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Effects of Furosemide and Oleic Acid On Thyroxine Binding to Isolated Pairs of Thyroxine Binding Serum Proteins

Deborah Hustead

ABSTRACT

EFFECTS OF FUROSEMIDE AND OLEIC ACID
ON THYROXINE BINDING TO ISOLATED
PAIRS OF THYROXINE BINDING SERUM PROTEINS

by

Deborah Hustead

Dosage dependent binding inhibition and redistribution of thyroxine (T_4) between three pairs of thyroxine binding serum proteins, TBG-TTR, TBG-albumin and TTR-albumin, by furosemide (0.0 to 1.0 mol/L) and oleic acid (0.0 to 0.10 mol/L) were studied using equilibrium dialysis with purified binding proteins at concentrations 1/200 of levels in normal human sera, separated across the dialysis membrane and competing for T_4.

Furosemide (1.0 mol/L) reduced TBG bound T_4 in both the TBG-TTR system and TBG-albumin system (>90% decrease) and inhibited T_4 binding to TTR in the TTR-albumin system (68% decrease) and to albumin in the TBG-albumin system (44% decrease). T_4 binding inhibition at 1.0 mol/L furosemide was accompanied by increases in free T_4 (FT_4): TBG-TTR system, 1063%; TBG-albumin system, 220%; and TTR-Albumin system, 334%.

Oleic Acid had no effect on T_4 binding to TBG in the TBG-TTR system, and minimal effect on T_4 binding to TBG in the TBG-albumin system. Significant inhibition of T_4 binding to TTR was observed in the TBG-TTR (47% decrease) and TTR-albumin (59% decrease) systems at 0.10 mol/L oleic acid concentration. Albumin-T_4 binding was inhibited in the TTR-albumin system. FT_4 increased in response to inhibition of binding at 0.10 mol/L in all oleic acid assays.

Repartitioning of T_4 between T_4 binding proteins occurred with both furosemide and oleic acid. In the furosemide assays, a decrease in TBG bound T_4 (38%) was coupled with an increase in TTR bound T_4 (29%) in the TBG-TTR system at 1.0 mol/L furosemide. In the TTR-albumin system, a concurrent decrease in TTR bound T_4 (68%) and increase in albumin bound T_4 (67%) was observed at 1.0 mol/L furosemide. Repartitioning of $[^{125}I]T_4$ between binding proteins by oleic acid was observed in the TBG-albumin system by a 3% decline in TBG bound T_4 and 64% increase in albumin bound T_4 at 0.10 mol/L oleic acid.

These data confirm that furosemide and oleic acid are potent inhibitors of T_4 binding to purified serum proteins *in vitro*. Increases in FT_4 concentrations resulting from displacement of T_4 from proteins occurred above physiologic concentrations of oleic acid in the TBG-TTR, TBG-albumin, and TTR-albumin systems. The finding that oleic acid displaced T_4 only from TTR and albumin suggests that oleic

acid cannot generate the inhibition of T_4 binding to TBG characteristic of NTI.

LOMA LINDA UNIVERSITY

Graduate School

EFFECTS OF FUROSEMIDE AND OLEIC ACID ON THYROXINE BINDING TO ISOLATED PAIRS OF THYROXINE BINDING SERUM PROTEINS

by

Deborah Hustead

A Thesis in Partial Fulfillment

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Science in Biology

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Each person whose signature appears below certifies that this thesis in their opinion is adequate, in scope and quality, as a thesis for the degree Master of Science.

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INTRODUCTION

Circulating inhibitors of thyroxine (T_4) binding may be responsible for the elevated serum free fraction of T_4 (free T_4/total T_4) in acute non-thyroidal illness (NTI) (1-6). Critically ill humans which are clinically euthyroid, but with abnormal serum thyroid hormone levels are referred to as non-thyroidally ill. Elevated FT_4, decreased total T_4 (TT_4), total triiodothyronine (TT_3), and free T_3 (FT_3) concentrations characterize NTI(7). In normal circulation, thyroxine is extensively bound to serum proteins. Thyroxine binding globulin (TBG) binds approximately 75%, transthyretin (TTR) 15%, and albumin 10% of circulating T_4 (8). Less than 0.1% of total T_4 in normal serum is free and unbound to serum proteins (7). The effective affinity constant (M^{-1}) of T_4 binding is 1.7×10^{10} for TBG, 2.3×10^{8} for TTR, and 6.2×10^{5} for albumin (9).

