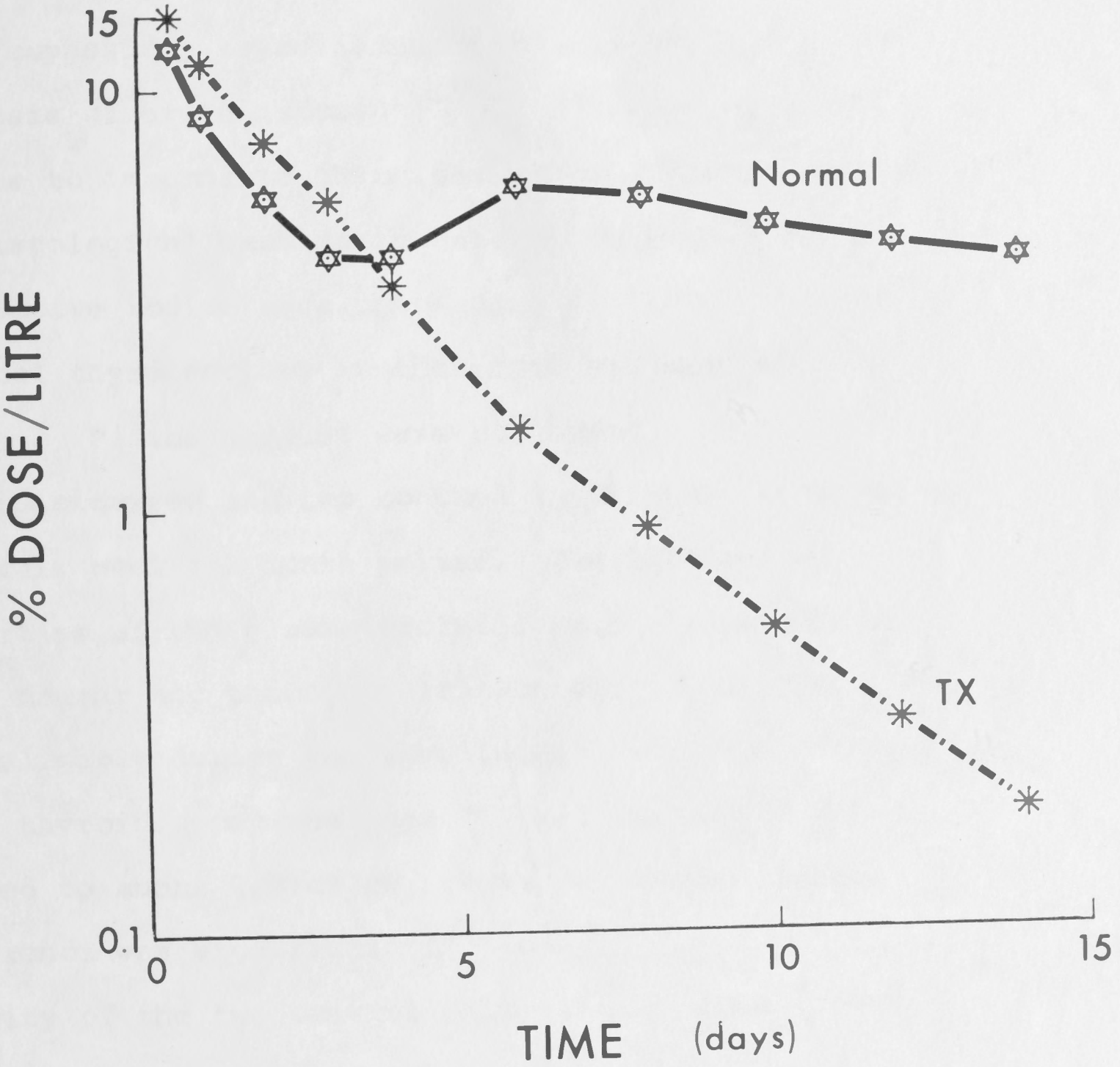


Figure 8.1. Plasma ^{131}I disappearance curves for a normal and a thyroidectomized ewe.

subsequently washed 3 times with 10% TCA. No appreciable protein-bound radioactivity could be demonstrated when the plasma of thyroidectomized animals was so treated. It should be pointed out however, that in other studies using supposedly thyroidectomized animals, appreciable synthesis of protein-bound (^{125}I) sometimes occurred. This was due to incomplete thyroidectomy as shown by anatomical and histological examination at post mortem. The use of radioactive iodide as a check on the completeness of surgical thyroidectomy is therefore recommended.

