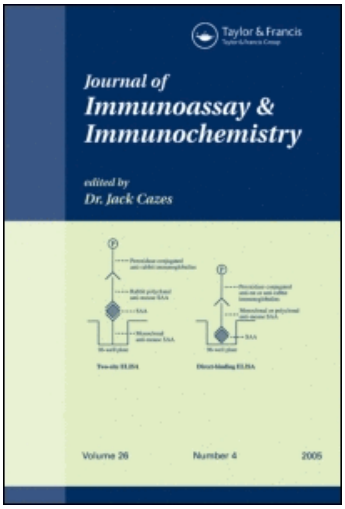


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# Study of Binding of Thyroxin-Conjugates to the Thyroxin-Binding Proteins

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# STUDY OF BINDING OF THYROXIN-CONJUGATES TO THE THYROXIN-BINDING PROTEINS

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## ABSTRACT

In this work we studied the effects of the molecular weight (M.W.) of thyroxin ($T_4$)-conjugates and the sample dilution factor on the binding potential (C) of serum $T_4$-binding proteins for these $T_4$-conjugates. We prepared six $T_4$-conjugates with great difference in molecular weight with proteins such as IgG, apoferritin, ferritin, transferrin, and thyroglobulin using p-benzoquinone as bifunctional reagent. The conjugates prepared were characterized in terms of their M.W., the molar ratio of $T_4$ to the carrier protein, and their affinity to bind with the anti-$T_4$ antibody. The binding potential values of serum $T_4$-binding proteins for the $T_4$-conjugates were determined, following appropriate mathematical interpretation of the results, obtained through an assay system containing $^{125}$I-labeled conjugated tracers, anti-$T_4$ antibody in great excess compared with the concentration of the tracers, and increasing concentration of $T_4$-binding proteins. We conclude that the M.W. of the conjugates is a parameter which significantly influences the binding of a conjugate to the $T_4$-binding proteins. Additionally, for conjugates of very high M.W. (> 650,000), zero C values were obtained using 20-fold sample dilution in the final incubation mixture.

(KEY WORDS: Free thyroxin, thyroxin-conjugated tracers, molecular weight of thyroxin-conjugate, conjugation method.)

## INTRODUCTION

The binding of thyroxin ($T_4$)-analogs to the serum $T_4$-binding proteins (albumin, TBG, prealbumin) raises significant analytical problems in the

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measurement of free thyroxin ($FT_4$) in serum samples by direct immunoassays (1, 2). Several approaches have been suggested in the literature to surpass these difficulties, such as the use of thyroxin conjugated to proteins ($T_4$-conjugates) (3) and the hapten-heterologous conjugates (4, 5). Amongst them, the $T_4$-conjugate tracers have been studied extensively and have been used for the development of commercially available immunoassay kits based on the labeled analog principle. However, contradicting results concerning the binding of $T_4$-conjugates to the $T_4$-binding proteins are not rare in the literature. As it has been reported the binding of $T_4$ conjugates with IgG, horseradish peroxidase (HRP), galactosidase, and chorionic gonadotropin to the $T_4$-binding proteins was insignificant (3, 6, 7, 8), whereas in other reports the binding of $T_4$-IgG and $T_4$-HRP was found to be significant (9-11). It has been speculated that these contradictory results can be ascribed, at least in part, to differences in the M.W. and the structure of the conjugates used in each case as well as to the individual assay conditions. However, to our knowledge, there is no detailed study in the literature concerning the effects of these parameters on the binding of $T_4$-conjugates to the $T_4$-binding proteins.



We report the results of our study concerning the effects of M.W. of the labeled $T_4$-conjugates as well as sample dilution factor on the binding of $T_4$-conjugates to the $T_4$-binding proteins. This effect was estimated by determining the binding potential (C) of serum $T_4$-binding proteins (12) for the conjugates since it allows quantitative comparisons. The binding potential was determined through the influence of $T_4$-binding proteins on the binding of labeled conjugates to the anti-$T_4$ antibody that was used in excess compared with labeled conjugate, following appropiate mathematical interpretation.