Serum TT_4 concentration is determined by serum FT_4 concentration. The anterior pituitary responds to decreases in serum FT_4 by secreting thyroid stimulating hormone (TSH). The thyroid gland releases T_4 into serum in response to TSH, resulting in elevated TT_4. Elevations in serum FT_4 inhibit TSH release, and consequently decrease TT_4 in serum. The coupled FT_4 elevation and decrease in TT_4 observed in acute NTI has been suggested to be caused in part by T_4 displacement from TBG, since total displacement of T_4 from

TTR and albumin is inadequate to produce the magnitude of T_4 attenuation observed in NTI (10-12).

Various drugs (13-16) and non-esterified fatty acids (NEFA) (10,17,18) have been reported to increase serum free T_4 *in vivo* and *in vitro*. Potent T_4 binding inhibition by oleic acid, a non-esterified fatty acid, coupled with its elevated serum concentration in NTI (19), has led to the hypothesis that it is an endogenous circulating inhibitor responsible for the low T_4 state of NTI (1,19-21). Albumin is the major binding entity of oleic acid in serum (12) and it modulates the interaction of NEFA with high affinity binders (4,10,22). The low serum concentration of albumin in NTI may promote increased free oleic acid interaction with binding sites on TBG and TTR and consequent T_4 displacement (23). Significant effects of free NEFA (FFA) on normal and NTI serum FT_4 levels have been shown to occur *in vitro* at NEFA:Albumin molar ratios exceeding 5:1 (22), and *in vivo* data indicate an effect on T_4 binding when this ratio is greater than 2 (23-25). Comparison of T_4 displacement by oleic acid in TBG deficient and normal serum *in vitro* have indicated that TTR bound T_4 is more sensitive to displacement by supraphysiologic concentrations of oleic acid than is TBG bound T_4 (22).

Of drug inhibitors, furosemide has potent T_4 displacing ability (13-16,26). Its frequent use in patients exhibiting anomalous T_4 levels in NTI requires that it be

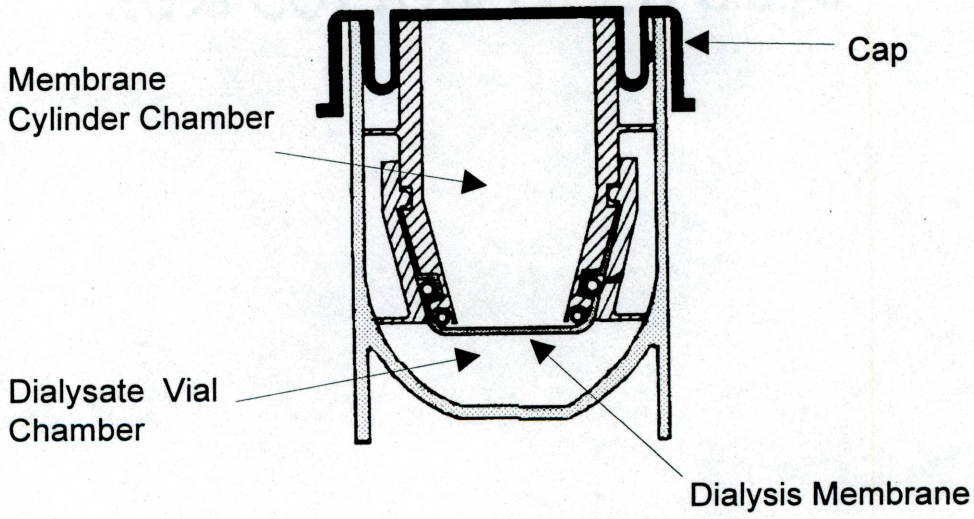
considered a potential causative factor in this syndrome (14). The dominant serum binding site of furosemide is albumin (27), for which it has the highest affinity, but it has been shown to displace T_4 from isolated TBG, TTR and Albumin *in vitro* (14). The abrupt increase in free furosemide at concentrations above the molar concentration of albumin in serum (27) and the direct competition of T_4 for furosemide binding sites on serum TBG (28) suggest that the decrease of serum albumin in NTI may result in increased furosemide interaction with TBG and increase free T_4 in NTI. Since the interactions of competitors with specific T_4 binding sites are difficult to elucidate in the system of multiple T_4 binding sites in serum, this study has employed an *in vitro* system of isolated serum proteins to test differential binding inhibition and redistribution of T_4 by the model inhibitors furosemide and oleic acid. Dose dependent effects of these inhibitors were tested in an equilibrium dialysis system in which purified binding proteins, separated across a dialysis membrane, compete directly for T_4 binding.

