THE THYROXINE-BINDING PROPERTIES OF SERUM PROTEINS.  
A COMPETITIVE BINDING TECHNIQUE 
EMPLOYING SEPHADEX G-25

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SUMMARY

A competitive binding technique is described for the estimation of the thyroxine (T₄)-binding properties of serum proteins in dilute blood serum and lymph. When used in conjunction with an assay for total T₄ the following parameters can be estimated: the number of functionally different T₄ binding proteins, their individual association constants and binding capacities for T₄, the amount of T₄ which is bound to each binding species, and the concentration of unbound (free) T₄.

Both human and sheep serum have three functionally different T₄-binding proteins. The association constants for the three human proteins were 9.5 × 10⁶, 1.6 × 10⁷ and 3.1 × 10⁶ l/mol for T₄-binding globulin (TBG), T₄-binding prealbumin (TBPA) and serum albumin, respectively. The corresponding sheep proteins, TBG, TBP-2 and albumin, had association constants of 8.9 × 10⁶, 1.4 × 10⁷ and 3.5 × 10⁶ l/mol. Human TBG had a mean binding capacity of 21.3 µg/100 ml and that of ovine TBG was 12.8 µg/100 ml. The other specific binding proteins (TBPA in man and TBP-2 in sheep) had mean binding capacities of 307 and 359 µg/100 ml respectively.

Two functionally different T₄-binding proteins were identified in rat serum.

INTRODUCTION

Qualitative and quantitative estimates of the thyroxine (T₄) binding properties of serum proteins in a number of species have generally been derived from experiments employing electrophoretic separation techniques (Robbins & Rall, 1957; Inada & Sterling, 1967; Refetoff, Robin & Fang, 1970). While such techniques have been invaluable clinically and have supplied considerable comparative information, the results obtained are unlikely to depict conditions in vivo closely (Gordon & Coutsofides, 1969).

This communication describes a technique which enables measurement of protein-bound thyroxine (T₄) and unbound (free) T₄ concentrations at equilibrium, over a wide range of total T₄ concentrations. The assay can be performed in physiological buffers at physiological temperature and pH. Analysis of the binding data gives estimates of the number of specific binding proteins, their association constants and binding capacities as well as an assessment of unbound T₄ concentration.

The method is similar in principle to that previously described for the study of testosterone binding to serum proteins (Pearlman & Crépy, 1967).

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MATERIALS AND METHODS

Materials

Normal human plasma was obtained from expired blood bank whole blood. The plasma was recalcified with 1 m-CaCl₂, allowed to clot and centrifuged. After dialysis overnight against 0.05 m-sodium phosphate-0.1 m-NaCl buffer, 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. Sheep blood samples were collected from the jugular vein of mature male and female Merino sheep and serum was separated. Rat blood was collected by abdominal arterial cannulation of mature Wistar rats. The blood was allowed to clot and serum separated.

Sephadex G-25, coarse grade, was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and was used untreated. [¹²⁵I]L-Thyroxine in 50% propylene glycol with an initial specific activity of 40–45 mCi/mg, containing 4–5 μg T₄/ml, was supplied by the Radiochemical Centre, Amersham, England. [¹²⁵I]Iodide free of stabilizer (A.A.E.C., Lucas Heights, Sydney) was diluted to approximately 2 mCi/ml with 0.05 m-NaOH. This solution was used to iodinate human serum albumin (Hoechst Australia Ltd, Sydney) by the iodine monochloride method of Helmkamp, Goodland, Bale, Spar & Mutschler (1960). L-Thyroxine sodium salt (BDH Chemicals Ltd, Poole, England) was dissolved 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 T₄/ml. This stock solution was diluted with 50% propylene glycol by weight to produce serial tenfold dilutions which were used as working standard solutions.

Several buffer systems were used during the development of the binding assay. The buffer system found to be least likely to change the properties of the binding proteins on dilution, and now used routinely, is 0.05 m-Na₂HPO₄-NaH₂PO₄, 0.10 m-NaCl, pH 7.4 (Sutherland, Brandon & Simpson-Morgan, 1975).