## MATHEMATICAL DERIVATIONS

The binding potential (C) of serum proteins for the tracer, defined as the sum of the individual protein concentration multiplied by the protein-binding constant for the tracer ($\Sigma KiPi$) (10) has been shown (12) to be equal to the ratio of the protein bound concentration of the tracer ($[P]_b$) to the free tracer concentration ([F]):

$$C = \frac{[P]_b}{[F]} \qquad (1)$$

At the equilibrium a system containing antibody (Ab), labeled conjugate or $T_4$ (tracer) and $T_4$-binding proteins (P) is described by the law of mass action (equation 2) whereas the distribution of the total tracer in the free, the serum-protein-bound and the antibody-bound portions is expressed by the equation 3.

$$K_{Ab} = \frac{[Ab]_b}{[Ab]\,[F]} \qquad (2)$$

$$[T]=[F]+[P]_b+[Ab]_b \qquad (3)$$

where $K_{Ab}$ is the antibody-binding constant for tracer, $[Ab]_b$ is the antibody-bound concentration of tracer, [Ab] is the concentration of free antibody-binding sites, [T] is total tracer concentration.

The $[Ab]_b$ and the [Ab] are described by the equations

$$[Ab]_b = b[T] \qquad (4)$$

$$[Ab] = [Ab]_T - b[T] \qquad (5)$$

where b is antibody-bound tracer fraction and $[Ab]_T$ is the total concentration of antibody binding sites.

When $[T] << [Ab]_T$ the second term of equation 5 can be neglected, since most of the antibody binding sites are unoccupied and approximately equal to the total sites.

Thus equation 5 becomes : $[Ab]=[Ab]_T$ (6)

Equations 1 and 4 inserted in equation 3 then give

$$[F]=\frac{(1-b)\,[T]}{1+C} \tag{7}$$

Equation 4, 6, 7 inserted in equation 2 then gives

$$\frac{b}{1-b} = \frac{K_{Ab}\,[Ab]_T}{1+C} \tag{8}$$

Since the $K_{Ab}\,[Ab]_T$ is the antibody binding potential (Co) the equation 8 becomes

$$\frac{b}{1-b} = \frac{Co}{1+C} \tag{9}$$

and without serum proteins (C=0), we get :

$$Co=\frac{bo}{1-bo} \tag{10}$$

where bo is the antibody-bound tracer fraction in the absence of serum.

Combing the equations 9 and 10, we obtain:

$$C=\frac{bo/(1-bo)}{b/(1-b)}-1 \tag{11}$$

Thus, in the case that the antibody binding sites are in great excess compared with the concentration of the labeled conjugate used, from equation 11, one can calculate the value of C at any serum protein concentration for each conjugate and for the $T_4$ by measuring the antibody-bound fraction of tracer in the absence (bo) and in the presence (b) of serum.

## MATERIALS AND METHODS

### Materials

DL-Thyroxin ($T_4$) sodium salt, horse spleen apoferritin, horse spleen ferritin, bovine thyroglobulin, gel filtration molecular weight markers, albumin reagent [BCG] and protein assay kit were obtained from Sigma Chemical Co (St.Louis, MO). Human serum IgG, human blood plasma transferrin were from Fluka Biochemica (Fluka Chemie AG, Buchs, Schweiz). p-benzoquinone was from Ferac Berlin. Rabbit antiserum to $T_4$ was from UCB-bioproducts (Brussels, Belgium). Free $T_4$-by Equilibrium Dialysis kit was bought from Nichols Institute Diagnostics (San Juan Capistrano, CA). Sephadex G-25, Sephadex G-75 and Sephacryl S-300 were obtained from Pharmacia (Uppsala, Sweden). $T_4$-solid RIA kit and sheep antiserum to rabbit γ-globulin were products of the I/R-R.P, N.C.S.R "Demokritos".