METHODS AND MATERIALS

Dialyis Cell Assembly and Treatment

Dialysis cells composed of a dialysate vial, membrane cylinder, and cap (Figure 1)(29) were modified to reduce the dialysate volume to 500 μL and used with equal retentate volumes (500 μL). Plastic components of the dialysis cells were obtained from Nichols Institute (San Juan Capistrano, CA). Dialysis membrane with MW cutoff 12-14 kD (VWR Scientific, Cerritos, CA) was used in the assembly of the membrane cylinder. The dialysis cell assembly, wash and storage protocol of Nelson and Tomei (29) were followed. The membrane compartment was pre-tested for leaks by sensitive radioimmunoassay (RIA) (2) of dialyzed human serum T_4. Membrane cylinders producing dialysate free T_4 values within two standard deviations of the mean were accepted for use in the inhibitor assay. Average FT_4 concentrations for tested cells was 1.58 ± 0.32 ng/dL. Between assay dialysis cell washes involved aspiration of remaining solution, tap water rinse, 2 h rinse in cell washing racks (29) in basin under running tap water, 18 h soak in 5% Radiac Wash Solution (Atomic Products Corp., Center Moriches, Long Island, NY) containing 500 mg/L sodium azide, cotton swabbing membrane cylinder, brushing dialysate vials and caps, tap water rinse, and three final rinses in distilled H_2O.

Figure 1. Inhibitor assay dialysis cell. Figure approximately 1.75 times actual size. From Nelson and Tomei (1988) (Clin Chem 34(9): 1737-1744).


Cap
Membrane
Cylinder Chamber
Dialysate Vial
Chamber
Dialysis Membrane

Dialysis Buffer

The dialysis buffer in which T_4 binding was studied was modeled after that reported by Nelson and Tomei (29) to resemble the chemical composition of the protein free matrix of normal serum. It contained gelatin to minimize adsorption of $[^{125}I]T_4$ to the dialyis cell and sodium azide to prevent bacterial growth. Neither of these reagents are known to affect T_4 binding to serum proteins. A liter of dialysis buffer contained sodium chloride, 5.625 g; monosodium glutamate, 561 mg; sodium lactate (60% solution), 1.0 mL; potassium chloride, 224 mg; magnesium sulfate, 246 mg; urea, 300 mg; gelatin, 500 mg; sodium azide, 520 mg; sodium phosphate (dibasic), 21.294 g; and sodium phosphate (monobasic), 3.622 g.

The buffer used in $[^{125}I]T_4$ purifications was a sodium phosphate solution prepared by the titration of 1L Na_2HPO_4 solution (0.150M) with approximately 175 mL NaH_2PO_4 (0.150M) to pH 7.4 at 37°C. Gelatin was added to 250 mL of this .150M sodium phosphate solution, which was then heated to 95°C with constant stirring to destroy any T_4 binding contaminants in the gelatin. All other reagents were dissolved at 20°C with constant stirring, brought to 1 L volume with sodium phosphate solution and pH adjusted to 7.4 at 37°C with sodium hydroxide or hydrochloric acid. Sodium phosphate (monobasic), sodium phosphate (dibasic),

sodium chloride, sodium lactate and potassium chloride were obtained from Fisher Scientific (Springfield, NJ), magnesium sulfate, urea, and sodium azide from J.T. Baker Chemical Co.(Phillipsburg,NJ), gelatin from Difco Labs (Detroit, MI) and monosodium glutamate from Nutritional Biochemical (Cleveland, OH).