Estimation of total T₄ concentration

Total plasma T₄ concentrations were measured by a modification of the method of Murphy & Pattee (1964). Serum was precipitated with 2 volumes of ethanol and duplicate 0.2 ml portions of the supernatant were evaporated to dryness. Sephadex G-25 (0.500 g) and 4.0 ml of a dilute (1:150) human plasma-tracer T₄ solution were added, the tubes shaken for at least 10 min at room temperature and an accurately weighed portion (approximately 1 ml) of the supernatant removed for counting. Unknown T₄ concentrations were read from a standard curve of c.p.m./ml of supernatant, versus T₄ concentration (0–15 ng), corrected for an ethanol extraction efficiency of 75.2%, and expressed as μg/100 ml plasma.

Serum albumin concentrations were measured by the method of Debro, Tarver & Korner (1957).

Principle of measurement of unbound and protein-bound T₄

Thyroxine is bound reversibly by Sephadex. When a constant amount of Sephadex is in contact with a constant volume of buffer solution containing T₄, at equilibrium the amount of T₄ included in the Sephadex (I), comprising both Sephadex-bound T₄ and T₄ dissolved in the included buffer, is related linearly to the amount of unbound T₄ (U) in the excluded volume (EV):

\[
I = \alpha U. \tag{1}
\]

When T₄-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
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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):

$$E = U + B.$$  \hfill (2)

If the total amount of $T_4$ present (T), the concentration of $T_4$ in the excluded volume ([E]), the excluded volume, and the constant $a$ 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:

$$T = I + U + B,$$  \hfill (3)

from (1)

$$U = (T - E)/a,$$  \hfill (4)

solving (2) and (4) for $U$

$$[U] = (T/EV - [E])/a,$$  \hfill (5)

$$[B] = [E] - [U].$$  \hfill (6)

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 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 equations:

$$[U] = [U^*] T/T^*$$

$$= (T^*/EV - [E^*]) T/aT^*,$$  \hfill (8)

$$[B] = [B^*] T/T^*$$

$$= [E^*] T/T^* - [U].$$  \hfill (9)

Measurement of radioactivity

Much preliminary experimentation was done using separate tubes to determine [U] and [B] for each amount of added $T_4$ as outlined by Sutherland & Simpson-Morgan (1974). This procedure was valuable in determining the optimal amounts of Sephadex and buffer and optimal dilutions of plasma for satisfactory analyses. However, the technique was too tedious for routine use. For this reason the following apparatus and procedure were developed.

The geometry of most well-type external crystal gamma scintillation counters is such that beyond a certain critical volume the count rate of a solution does not increase with an increase in the volume counted. An apparatus was devised to exploit this phenomenon in studying the binding of $T_4$ to proteins using Sephadex as a competitive binding agent. Using this apparatus the radioactivity in the EV could be measured each time the system came to equilibrium after additional $T_4$ was added.

The vessel used is shown in Fig. 1. It consists of a flat-bottomed round flask of about 85 ml capacity, fitted with a B10 and B29 ground-glass socket. A stopper was machined from Teflon or nylon to fit 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 G-25 was weighed accurately into the flask. Plasma to be studied was diluted 1/150 with a suitable buffer and $[^{125}I]T_4$ added to give a total initial concentration of exogenous $T_4$ of 0.1 ng/ml diluted plasma (=0.01–0.02 $\mu$Ci/ml). Ten millilitres of the diluted plasma were pipetted into the counting tube, and 20 ml added 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 dilute plasma in it poured into the flask. The smaller socket was stoppered, and the flask was shaken, with the counting tube horizontal, in a Warburg apparatus at 37 °C for 2 h. The Sephadex was then allowed to settle while the flask remained in the bath, and the supernatant 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 \( \text{T}_4 \) added and the flask shaken in the water bath for 7–10 min, 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 bore 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 \( \alpha \) was determined by adding \([^{125}\text{I}]\text{T}_4 \) (10 \( \mu l \) in 50 % propylene glycol) 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 \([^{125}\text{I}]\text{T}_4 \) into 10 ml of buffer in a counting tube and measuring the radioactivity in the same apparatus. The excluded volume was determined by the dilution of added \( ^{131}\text{I} \)-labelled albumin in the supernatant.