Plasma samples were collected from the four thyroidectomized and two control animals at fortnightly intervals over a 2 month period. The T_4 binding properties of the plasma proteins were assayed in the usual manner and the concentration of TBG was found to fall slightly during the sampling period. Eight weeks after thyroidectomy the mean TBG binding capacity was reduced to about 2/3rds of its pre-treatment value. This difference was significant ($P < 0.05$). The TBG binding capacity of the two control animals increased slightly during the sample period (Figure 8.2).

Thyroidectomy had virtually no effect on the binding of T_4 to TBP-2 and albumin as assessed by changes in the TBP-2 binding capacity and K_n (Figure 8.2). The latter finding was confirmed by measurement of plasma albumin concentrations which did not change significantly.

The effect of administration of sex steroids on the concentration of plasma T_4BPs in sheep

In a preliminary experiment designed to assess

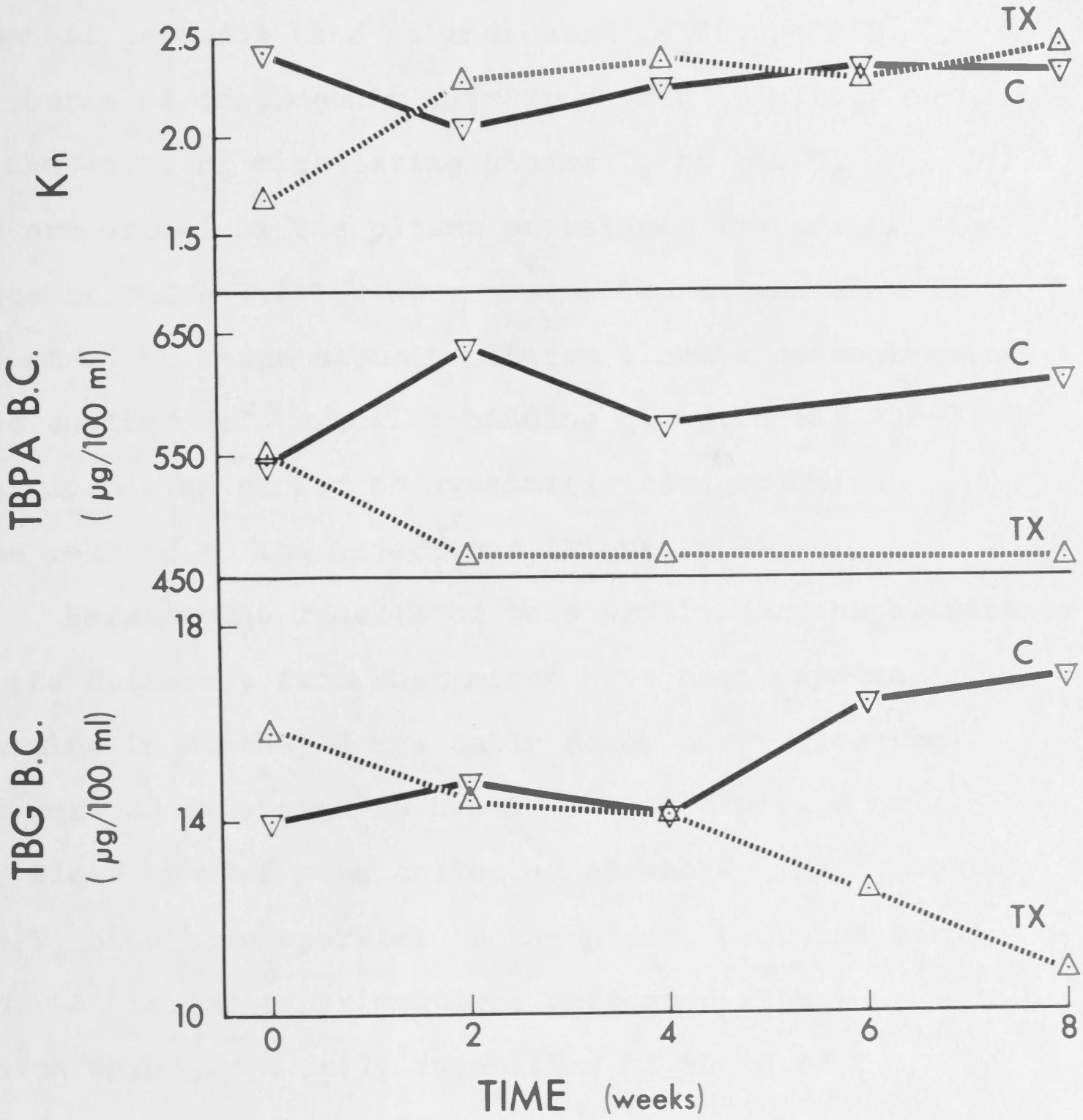


Figure 8.2. The effect of thyroidectomy on the concentration of plasma thyroxine binding proteins in sheep. Data points are the mean values of four thyroidectomized (TX) and two normal control (C) animals.