Purification of TBG-[^{125}I]T_4 and TTR-[^{125}I]T_4

[^{125}I]T_4 was obtained from New England Nuclear, Boston, MA. Radioiodide contamination of tracer was removed by predialysis as suggested by Schussler and Plager (30) immediately prior to use in T_4 binding inhibitor assays. On arrival of tracer, TTR-[^{125}I]T_4 and TBG-[^{125}I]T_4 solutions were prepared with [^{125}I]T_4 (~40 μCi/mL) human TTR (0.53 g/L) (9.8×10^{-6} mol/L) (Sigma Chemical Co.) and human TBG (0.0432 g/L) (8.0×10^{-7} mol/L) (Calbiochem-Behring, La Jolla, CA) in PBS Buffer pH 7.4 (Sigma Chemical Co.). Quantitation of residual unlabeled T_4 on the isolated human proteins was not performed. Five hundred μL volumes of TBG-[^{125}I]T_4 and TTR-[^{125}I]T_4 were suspended in membrane cylinders and separately dialyzed against 1 Liter Dulbecco's PBS (Sigma Chemical Co.) containing 520 mg/L sodium azide (Baker) pH 7.4 at 6°C. The dialysate was changed every 24 hours. Maximum removal of radioiodide was indicated by a plateau in dialyzed radioactivity of 24 hr aliquots, reached after 5-7

days dialysis. Fifty μL aliquots of TBG-[^{125}I]T_4 and TTR-[^{125}I]T_4 solutions were diluted to 1 mL and added in 25 μL amounts to the T_4 inhibitor assay. Twenty-five μL aliquots of the protein[^{125}I]T_4 reagents were counted in triplicate to determine cpm added to the assay. [^{125}I]T_4 concentrations (mmol/mL) added to assays were as follows: furosemide TBG-TTR, 6.1 X 10^{-3}; furosemide TBG-albumin, 2.3 x 10^{-3}; furosemide TTR-albumin, 1.5 X 10^{-2}; oleic acid TBG-TTR, 8.2 X 10^{-3}; oleic acid TBG-albumin, 1.3 X 10^{-2}; and oleic acid TTR-albumin, 7.1 X 10^{-2}.

T_4 Inhibitor Assay

The ability of furosemide and oleic acid to displace and redistribute [^{125}I]T_4 between two competing purified human T_4 binding serum proteins was tested in the equilibrium dialysis system. T_4 binding protein pairs were: TBG and TTR, TBG and albumin, and TTR and albumin. Assay concentrations of purified human thyroxine binding proteins were 1/200th normal serum concentrations: TBG; 0.108 mg/L (2.0 X 10^{-9} mol/L), TTR; 1.325 mg/L (2.4 X 10^{-8} mol/L), and serum albumin; 0.0207 g/L (3.1 X 10-7 mol/L). Tracer quantities of [^{125}I]T4 were used in the assays (20,000-150,000 cpm). Furosemide (Sigma Chemical Company) assay concentrations were 0.00, 0.001, 0.01, 0.10, and 1.00 mol/L; oleic Acid (Sigma Chemical Company) assay

concentrations were 0.00, 0.01, 0.03, and 0.10 mol/L. Furosemide and oleic acid solutions (0.1M) were serially diluted with assay buffer to achieve assay concentrations. Furosemide was dissolved in acetone (VWR Scientific) and oleic acid was dissolved in 67% ethanol (VWR Scientific). Final assay concentrations of acetone and ethanol were ≤ 1%.

The assay design employed two groups of dialysis cells per pair of T_4 binding proteins. In group A" dialysis cells, individual proteins of the pair being tested were added to opposite sides of the dialysis membrane in 25 µl aliquots. In "group B" dialysis cells, both proteins were added to the same side of the membrane. Triplicate dialysis cells were set up per group for each pair of proteins tested. All cells were incubated at 37° C in a temperature controlled chamber (Sheldon Mfg, Inc., Portland, OR) for 18 hours. Triplicate aliquots (100 µL) of dialysate (D) and retentate (R) chambers were counted with a TM Analytic triple well gamma counter. All aliquots were counted to ≥10,000 cpm. Background counts were subtracted from each aliquot cpm.