**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 \( \alpha \), \([U]\) 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 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 \( \alpha \) according to equation (10).
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below. The total amount of radioactivity added (T*), 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 (namely 30 for the assay procedure and 10 for measuring x). By substituting [E*] for [U*] in equation (6) x can be calculated according to
\[ x = (T*/EV - [E*])/[E*]. \] (10)

Analysis of errors
A complete analysis of errors in this system is complex but is important in relation to fitting models to the estimated concentrations of unbound and bound T4. As far as the radioassay is concerned, it is possible to show that the estimate of [U*] is considerably more precise than [B*] and that neither of these changes greatly over the range of T4 additions used. Since the estimates of [U*] and [B*] are converted to absolute concentrations by multiplying by T/T*, and since in any assay T* remains constant, the likely errors in estimates of [U] and [B] will be related more or less directly to the total amount of T4 added.

Fitting the binding data
A considerable amount of evidence from electrophoretic studies suggests that T4 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.
\[ [B] = \sum C_i[U]/(K_i + [U]). \] (11)
This assumes that T4 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 three T4-binding proteins in human plasma: T4-binding globulin (TBG), T4-binding prealbumin (TBPA) and albumin. Moreover the dissociation constant for T4-albumin is much higher than for TBG or TBPA (Robbins & Rall, 1967; Hamada, Nakagawa, Mori & Torizuka, 1970). It should be possible therefore to study binding over a range of [U] which would contain enough information to calculate the binding parameters for TBG and TBPA, but [U] would remain small relative to the dissociation constant for T4-albumin. The denominator (K_i + [U]) in the term in equation (11) corresponding to albumin would not change appreciably from K_n, and over this range of [U] albumin-bound T4 (B_\text{album}) would be given by
\[ B_{\text{album}} = C_{\text{album}}[U]/K_{\text{album}} \]
\[ = K_n[U]. \] (12)
This is equivalent to the non-specific binding in the formulation of Baulieu & Raynaud (1970), but it should be pointed out that for any binding protein, even so-called specific binding proteins, such a relationship holds while [U] is small relative to the dissociation constant.

For human plasma the model fitted to the binding data is given by (13):
\[ [B] = C_1[U]/(K_1 + [U]) + C_2[U]/(K_2 + [U]) + K_n[U]. \] (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 & Smith, 1966; Baulieu & 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 $K_a$ ($c_1$, $c_2$, $k_1$, $k_2$ and $k_a$ respectively) to improve the fit were calculated by fitting the multiple regression:

$$Y = c_1X_1 + c_2X_2 + k_1X_2 + k_2X_4 + k_aX_a$$  \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 $K_a$ 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 $X_a$ are given by:

$$X_1 = [U]/(K_1 + [U])T, \quad X_2 = [U]/(K_2 + [U])T, \quad X_3 = -C_1[U]/(K_1 + [U])^2T,$$

$$X_4 = -C_2[U]/(K_2 + [U])^2T, \quad X_a = [U]/T.$$

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 proved 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 (Digital Equipment Corporation) running a 7 user FOCAL system 'LIBRA'. Each user in this system has only about 800 words of core storage for his program and data. The routine used two separate FOCAL programs chained together and could handle a maximum of 18 pairs of data points. These details are given only to emphasize that fitting of such binding data can be carried out with a small computer. Complete solution takes about 5 min.

**RESULTS**

*Estimation of $\alpha$*

The mean $\alpha$ value was $7.362 \pm 0.083$ (s.d.) ($n = 6$) and was independent of the concentration of added $T_4$. 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 in the calculation of $\alpha$. Simulation studies have shown that substantial errors in the calculation of $\alpha$ result in unchanged estimates of the binding capacities of the specific $T_4$-binding proteins.