the effects of oestrogen on plasma T_4BP concentrations in sheep, one ewe received a series of intramuscular injections of stilboestrol over a four week period. The experimental protocol used is indicated in Figure 8.3. Such a course of oestrogenic injections had no effect on either the level of circulating plasma T_4 or the T_4 binding properties of the plasma proteins. The association constants of TBG and TBP-2 were unaffected as was the binding of T_4 to serum albumin. Serum albumin concentrations remained unaltered. Thyroxine-binding globulin and TBP-2 binding capacities showed no systematic changes which could be related to the injections (Figure 8.3).

Because the results of this preliminary experiment were quite different from what might have been expected from results in humans, large daily doses of stilboestrol (10 mg) were administered to two ewes over a period of 4 weeks. Blood samples were collected at weekly intervals and the T_4 binding properties of the plasma proteins were assayed. A similar experiment was performed with two ewes which were given daily injections of 50 mg of testosterone propionate. Administration of such large doses of steroid was associated with a slight decline in the total plasma T_4 levels in oestrogen treated sheep but not in testosterone treated animals (Table 8.1).

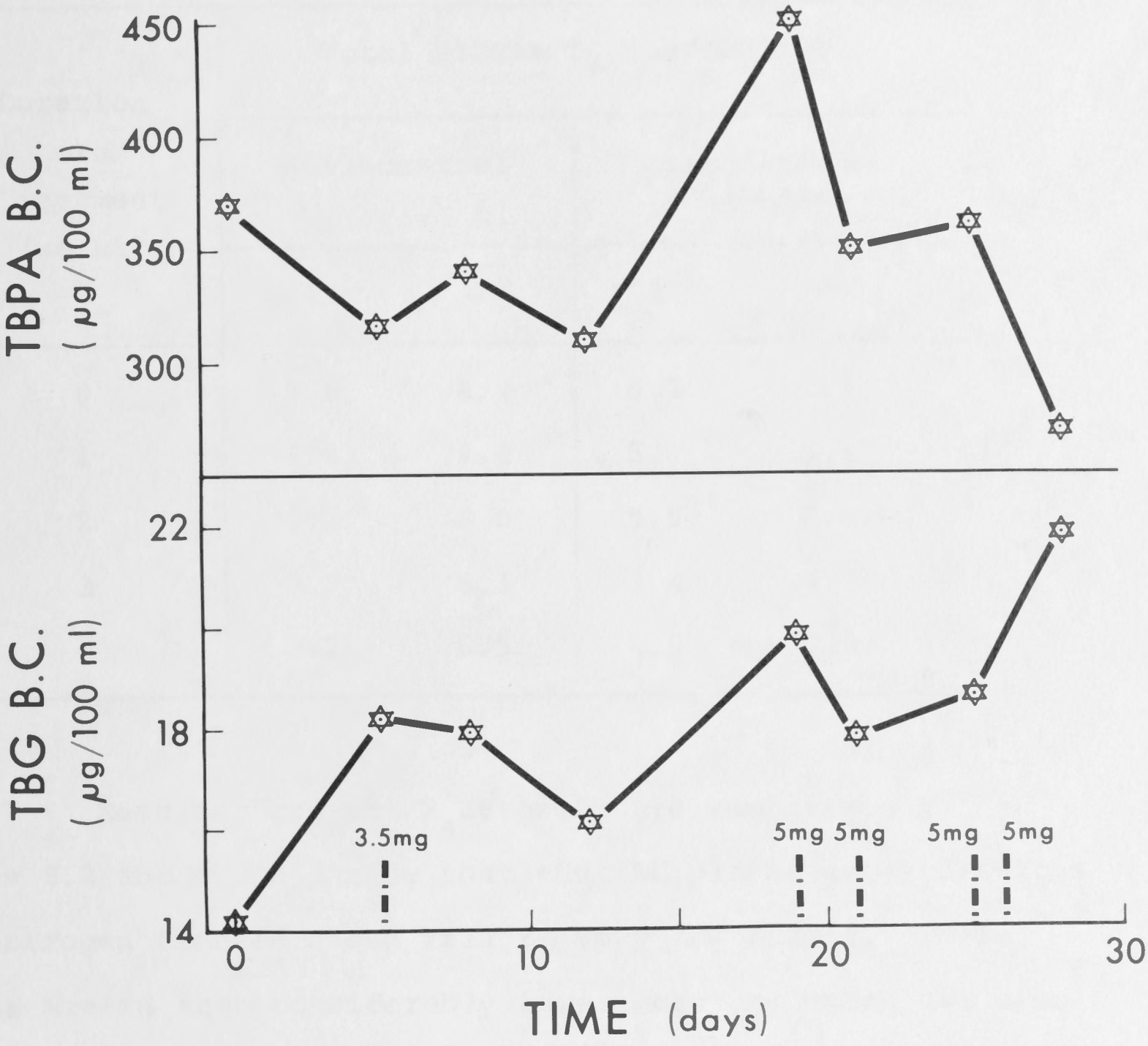


Figure 8.3. The effect of stilboestrol administration on the T_4 binding capacity of plasma TBG and TBP-2 in a ewe. The time and quantity of injected material is shown on the abscissa.

Table 8.1. Total plasma T_4 concentrations in four sheep receiving daily doses of 10 mg stilboestrol or 50 mg testosterone propionate.

Duration of Treatment (weeks)	Total Plasma T_4 (µg/100 ml)			
	Stilboestrol		Testosterone Propionate	
	1	2	3	4
0	9.8	8.4	6.3	7.4
1	8.4	8.4	5.7	6.6
2	7.2	8.0	5.5	6.6
3	7.7	6.3	5.4	4.4
4	7.2	6.5	6.6	7.8