### Human Serum not Containing $FT_4$.

Human serum not containing $FT_4$ (zero standard) was prepared by the method of activated charcoal (13). After removal of the $T_4$ sodium azide was added as preservative at a final concentration of 0.5 g/L. No $FT_4$ was detectable in this preparation using Free $T_4$-by equilibrium dialysis kit. When required the zero standard was diluted with 0.1 mol/L phosphate buffer, pH 7.4, containing 1g/L of gelatine. The concentration of albumin was determined by bromocresol green [BCG] method in each dilution and in the undiluted zero standard.

### Preparation of $T_4$-protein Conjugates.

$T_4$ was conjugated to the proteins following the p-benzoquinone (PBQ) method (14) slightly modified. Briefly 3 mg of IgG and 2 mg of either transferrin or ferritin

or apoferritin or thyroglobulin were dissolved in 0.2 mL of 0.25 mol/L phosphate buffer, pH 6.0, containing 0.15 mol/L NaCl. Appropriate volume of freshly prepared PBQ solution (30 g/L) in ethanol was added to each one of the above solutions. The volumes added were 0.05 mL for transferrin and ferritin solutions, 0.04 mL for IgG solution, 0.015 mL for apoferritin solution and 0.01 mL for thyroglobulin solution. After addition of the PBQ solution the mixtures were incubated for 60 min in the dark at room temperature. The excess of PBQ was removed by passage through a Sephadex G-25 column (1X30 cm) equilibrated with 9 g/L NaCl. The column was eluted with the same solution and fractions of 0.6 mL were collected. The fraction containing the protein-PBQ (0.6 mL) is collected and 0.06 mL of a $T_4$ solution (1 g/L) in 0.1 mol/L carbonate buffer, pH 9.2 was added and the mixture was incubated overnight at room temperature. Then, it was applied to Sephadex G-75 column (1X40 cm) that had been equilibrated with 0.05 mol/L carbonate buffer, pH 9.2, containing 0.5 g/L sodium azide. The column was eluted with the same buffer and 0.6 mL fractions were collected. The protein elution profile was estimated by measuring the optical density of the fractions at 280nm while the $T_4$ elution profile was obtained by determining the $T_4$ using the $T_4$-solid RIA kit. The fractions with the maximum optical density and the highest value of $T_4$ were collected and stored at 4 $^{o}$C. Especially, for $T_4$-Transfferin conjugate, the fraction obtained from the Sephadex G-75 colunm was then applied to a Sephacryl S-300 colunm (1X100 cm) that had been equilibrated and eluted with 0.05 mol/L carbonate buffer, pH 9.0. Two fractions containing $T_4$-Transferrin conjugates with different M.W.s called thereafter $T_4$-Transferrin-1 and $T_4$-Transferrin-2, were obtained.

Characterization of the Conjugates.

Determination of molar ratio of $T_4$ to the carrier protein, the concentrations of the protein-PBQ derivatives, and of the $T_4$-conjugates were determined by the method of Lowry (15) using standard solutions of the respective proteins. Both the $T_4$-conjugates and the protein-PBQ derivatives were then diluted to a final concentration of 0.25 mg/mL and the absorbance of those solutions was determined at 280 nm. The concentration of the $T_4$ coupled on the proteins in the $T_4$-conjugates solutions was determined by substracting the absorbance of the protein-PBQ solution from the absorbance of the corresponding $T_4$-conjugate solution and dividing the subtract by the molar absorptivity of $T_4$ (3900 L $mol^{-1}$ $cm^{-1}$). Then the molar ratio of the $T_4$ to the carrier protein can be calculated by dividing the estimated $T_4$ concentration by the protein concentration of $T_4$-conjugate.

The molecular weight of the conjugates was estimated by means of gel filtration chromatography using a column (1X100 cm) of Sephacryl S-300 that had been equilibrated with 0.05 mol/L carbonate buffer, pH 9.0. The column was eluted with the same buffer and the elution of the M.W. markers and conjugates have been followed by readings at 280 nm. The elution volume of the M.W. markers and of conjugates was determined by measuring the volume of effluent collected from the point of sample application to the center of the effluent peak.