When $\alpha$ was assessed using $[^{131}I]L-3,3',5$-tri-iodothyronine ($T_3$) the value was $8.65$. This indicated that tracer impurities of $T_3$ would lead to insignificant errors in $\alpha$. In contrast, large amounts of labelled iodide impurity could lead to appreciable errors in $\alpha$ since $65\%$ of iodide is distributed in the excluded volume. For this reason care was taken to use only fresh isotope for this calculation. If a minor underestimation of $\alpha$ did occur due to iodide contamination, this would be accompanied by an underestimation of the association constants. With the addition of protein to the system these sources of error become less significant and can be readily corrected for when the percentage iodide contamination is measured.

*Thyroxine-binding proteins in human serum*

Twelve separate estimates of the concentration of $^{131}I$-labelled human serum albumin in the supernatant of the assay system gave a mean value of $22.32 \pm 0.53$ (s.d.) ml for the
excluded volume. This resulted in a final dilution of plasma proteins of 1:111.6 when the initial dilution in buffer was 1:150. Thyroxine was added progressively to such diluted samples of plasma to give cumulative additions of 0, 10, 20, 40, 80, 120, 200, 300, 500, 700 ng, and 1, 2, 4.5, 7 and 10 μg T₄. The concentrations of unbound and protein-bound T₄ were calculated after each addition. Using this data it was possible to get an apparently adequate fit to a model consisting of two specific binding proteins (TBG and TBPA) and one non-specific binding protein (albumin).

Table 1. Estimates of errors due to radioassay

<table>
<thead>
<tr>
<th>Counts/400 s</th>
<th>Calculated partition of [¹²⁵I]T₄ in supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unbound</td>
</tr>
<tr>
<td>Background</td>
<td>2500</td>
</tr>
<tr>
<td>Starting dilute plasma</td>
<td>265000</td>
</tr>
<tr>
<td>Supernatant after equilibration with Sephadex:</td>
<td></td>
</tr>
<tr>
<td>Expected counts if all protein bound</td>
<td>366000</td>
</tr>
<tr>
<td>Expected counts if all unbound</td>
<td>43700</td>
</tr>
<tr>
<td>Measured counts with no added T₄</td>
<td>268500</td>
</tr>
<tr>
<td>Measured counts with 10 μg added T₄</td>
<td>121000</td>
</tr>
</tbody>
</table>

* All values of unbound and bound thyroxine (T₄) were calculated using a value of 22.32 ml for the excluded volume and 7.362 for α.

Some pertinent details of the radioassay are presented in Table 1, where it can be seen that the uptake of T₄ by Sephadex was considerable, as would be expected from the value of α. The difference between the measured amounts of [¹²⁵I]T₄ in the supernatant, and what would be expected if all the T₄ remained in the supernatant, could be measured with high precision in the standard 400 s counting period. Since the estimates of unbound and bound [¹²⁵I]T₄ in the supernatant depend on these differences, it is not surprising that the actual errors in these estimates, which could result from counting errors, are relatively small and do not change greatly over the range of added T₄. These errors are also shown in Table 1. It is also worth noting that at the lowest T₄ concentration, the amount of T₄ taken up by the Sephadex exceeds the amount added as [¹²⁵I]T₄ so that the range of unbound concentrations spans that in the undiluted plasma.

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 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). 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 K₁ or K₂ above (or below) the mean value was accompanied by a C₁ or C₂ value above (or below) the corresponding mean C value. With replicate analyses the mean value of K is likely to be a more precise estimate of the true value of K than any individual estimate of K. For this reason C₁, C₂ and Kₙ were recalculated using the mean values of K₁ and K₂. These values are shown in parentheses in Table 2. While the mean values of C₁, C₂ and Kₙ were unaffected by the use of mean K₁ and K₂ values, the coefficients of variation for C₁ and C₂ were reduced and that for Kₙ was slightly increased.

Assuming that it is valid to extrapolate from diluted to undiluted plasma by correcting C₁, C₂ and Kₙ for dilution, estimates of the association constants and binding capacities
of TBG and TBPA in undiluted plasma were derived. The association constant of albumin was calculated from $K_n$ corrected for dilution, the measured albumin concentration, and assuming one binding site per albumin molecule.