Results from the T_4BP assay are summarized in Tables 8.2 and 8.3 and show that the TBG plasma concentrations in oestrogen treated sheep fell to very low levels. These levels are in fact considerably lower than any which had been encountered previously in the study of large numbers of normal sheep. Because the binding capacity of TBG was decreased proportionately greater than total T_4 concentration, the lowest concentration of unbound T_4 measured in the assay, and subsequently used in the fitting procedure, was much higher than had been measured previously. This probably made the fitting procedure less reliable, particularly with respect to TBG binding. Nevertheless, the association constants for TBG rose to levels higher than those

Table 8.2. The thyroxine-binding properties of plasma proteins in samples collected at weekly intervals from two sheep receiving daily intramuscular injections of 10 mg stilboestrol.

Duration of treatment (weeks)	TBG		TBP-2		Albumin
	K_{assoc} ($M^{-1}x10^{9}$)	Binding Capacity (µg/100ml)	K_{assoc} ($M^{-1}x10^{8}$)	Binding Capacity (µg/100ml)	K_n
Sheep 1					
0	10.0	22.0	1.70	460	2.63
1	11.3	15.9	1.78	514	2.53
2	29.9	4.0	0.92	439	1.94
3	41.0	4.6	1.54	582	2.18
4	69.0	2.0	0.71	440	2.63
Sheep 2					
0	13.5	16.6	2.14	412	3.04
1	13.4	13.3	1.96	633	2.51
2	14.3	8.5	1.15	782	2.27
3	33.4	4.7	1.70	650	2.13
4	22.6	3.1	1.10	427	3.02

Table 8.3. The thyroxine-binding properties of plasma proteins in samples collected at weekly intervals from two sheep receiving daily intramuscular injections of 50 mg testosterone propionate.

Duration of treatment (weeks)	TBG		TBP-2		Albumin
	K_{assoc} ($M^{-1}x10^{9}$)	Binding Capacity (µg/100ml)	K_{assoc} ($M^{-1}x10^{8}$)	Binding Capacity (µg/100ml)	K_n
Sheep 3					
0	11.1	17.1	3.51	289	2.81
1	10.0	16.9	2.07	438	2.65
2	9.7	13.1	0.99	486	1.46
3	8.9	13.6	1.41	500	2.55
4	12.6	9.6	1.02	477	2.05
Sheep 4					
0	6.1	29.7	2.02	408	2.58
1	7.8	22.2	1.81	481	2.57
2	12.0	12.0	1.18	420	1.74
3	5.5	17.3	1.31	516	2.92
4	10.0	11.2	0.87	542	2.27

determined in any other samples of sheep plasma (Table 8.2).