Radioiodination of $T_4$-conjugates.

The conjugates were radioiodinated by standard procedure with chloramine-T. The free iodide was removed by a Sephadex G-75 column (1X40 cm). The column was eluted with 0.1 mol/L phosphate buffer, pH 7.4, containing 1g/L of gelatin and 0.5 g/L of sodium azide. The fraction containing $^{125}I$-labeled conjugates

(and $^{125}$I-$T_4$) was diluted with the same buffer at approximately 0.75 μCi/mL, to provide 130,000-140,000 cpm per 100 μL of the tracer solution.

Determination of Total Antibody Binding sites.

Fifty μL of a dilution series of $T_4$ with concentrations ranging between 20-500 nmol/L in 0.1 mol/L phosphate buffer, pH 7.4, containing 1g/L of gelatin were incubated with 100 μL of anti-$T_4$ antiserum (diluted 100-fold with the same buffer) and 100 μL of $^{125}$I-$T_4$ ($5x10^{-10}$ mol/L) for 30 min at room temperature. One mL of sheep antiserum to rabbit γ-globulin was added (second antibody, diluted 33-fold) and the mixture was incubated for 15 min at room temperature. The mixture was centrifuged for 15 min at 1850 X g, the supernatant was decanted, and the radioactivity of the precipitate, representing the bound fraction of tracer to the antibody, was determined. The concentration of antibody binding sites was determined by plotting the reciprocal of bound antigen versus the reciprocal of free antigen according to the method of Steward and Petty (16).



Determination of Serum and Antibody Binding Potential for the Labeled $T_4$-conjugates.

Fifty μL of either 0.1 mol/L phosphate buffer, pH 7.4 containing 1 g/L of gelatin or zero standard or dilutions of it were incubated with 100 μL of $^{125}$I-labeled $T_4$-conjugate or $^{125}$I-labeled $T_4$ (tracer) and 100μL of anti-$T_4$ antiserum (diluted 100-fold with 0.1 mol/L phosphate buffer pH 7.4, containing 1g/L of gelatin), for 30 min at room temperature before the separation of the free from the antibody bound fraction of the tracers. The separation was performed by the second antibody method described for the determination of the antibody binding

sites. Blank values were obtained for each one of the zero standard dilutions and for the buffer replacing the anti-$T_4$ antiserum by the antibody diluent. The antibody-bound tracer fraction (b) at each concentration of serum and in the absence of serum (bo) was determined after correction for blank values. The serum and antibody binding potentials, C and Co, respectively, were calculated with use of equations 11 and 10.

The relative antibody-binding constants for $T_4$-conjugates with respect to $T_4$ ($K_{Ab,conj}/K_{Ab,T4}$) were obtained by dividing the antibody binding potential for each conjugate ($Co,_{conj}$) by the antibody binding potential for $T_4$ ($Co,_{T4}$) both determined in absence of serum as described above. These parameters are equal to $K_{Ab,conj}$ $[Ab]_T$, and $K_{Ab,T4}$ $[Ab]_T$, respectively. Since the concentration of the total antibody binding sites used throughout this study was the same for all the tracers, the ratio $Co,_{conj}/Co,_{T4}$ was equal to the ratio $K_{Ab,conj}/K_{Ab,T4}$.



## RESULTS

The objective of this study was to determine the effects of the M.W. of the $T_4$-conjugates and of the sample dilution factor on the binding of $T_4$-conjugates to the $T_4$-binding proteins. For this reason we prepared conjugates of $T_4$ with proteins of different M.W. following same reaction conditions in all cases and using PBQ as bifunctional reagent. After purification, characterization (with respect to the M.W and the molar ratio) and radioiodination of $T_4$-conjugates we determined the relative antibody binding constant for each one of them. From the conjugates prepared, we selected for our study those presenting great difference in their M.W. and more or less similar antibody-binding constant. Finally, in order to achieve quantitatively comparable results and thus to investigate the effect of M.W. of the

conjugates and of serum dilution, we determined the binding potential of serum (C) for each one of $T_4$-conjugates.