Table 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

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>( K_1 ) (100 ml/ng)</th>
<th>( C_1 ) (ng/100 ml)</th>
<th>( K_2 ) (100 ml/ng)</th>
<th>( C_2 ) (ng/100 ml)</th>
<th>( K_n )</th>
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<td>2419</td>
<td>1.49</td>
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<td>(180)</td>
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<td>(2416)</td>
<td>(2416)</td>
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<tr>
<td>± S.D.</td>
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<td>±18</td>
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<tr>
<td></td>
<td>(10)</td>
<td>(10)</td>
<td>(307)</td>
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<td>c.v. (%)</td>
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Properties of plasma $T_4$-binding proteins in subjects of differing clinical thyroid status

Plasma samples from three normal, two hypothyroid, two hyperthyroid and one pregnant human subject were analysed as described. The results extrapolated to undiluted plasma are summarized in Table 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 $T_4$-binding proteins 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 association constants of TBG, TBPA and albumin in these samples were $9.5 \times 10^8$, $1.6 \times 10^8$ and $3.1 \times 10^8$ l/mol, respectively. The TBG binding capacity in the one pregnant subject was 44.5 $\mu$g/100 ml which was significantly higher than the mean value of $21.3 \pm 3.2 \mu$g/100 ml for the other seven subjects. The mean value for the TBPA binding capacity was $307 \pm 78 \mu$g/100 ml.

Since total $T_4$ concentration, $K_1$ and $K_2$ were measured and $C_1$, $C_2$ and $K_n$ corrected for dilution could be calculated, it was possible to evaluate the concentration of unbound $T_4$ in undiluted plasma and calculate the distribution of bound $T_4$ between its three binding proteins. The unbound $T_4$ concentrations agreed with the clinical status and the one pregnant subject had an unbound $T_4$ concentration at the lower end of the range seen in the three 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.
### Table 3. The thyroxine (T₄)-binding properties of human plasma proteins from subjects of differing clinical thyroid status: data are expressed as the mean ± S.D.

<table>
<thead>
<tr>
<th>Thyroid status</th>
<th>Total $T_4$ (µg/100 ml)</th>
<th>TBG $K_{assoc}$ (M⁻¹ × 10⁸)</th>
<th>BC (µg/100 ml)</th>
<th>TBPA $K_{assoc}$ (µg/100 ml)</th>
<th>Albin</th>
<th>Concn (g/100 ml)</th>
<th>K₄ (M⁻¹ × 10⁸)</th>
<th>Distribution of total T₄ (%)</th>
<th>Unbound $T_4$ (ng/100 ml)</th>
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</thead>
<tbody>
<tr>
<td>Normal ($n = 3$)</td>
<td>6-1</td>
<td>± 1-7</td>
<td>9-53</td>
<td>± 1-1</td>
<td>21-3</td>
<td>± 3-1</td>
<td>1-61</td>
<td>258</td>
<td>73-3</td>
</tr>
<tr>
<td>Hypothyroid ($n = 2$)</td>
<td>3-2</td>
<td>± 0-5</td>
<td>10-55</td>
<td>± 0-06</td>
<td>21-5</td>
<td>± 5-9</td>
<td>1-62</td>
<td>406</td>
<td>70-1</td>
</tr>
<tr>
<td>Hyperthyroid ($n = 2$)</td>
<td>8-9</td>
<td>± 0-4</td>
<td>9-30</td>
<td>± 0-71</td>
<td>21-2</td>
<td>± 2-5</td>
<td>1-44</td>
<td>272</td>
<td>72-1</td>
</tr>
<tr>
<td>Pregnant ($n = 1$)</td>
<td>7-3</td>
<td>± 0-4</td>
<td>7-9</td>
<td>± 0-71</td>
<td>44-5</td>
<td>± 2-5</td>
<td>1-92</td>
<td>326</td>
<td>80-1</td>
</tr>
</tbody>
</table>

Abbreviations: BC, thyroxine-binding capacity; TBG, thyroxine-binding globulin; TBPA, thyroxine-binding prealbumin.