This situation should be contrasted with that in testosterone treated sheep where the concentration of TBG declined to a lesser extent during the course of the treatment but the association constants remained within the previously measured range for untreated sheep as can be seen in Table 8.3. Neither of the sex hormones used had any appreciable effect on T_4 binding to either TBP-2 or albumin.

The interpretation of changes in TBG binding capacities was complicated by the fact that during the period when these experiments were carried out, the mean binding capacity of TBG in two normal control sheep declined from 17.0 to 7.3 µg/100 ml and this period coincided with the onset of regular oestrous cycles in other ewes housed in the same area.

DISCUSSION

The results presented in this chapter suggest that thyroidectomized sheep could be useful experimental animals. Despite the total absence of circulating thyroid hormones such animals remained healthy for several months when kept indoors. The plasma concentration of TBG was consistently reduced following thyroidectomy but the other two T_4BPs (TBP-2 and albumin) were unaffected. This result is at variance with results reported in humans where hypothyroidism leads to unchanged or increased TBG

levels in the blood (Oppenheimer et al., 1963; Inada and Sterling, 1967; Gordon et al., 1971).

It might be thought that thyroidectomy with its consequent depletion of thyroid hormones, would be a useful way of decreasing the proportion of unbound T_4 in experiments similar to those reported in Chapter 7. However the proportion of T_4 which would be unbound following its injection into thyroidectomized sheep would not be very different from that in normal sheep if the levels of the three T_4BPs remained unchanged. With the decrease in TBG concentration following thyroidectomy the proportion of tracer doses of T_4 which would be unbound following injection would be higher than those in normal animals.

The apparent effects of oestrogenic hormones on the levels of TBG in sheep are also different from those reported in humans. When injections of 5 mg stilboestrol were given at various intervals to a ewe there was no consistent effect on the plasma concentration of TBG or the other T_4BPs. However, when stilboestrol was given in massive daily injections the concentration of TBG fell drastically. This result is very different from those reported in humans where it has been shown that administration of oestrogens to men as well as women consistently increased plasma levels of TBG (Dowling et al., 1956). It is interesting that concomitant with the fall in TBG concentration following oestrogen administration to sheep, the affinity of TBG for T_4 appeared to increase. There is no comparable data for the effects

of oestrogens on TBG affinity in humans but this phenomenon is worthy of further investigation.

Similarly the apparent effect of testosterone propionate when given to ewes was to decrease the concentration of TBG while the levels of plasma T_4 were unchanged. These results were difficult to interpret as normal untreated animals showed a similar reduction in TBG concentration over the same period. The latter decline in TBG levels was accompanied by a reduction in total plasma T_4 concentration. There is some uncertainty as to the effect of androgens on thyroxine binding protein concentrations in the human. Engbring and Engstrom (1959) reported that testosterone propionate decreased PBI levels in humans. This decline in PBI was accompanied by a fall in TBG concentration in only 3 of 9 euthyroid males studied. Recently Barbosa, Seal and Doe (1971) reported that testosterone propionate had no effect on TBG concentration whereas other anabolic steroids, e.g. 17α-methyl-testosterone reduced the level of TBG to about 50% of the normal level. It seems likely that testosterone propionate has a negligible effect on the concentration of plasma TBG in both humans and sheep.

The experiments reported in this chapter have contributed nothing to an understanding of the role of plasma T_4BPs as it was hoped they might. Results presented in earlier chapters have indicated that the differences between species, with regard to the T_4BPs of plasma, are somewhat less than would be suggested by earlier electrophoretic studies (e.g. Refetoff et al., 1970). Thus by using the method of assay developed during

the course of the work reported in this thesis, it was possible to show that in all species studied so far (human, sheep, rat and rabbit), the protein most constant between species is one which has properties similar to those of human thyroxine-binding prealbumin but which in other species could not be resolved electrophoretically. Sheep plasma was shown to contain T_4BPs which are functionally very similar to those of human plasma. This similarity between the two species applies only to the number of specific binding proteins and their affinity for T_4. Mechanisms involved in the control of thyroxine-binding protein concentrations in the two species appeared to vary considerably as was exemplified by the differential effects of thyroid and oestrogenic hormones on the plasma concentrations of human and sheep TBG.