Preparation and Characterization of the $T_4$-conjugates.

The preparation of the $T_4$-conjugates using PBQ as bifunctional reagent was found to be reliable, repeatable, and provided conjugates that were stable for at least 6 months when stored at 4°C.

The $T_4$-conjugates were characterized in terms of M.W., relative $K_{Ab}$ and molar ratio of $T_4$ per conjugate molecule (Table 1). Elution profiles of the $T_4$-conjugate preparations obtained from Sephacryl S-300 column indicated that almost all of the preparations (except $T_4$-Transferrin) were homogeneous. As it is shown in Table 1, the M.W.s of $T_4$-conjugates with thyroglobulin, ferritin and apoferritin were almost equal to those of the parent molecules, whereas the M.W. of $T_4$-IgG was estimated to be approximately 1,000,000, indicating that it was a polymer of IgG (seven IgG per molecule of conjugate). Considering the $T_4$-Transferrin preparation we found that it was possible to isolate two distinct fractions containing $T_4$-conjugate using the Sephacryl S-300 colunm. We found that the one fraction contained a conjugate with a M.W. of 210,000 ($T_4$-Transferrin-1) indicating that it was mainly a dimer of transferrin, whereas the second fraction contained a conjugate ($T_4$-Transferrin-2) corresponding to a transferrin monomer with a M.W. of 90,000 .

We also found that the molar ratio of $T_4$ to the carrier protein was not necessarily correlated with the $K_{Ab}$. For example $T_4$-Transferrin-2 with molar ratio of 5 provided almost 3-fold higher relative $K_{Ab}$ compared with that of $T_4$-Apoferritn with molar ratio of 7, whereas same relative $K_{Ab}$ values were

TABLE 1

Characterization of $T_4$-conjugates.

| $T_4$-conjugates | bo[a] | Relative binding constant to anti-$T_4$[b] | M.W. of conjugate | Molar ratio[c] |
|---|---|---|---|---|
| $T_4$-IgG | 0.37 | 58.7 | 1000 000 | 28 |
| $T_4$-Thyroglobulin | 0.34 | 51.5 | 669 000 | 12 |
| $T_4$-Ferritin | 0.32 | 47 | 450 000 | 28 |
| $T_4$-Transferrin-1 | 0.32 | 47 | 210 000 | 9 |
| $T_4$-Transferrin-2 | 0.30 | 43 | 90 000 | 5 |
| $T_4$-Apoferritin | 0.13 | 15 | 450 000 | 7 |
| $T_4$ | 0.50 | 100 | - | - |

a: bo, antibody-bound tracer fraction in absence of serum
b: $K_{Ab\cdot conj}/K_{Ab\cdot T4}$ X100
c: $T_4$ molecules per molecule of conjugate



obtained for $T_4$-Transferrin-1 and $T_4$-Ferritin with molar ratios of 9 and 28, respectively (Table 1).

Binding Potential of Serum Proteins for the $T_4$-conjugates.

For the determination of the serum binding potential (C), we used great excess of the antibody binding sites ($6x10^{-9}$ mol/L) compared with the concentration of the labeled conjugates (ranged between $2x10^{-11}$ to $6x10^{-11}$ mol/L) in order to meet the requirements described in the Mathematical Derivation section.