### Table 4. The thyroxine (T₄)-binding properties of sheep and rat serum proteins: data are expressed as the mean ± S.D.

<table>
<thead>
<tr>
<th></th>
<th>Total $T_4$ (µg/100 ml)</th>
<th>TBG $K_{assoc}$ (M⁻¹ × 10⁸)</th>
<th>BC (µg/100 ml)</th>
<th>TBP-2 $K_{assoc}$ (µg/100 ml)</th>
<th>Albin</th>
<th>Concn (g/100 ml)</th>
<th>K₄ (M⁻¹ × 10⁸)</th>
<th>Distribution of total T₄ (%)</th>
<th>Unbound $T_4$ (ng/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep ($n = 16$)</td>
<td>6-2</td>
<td>± 2-7</td>
<td>8-9</td>
<td>± 2-9</td>
<td>12-8</td>
<td>± 4-8</td>
<td>1-39</td>
<td>359</td>
<td>53-1</td>
</tr>
<tr>
<td>Rat ($n = 7$)</td>
<td>4-0</td>
<td>± 2-3</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>3-64</td>
<td>317</td>
<td>85-1</td>
</tr>
</tbody>
</table>

Abbreviations: BC, thyroxine-binding capacity; TBG, thyroxine-binding globulin; TBP-2, a specific thyroxine-binding protein.
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 suggest that there are only two T4-binding proteins in sheep serum (TBG and albumin), fitting a model with one specific and one non-specific binding protein (S1 + 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 proteins (S1 + S2 + NS) gave a good fit to the data as is demonstrated in Fig. 2. The binding parameters extrapolated to undiluted serum are shown in Table 4. In all samples studied there appeared to be at least three T4-binding proteins. The most avid T4-binding protein, which probably corresponds to TBG of electrophoresis had a mean association constant of $8.9 \times 10^9$ l/mol 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 TBP-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 T4 in ovine plasma was $3.5 \pm 2.0$ ng/100 ml. The distribution of total T4 between the various binding proteins differed from that of the human samples due to the lower binding capacity of ovine TBG (Table 4).

Thyroxine-binding proteins in rat serum

The binding of T4 by rat serum was considerably different from that seen with human and sheep serum (Fig. 3). Data were 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 $3.64 \times 10^8$ l/mol and a mean capacity of 317 μg/100 ml (Table 4). Non-specific binding

![Graph](image-url)
Serum thyroxine-binding proteins

![Graph](image)

**DISCUSSION**

Conventional methods of measuring the T₄-binding properties of proteins in whole serum have involved electrophoretic separation techniques which yield estimates of the number of different binding proteins and their T₄ binding capacities. Such methods cannot yield accurate estimates of the in-vivo association constants for the specific binding proteins since these values can only be derived when the association constant of albumin has been assessed by some other means, e.g. equilibrium dialysis studies (Robbins & Rall, 1967; Hamada et al. 1970). In addition, the distribution of labelled T₄ in the electrophoretograms is influenced by a number of in-vitro factors: the ionic strength, ionic composition and pH of the buffer system which dilutes the binding proteins during electrophoresis, the affinity of the supporting medium for T₄, and the temperature at which the electrophoretic separation takes place (Gordon & Coutsoftides, 1969). Experiments in our laboratory have also demonstrated that the ionic strength, ionic composition, pH and temperature of the diluent buffer influences T₄ binding in the present assay system probably by affecting the avidity with which T₄ is bound (R. L. Sutherland & M. W. Simpson-Morgan, unpublished results).