Determination of Protein Bound and Free $[^{125}I]T_4$

Total cpm was determined for each dialysis cell as the sum of total dialysate cpm plus total retentate chamber cpm at equilibrium. Total cpm in dialysates and retentates were

calculated from the average cpm of triplicate aliquots of each. Percent free [^{125}I]T_4 was calculated as the ratio of average dialysate aliquot cpm to total cpm for each "group B" dialysis cell (both proteins on the same side of the membrane). To determine protein bound cpm in "group A" cells (one protein on each side of the membrane) FT_4 average cpm was subtracted from the average cpm on both sides of the membrane. The percentage of protein bound [^{125}I]T_4 was calculated as the ratio of protein bound cpm to total cpm for each "group A" dialysis cell.

Change in Free T_4, Bound T_4, and Bound / Free T_4 Ratio:

Triplicate determinations of the percent [^{125}I]T_4 protein bound and free were used to determine mean and standard error at each inhibitor concentration. Change in FT_4, protein bound T_4, and the bound/free ratio of T_4 for each inhibitor concentration were calculated as a ratio of values at zero inhibitor concentration. These data were normalized by an arcsin transformation. ANOVA and Scheffe's Multiple Range Comparison tests were then used to determine statistical significance (P=0.05) of results.

[^{125}I]T_4 Recovery

The percent of tracer recovery at high inhibitor concentration was calculated as the ratio of average total

cpm at highest inhibitor concentration to average total cpm at lowest inhibitor concentration. Tracer recovery from dialysis cells with no inhibitor present was determined as the ratio of total cpm recovered (n=33) to total cpm added (n=3). By this estimation, tracer recovery was 97%. Percent tracer loss at highest inhibitor concentration, therefore, was approximated as 100% minus percent recovery.

RESULTS

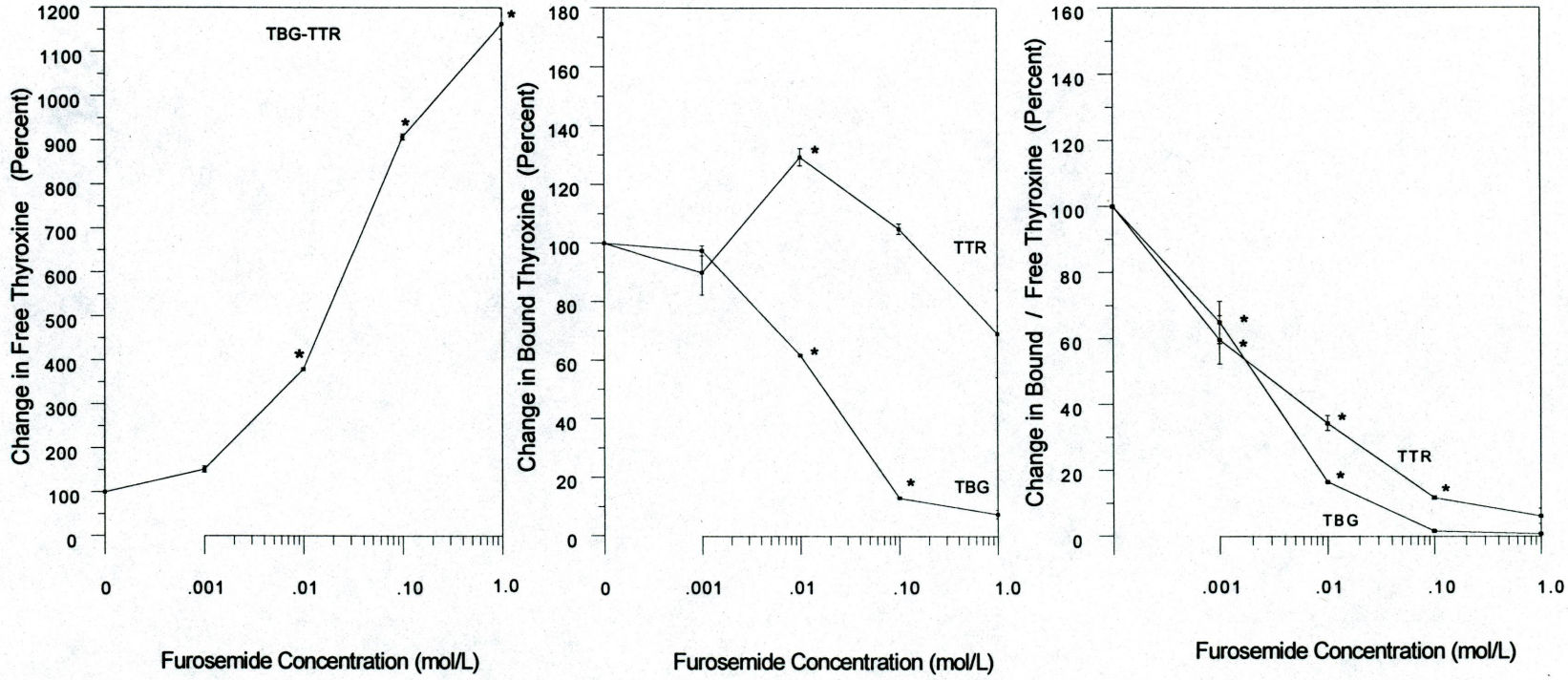
Effects of furosemide on [^{125}I]T_4 binding to TBG