As was expected the binding of the $T_4$-conjugates prepared with the antibody was significantly less affected by the presence of serum $T_4$-binding proteins than that of $T_4$. The influence of increasing concentration of the $T_4$-binding proteins on the binding of the labeled conjugates and of the $^{125}I$-$T_4$ to anti-$T_4$ antibody is presented in Figure 1. We found that the binding of $T_4$-IgG conjugate with the antibody was scarcely affected by concentrations of $T_4$-binding proteins ranging between 1 and 9.6 mg/mL in the final incubation mixture, whereas the binding of the other conjugates to anti-$T_4$ antibody was affected to different degrees by increasing the concentration of $T_4$-binding proteins. Especially for the $T_4$-Transferrin-1, $T_4$-Transferrin-2 and $T_4$-Ferritin conjugates we observed a sharp decrease of their binding to the anti-$T_4$ antibody at the lowest serum protein concentration used in this study, whereas the decrease observed using higher concentrations of serum proteins was smoothed down. For those conjugates that bind in a certain degree to the $T_4$-binding proteins the sharp decrease observed at the lower serum protein concentration was, to some extent, expected. This decrease can be ascribed to the fact that even at this serum dilution the $T_4$-binding proteins contained in the incubation mixture were in great excess compared with the concentration of $T_4$-conjugate, and thus they presented a decreased pattern similar to that observed for $^{125}I$-$T_4$.

Using these results we calculated the binding potential of serum $T_4$-binding proteins for each conjugate by equation 11. The serum binding potentials for the conjugates, calculated at the highest protein concentration used, are presented in Table 2. The C value for the $T_4$-IgG conjugate was very low, compared with the C value calculated for all the other conjugates prepared, and it was 150-fold lower than the C value for $T_4$. The serum binding potential for the conjugates was

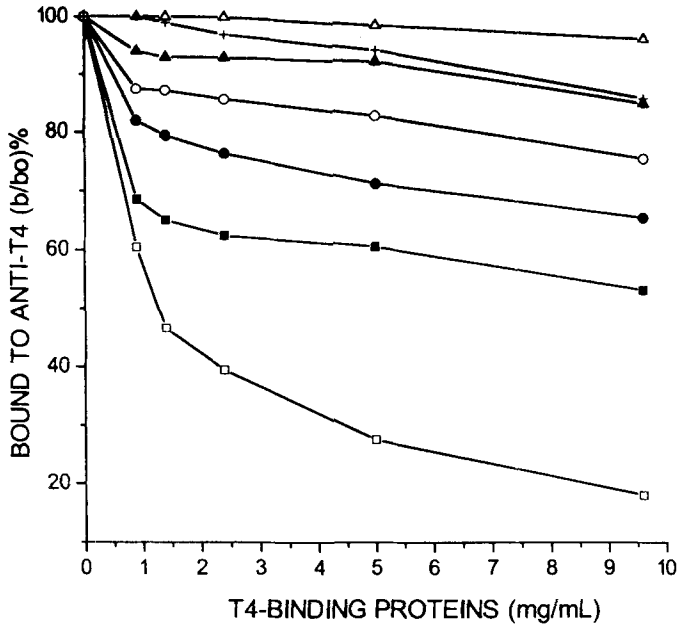


FIGURE 1. Effect of increasing concentrations of $T_4$-binding proteins on the binding of $T_4$-conjugate tracers and of labeled $T_4$ to anti-$T_4$ antibody. The concentration of the $T_4$-binding proteins is approximately equal to the concentration of total albumin. (□) $T_4$; (■) $T_4$-Transferrin-2; (●) $T_4$-Transferrin-1; (○) $T_4$-Ferritin; (▲) $T_4$-Apoferritin; (+) $T_4$-Thyroglobulin; (Δ) $T_4$-IgG.



decreased as the M.W. was increased with the exception of $T_4$-Apoferritin which had a significantly lower $K_{Ab}$ compared to the other conjugates, however. Moreover, as it is shown in Figure 2 the C values determined for the conjugates with similar $K_{Ab}$, at three different serum protein concentrations, are inversely proportional to the log of the M.W. of the conjugates. This finding was also true for the other serum protein concentrations used in this study (data not shown).