In order to measure association constants which will be valid in vivo, one must use equilibrium techniques under conditions which closely mimic the physiological state. Plasma diluted in 0-05 m-phosphate, 0-10 m-NaCl, pH 7-4, and assayed at 37 °C has proved a convenient system which appears unlikely to deviate considerably from the conditions of T₄ binding in vivo. Like Spaulding & Gregerman (1972), we have shown that increases in chloride ion concentration cause a significant decline in T₄ binding while increases in phosphate concentration above 0-05 m caused minimal changes in T₄ binding parameters (Sutherland et al. 1975). The former observation necessitated the inclusion of physiological levels of chloride in the assay buffer. Phosphate at a concentration of 0-05 m may lead to a slight deviation from in-vivo conditions since this level of phosphate reduced T₄ binding.

was probably due to albumin. The association constant of rat albumin for T₄ was 6·25 ± 2·11 × 10⁵ l/mol which is twice the corresponding value found for ovine and human albumin.
from that seen at physiological levels of phosphate or bicarbonate (Woebel & Ingbar, 1968; Spaulding & Gregerman, 1972).

It has long been realized that if equilibrium concentrations 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). Earlier forms of analysis of binding data relied on linear transformations and graphical techniques (Lineweaver & Burk, 1934; Eadie, 1942; Scatchard, 1949). These are liable to systematic errors and are not adequate for situations involving a number of binding species (Baulieu, Raynaud & Milgrom, 1970; Westphal, 1971). With the availability of modern computer techniques it is now possible to fit non-linear functions over a wide concentration range (Baulieu & Raynaud, 1970). The present method of analysis is similar to that of Baulieu & Raynaud (1970) but we have not used the proportional graph to get initial estimates of binding affinities; these were found by trial and error using published values. The proportional graph, however, has been useful for assessing the number of saturable binding proteins present and to indicate whether the range of concentrations was great enough to permit an adequate solution. This latter point is important since incorrect solutions can arise if the specific $T_4$ binding proteins are not saturated completely as was the case in the solution presented by Sutherland & Simpson-Morgan (1974).

The results presented in this paper demonstrate the applicability of this technique to sera of different species and of different clinical thyroid states. Because of the small numbers in each group it is unlikely that the data are truly representative of the group but 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 realized that the various estimates were made under varying conditions of pH, temperature and buffer composition (Tabachnick, 1964, 1967; Green, Marshall, Pensky & Stanbury, 1972; Pages, Robbins & Edelhoch, 1973). Our observations agree with previous findings that the binding capacity of TBG is substantially increased during pregnancy (Refetoff, Hagan & Selenkow, 1972) but is little affected by hypothyroidism (Inada & Sterling, 1967), and that the concentration of unbound $T_4$ is at the lower end of the normal range during pregnancy, is lowered in hypothyroidism and elevated in hyperthyroidism (Sterling & Brenner, 1966; Thorson, Wilkins, Schaffrin, Morrison & McIntosh, 1972). The levels of unbound $T_4$ found in the two hyperthyroid patients were much lower than other reported values for 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 present estimates of the distribution of $T_4$ between its binding proteins do not differ greatly from those of Woebel & Ingbar (1968) and Hamada et al. (1970) but are different from earlier estimates (Ottendorf & Surks, 1964; Robbins & Rall, 1967).

The observation that sheep serum contains three functionally distinguishable $T_4$-binding proteins is at variance with electrophoretic data which have demonstrated only two $T_4$-binding proteins (Farer, Robbins, Blumberg & Rall, 1962; Refetoff et al. 1970). This illustrates the ability of the present technique to detect functionally different $T_4$-binding proteins and to measure their affinities and capacities for $T_4$. 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 $T_4$-binding proteins in the rat thus differing from results obtained with 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.
Serum thyroxine-binding proteins

The competitive binding technique described in this paper 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 radioactive 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 conditions in vivo or altered to yield information about mechanisms underlying the binding reactions. Apparently, similar competitive binding methods which have been described previously have employed ion exchange resins, dextran-coated charcoal and Sephadex columns (Keane, Pegg & Johnson, 1969; Roberts & Nikolai, 1969; Refetoff et al. 1972; Elewaut, 1973). In our hands ion exchange resins take too long to reach equilibrium, bind $T_4$ too strongly and bind $T_4$-binding proteins themselves. Sephadex columns yield data under non-equilibrium conditions and such data are unsuitable for kinetic analysis (Simpson-Morgan & Sutherland, 1974).

REFERENCES