Radical attenuation of [^{125}I]T_4 binding to isolated TBG was observed at assay furosemide concentrations of 0.01, 0.10 and 1.00 mol/L in the TBG-TTR (Figure 2B) and TBG-Albumin (Figure 3B) assays. A 50% decrease in the average percent total [^{125}I]T_4 bound to TBG occurred at 0.01 mol/L furosemide concentration in the TBG-TTR assay and 0.013 mol/L in the TBG-albumin assay. Furosemide concentration of 1.0 mol/L produced almost complete inhibition of T_4 binding to TBG in both assays. Decreases in [^{125}I]T_4 bound to TBG of 93 and 91 percent were observed in the TBG-TTR (Figure 2B) and TBG-albumin (Figure 3B) assays, respectively, in the range of furosemide concentrations studied. In both assays the reduction in TBG bound T_4 was accompanied by a decrease in apparent affinity of TBG for T_4 (Figure 2C)(Figure 3C).

Effects of furosemide on [^{125}I]T4 binding to TTR

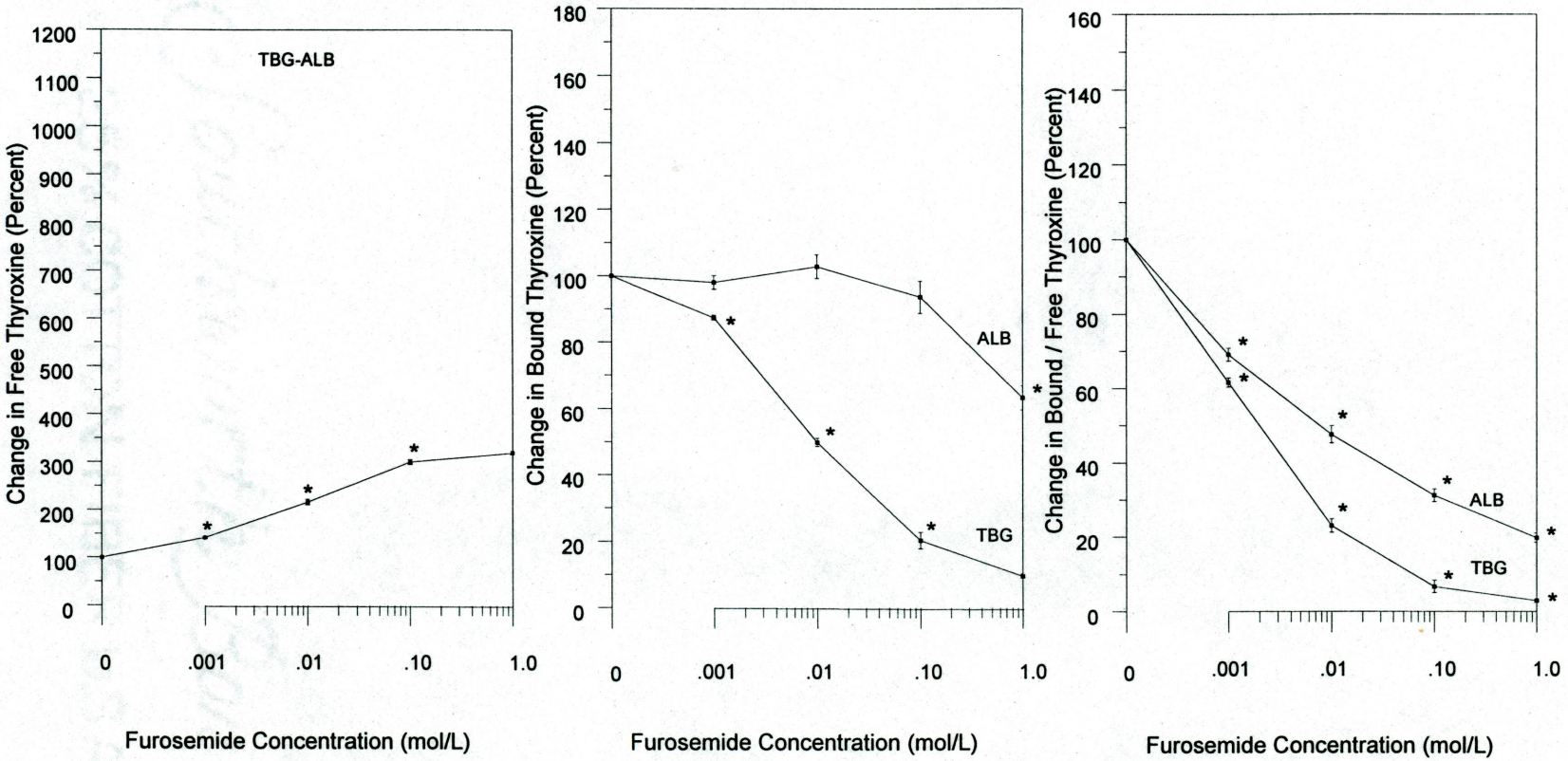
Furosemide significantly reduced [^{125}I]T_4 binding to isolated TTR in the TTR-albumin assay at concentrations greater than 0.01 mol/L. In that assay, furosemide concentrations of 0.10 and 1.0 mol/L produced 37% and 68% reductions in the ratio of TTR bound [^{125}I]T_4 to percent [^{125}I]T_4 bound with no furosemide present (Figure 4B). The decrease in TTR bound T_4 was associated with a decrease in

Figure 2. Effect of serial increments of furosemide on change in $[^{125}I]T_4$ distribution between isolated human thyroxine binding serum proteins thyroxine binding globulin (TBG) and transthyretin (TTR). Change in percent free thyroxine (A), percent bound to TBG and TTR (B), and the ratio of free to bound thyroxine (C) at each inhibitor concentration is expressed as a percentage of corresponding values at zero inhibitor concentration. Assay protein concentrations were 1/200th of normal serum concentrations: TBG (0.108 mg/L) and TTR (1.325 mg/L). $[^{125}I]T_4$ was added in tracer quantity (6.1×10^{-3} mmol/L). Each point is the mean of triplicate determinations and error bars denote standard error of the mean. A * indicates significance (P=0.05) by ANOVA and Scheffe's Multiple Range Comparison.


Change in Free Thyroxine (Percent)
TBG-TTR
Furosemide Concentration (mol/L)
Change in Bound Thyroxine (Percent)
TTR
TBG
Furosemide Concentration (mol/L)
Change in Bound / Free Thyroxine (Percent)
TTR
TBG
Furosemide Concentration (mol/L)