SUMMARY OF

EXPERIMENTAL RESULTS

1. Sephadex G-25 has a high affinity for thyroxine (T_4) and triiodothyronine (T_3) which makes it an ideal competitive binding agent for studying thyroid hormone-protein interactions at thermodynamic equilibrium.

2. With pairs of protein-bound and unbound T_4 concentrations over a suitable range, the affinities and binding capacities of T_4 binding proteins (T_4BPs) in mixtures such as plasma and lymph can be estimated. Graphical methods used previously for this purpose can lead to erroneous results.

3. Human plasma was shown to have three functionally different T_4BPs namely; TBG, TBPA and serum albumin. Their mean association constants for T_4 were 9.5×10^9, 1.6×10^8 and 3.1×10^5 M^{-1} respectively. The mean T_4 binding capacity of TBG was 21.3 µg/100 ml of plasma and that of TBPA was 307 µg/100 ml.

Sheep plasma was also found to have three functionally different T_4BPs even though only two can be shown on electrophoresis. Sheep TBG, TBP-2 and albumin had mean association constants for T_4 of 8.9×10^9, 1.4×10^8 and 3.5×10^5 M^{-1} respectively. The mean T_4 binding capacity of TBG was 12.8 µg/100 ml and that of TBP-2 was 359 µg/100 ml.

Rat plasma had two functionally different T_4BPs. The mean association constants for T_4 were 3.6×10^8 and 6.3×10^5 M^{-1} and the mean T_4 binding capacity of the most avid binding protein was

317 µg/100 ml. The existence of two different T_4BPs in rat plasma was confirmed by gel filtration studies.

4. The binding of T_4 to plasma proteins is affected by the ionic strength, ionic composition, pH and temperature of the assay medium. A diluent for plasma in which binding is not likely to differ greatly from that in vivo was determined to be 0.05 M sodium phosphate, 0.10 M sodium chloride, pH 7.4 at 37°.

5. The presence of merthiolate in the assay medium inhibits the binding of T_4 to plasma proteins causing an increase in the concentration of unbound T_4.

6. Barbiturate ions inhibit the binding of T_4 to all three T_4BPs in human plasma. Binding of T_4 to TBG is less affected than T_4 binding to TBPA and albumin. Barbital is not a specific inhibitor of T_4 binding to TBPA as was previously thought.

7. Exposure of both human and sheep plasma to 60° for 1 hour destroyed the ability of TBG to bind T_4.

8. Binding of T_4 to pure ovine serum albumin (OSA) and human serum albumin (HSA) was found to be affected by similar factors to those affecting binding in whole plasma. Affinities of purified OSA and HSA for T_4 were similar to those determined for albumin in analyses of

whole ovine and human plasma.

Triiodothyronine was bound to OSA and HSA with 1/3rd of the affinity of T_4.

9. In sheep T_4 was found to pass across capillaries more rapidly than albumin; this was not due to preferential transcapillary movement of T_4BPs. The same T_4BPs present in sheep plasma were found in all samples of lymph collected from various regions of the body. Lymph/plasma concentration ratios for all T_4BPs were similar to each other and to those for albumin and T_4. Concentrations of unbound T_4 in plasma and lymph were similar. It was concluded that T_4 moved across capillary endothelium primarily as the unbound moiety.

10. Increasing the proportion of thyroid hormone that was unbound, either by administration of loading doses of T_4 or by using tracer T_3 resulted in an increased rate of equilibration between plasma and lymph. The final distribution of T_4 between plasma and interstitial fluid was unaffected by the level of T_4 injected but the distribution between the extracellular and intracellular pools was affected by the total amount of hormone administered.

11. Surgical thyroidectomy resulted in a significant decline in plasma TBG concentration in sheep. Eight weeks after surgery the TBG levels had fallen to approximately 2/3rds of their pretreatment concentration.

The other two T_4BPs were unaffected.

12. Administration of stilboestrol (10 mg/day) to adult ewes resulted in a marked decline in the level of plasma TBG. This was accompanied by an increase in the affinity of TBG for T_4. Testosterone propionate (50 mg/day) appeared to have no effect on the concentration of circulating T_4BPs in ewes.

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APPENDIX

APPENDIX

Uncorrected [B] and [U] values taken from Figure 1 of Elewaut (1973).

[B]/[U]	[B] µg/100 ml	[U] µg/100 ml
9.14	9.6	1.05
6.58	18.3	2.78
5.38	27.1	5.04
4.13	41.0	9.93
2.93	82.0	28.0
2.20	143.0	65.0
1.64	190.0	116.0