The results presented in Figure 2 indicate that the dilution of the sample could cause a decrease of the binding potential of serum proteins for a conjugate, although

TABLE 2

Binding potential of $T_4$-binding proteins calculated for the conjugates at the maximun protein concentration used in this study.

| $T_4$-conjugates | Binding potential of serum $T_4$-binding proteins (C) |
|---|---|
| $T_4$-IgG | 0.06 |
| $T_4$-Thyroglobulin | 0.25 |
| $T_4$-Ferritin | 0.47 |
| $T_4$-Transferrin-1 | 0.77 |
| $T_4$-Transferrin-2 | 1.24 |
| $T_4$-Apoferritin | 0.22 |
| $T_4$ | 9.00 |



to a lesser degree compared with the decrease that could be obtained by increasing the M.W. of the conjugate. In fact, the C value obtained for $T_4$-Transferrin-2 at 1 mg/mL of serum protein concentration corresponded to 63% of the C value obtained at 9.6 mg/mL, whereas by increasing the M.W. of the conjugates from 90,000 to 1,000,000 (11-fold increase) the C value of the conjugate was decreased approximately 21-fold at 9.6 mg/mL of serum protein concentration. However considering the conjugates presenting low C values, $T_4$-IgG and $T_4$-Thyroglobulin, practically zero C values were obtained for serum protein concentrations of equal or less than 2.4 and 1.4 mg/mL, respectively.

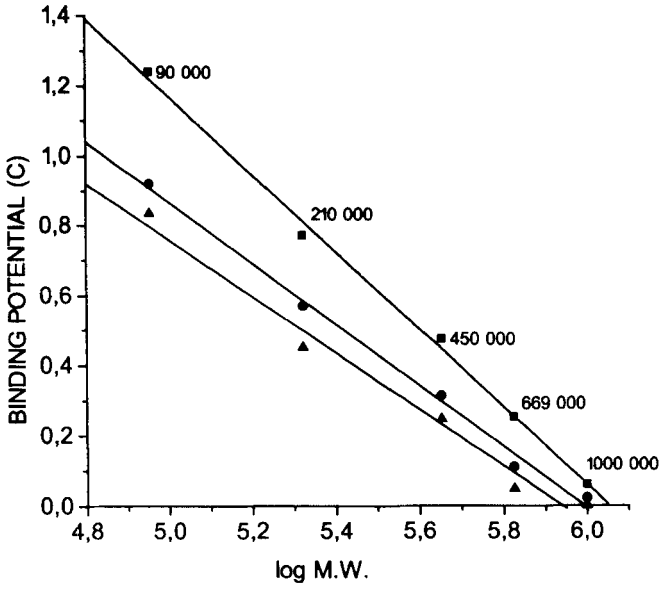


FIGURE 2. Plot of the binding potential values (C) of serum $T_4$-binding proteins determined in three different serum protein concentrations in the final incubation mixture against the log of the M.W. of $T_4$-conjugates. (■) 9.6 mg/mL; (●) 5.0 mg/mL; (▲) 2.4 mg/mL



## DISCUSSION

The development of $T_4$-conjugate tracers that bind slightly or not at all to the $T_4$-binding proteins is suggested that could lead to a significant advance in immunoassays for measurement of $FT_4$ by means of the labeled-analog method (2). However, the results reported for some $T_4$-conjugate tracers are to some extent confusing (8-11). This is possibly due, to the fact that these $T_4$-protein conjugates are not easily compared, since the methods for their preparation as well as the assay conditions used for their study were considerably different. Additionally, no detailed characterization of these conjugates has been described.

We present the results of our study concerning the effects of the M.W. of the conjugates and of the sample dilution on the binding of $T_4$-conjugates to the $T_4$-binding proteins. This effect was estimated by determining the binding potential (C) of serum $T_4$-binding proteins for the labeled conjugates. As it has been reported (10) the calculated binding potential of serum is a very important parameter from the point of view of the utility of the labeled $T_4$-conjugate as a possible tracer for use in a $FT_4$ assay. The magnitude of this parameter, and consequently the $FT_4$ assay performance, is affected by variations in serum $T_4$-binding proteins concentrations, by inherited changes of the binding affinities of these proteins, and by the presence of binding competitors. Thus, the C value for a tracer must be as low as possible (optimal value is zero) to overcome the above interferences in the assay results (2).



For the needs of our study we prepared conjugates of $T_4$ with proteins of different M.W. using the same conjugation conditions, and we carried out the binding studies under the same assay conditions to facilitate comparisons. Additionally the different serum protein concentrations used in the final reaction mixture were selected so as to cover a wide range of protein concentrations used in a certain number of published $FT_4$ immunoassays.

Considering the effect of the M.W. of the conjugates on the serum binding potential (C), the results of our study based on comparisons carried out using $T_4$-conjugates with similar affinity for anti-$T_4$ antibody indicated that there was a strong relationship between the C and the M.W. of the conjugates since we found that the C values were inversely proportional to the log of M.W. of the conjugates. Thus, increasing the M.W. of the conjugates from 90,000 to 1,000,000 the C value was decreased 21-fold at a serum concentration of 9.6 mg/mL.

As it was expected when serum protein concentration was decreased (increasing dilution of serum) the C value was also decreased. However, even when a 50-fold dilution of serum was used in the final incubation mixture the calculated C value of serum proteins for $T_4$-Transfferin-1, $T_4$-Transferrin-2 and $T_4$-Ferritin were found to be significantly higher compared with the C value obtained for $T_4$-IgG conjugate at only 5-fold final serum dilution (data not shown). The same findings were obtained for $T_4$-Thyroglobulin conjugate compared with $T_4$-Transferrin-1, $T_4$-Transferrin-2. This indicated that although the sample dilution in the final incubation mixture can be helpful, significant increase of the M.W. of a conjugate should be a much more effective way to decrease the undesirable binding of the labeled conjugate to the $T_4$-binding proteins. Nevertheless, using conjugates with M.W. higher than 650,000, we found that it was possible to achieve practically zero C values with 20-fold sample dilution in the final incubation mixture, thus avoiding high dilutions of the sample which is not recommended for a valid $FT_4$ assay (4).

Considering the results obtained using $T_4$-Ferritin and $T_4$-Apoferritin conjugates with molar ratios of 28 and 7 respectively, we observed that when the molar ratio was decreased 4-fold the C value decreased 2-fold, whereas a greater decrease (approximately 3-fold) of the binding of the conjugates to the antibody was observed. Thus, it seems that the molar ratio lacks the flexibility to allow convenient manipulations for development of $FT_4$-immunoassay. However, since these results are isolated only across two conjugates, further examination of this parameter, using a greater number of conjugates, will be informative.

Amongst the $T_4$-conjugates prepared, it was observed that the $T_4$-IgG was the one providing very low binding to the serum $T_4$-binding proteins and satisfactory binding to the antibody. Using this conjugate we developed an immunoassay for the

measurement of $FT_4$ in human serum. A 5-fold sample dilution in the final incubation mixture and 200-fold diluted anti-$T_4$ antibody was used. Preliminary results indicated that this assay is accurate (data not shown), sensitive (0.7 pmol/L), reproducible (inter- and intra-assay CVs 8.5-13.2% over the range of the standards) with a dynamic range of 1-120 pmol/L. This assay is now under evaluation.

In conclusion, we studied the effects of the M.W. of the $T_4$-conjugates and of the sample dilution factor in the final incubation mixture on the binding potential (C) of serum $T_4$-binding proteins. We found that $T_4$-conjugates presenting similar affinity to bind with the anti-$T_4$ antibody, the C was inversely proportional to the log of the M.W. of the conjugates. When the M.W. of the conjugates was greater than 650,000, very low to near zero C values were obtained. For the laters, we found that with relatively small sample dilution in the final incubation mixture (approximately 20-fold) the undesirable binding with the serum $T_4$-binding proteins was practically eliminated. Amongst the conjugates prepared, the $T_4$-IgG appeared to be the most promising for development of $FT_4$ immunoassay according to our preliminary results. These findings should be of significant assistance for designing new optimized $T_4$-conjugates and assay formats for the development of $FT_4$ immunoassays essentially independent of the presence of serum $T_4$-binding proteins.



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