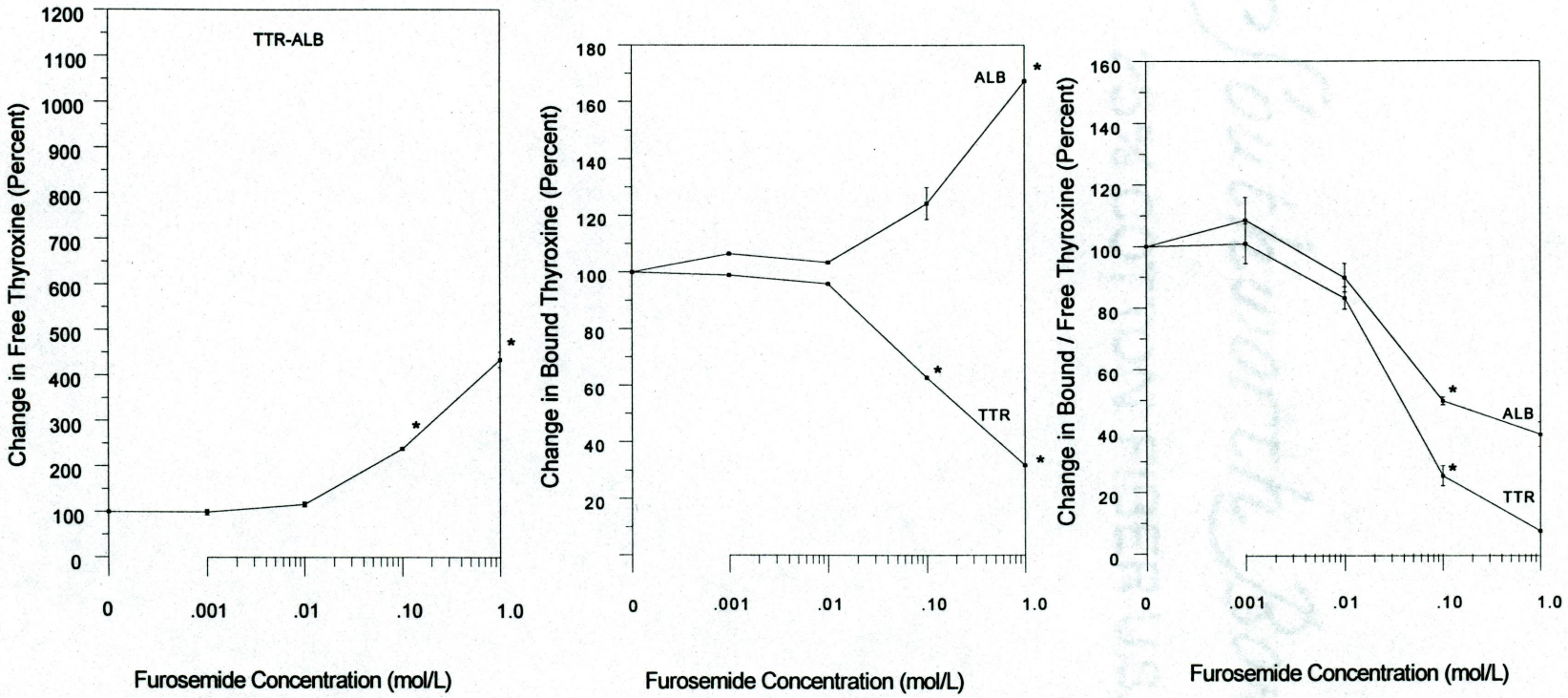
A B C

Figure 3. Effect of serial increments of furosemide on change in $[^{125}I]T_4$ distribution between isolated human thyroxine binding serum proteins thyroxine binding globulin (TBG) and human serum albumin (ALB). Change in percent free thyroxine (A), percent bound to TBG and ALB (B), and the ratio of free to bound thyroxine (C) at each inhibitor concentration is expressed as a percentage of corresponding values at zero inhibitor concentration. Assay protein concentrations were 1/200th of normal serum concentrations: TBG (0.108 mg/L) and ALB (0.207 g/L). $[^{125}I]T_4$ was added in tracer quantity (2.3×10^{-3} mmol/L). Each point is the mean of triplicate determinations and error bars denote standard error of the mean. A * indicates significance (P=0.05) by ANOVA and Scheffe's Multiple Range Comparison.


TBG-ALB
Change in Free Thyroxine (Percent)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
0
.001
.01
.10
1.0
Furosemide Concentration (mol/L)
Change in Bound Thyroxine (Percent)
180
160
140
120
100
80
60
40
20
0
ALB
TBG
Furosemide Concentration (mol/L)
Change in Bound / Free Thyroxine (Percent)
160
140
120
100
80
60
40
20
0
ALB
TBG
Furosemide Concentration (mol/L)

A B C

Figure 4. Effect of serial increments of furosemide on change in $[^{125}I]T_4$ distribution between isolated human thyroxine binding serum proteins transthyretin (TTR) and human serum albumin (ALB). Change in percent free thyroxine (A), percent bound to TTR and ALB (B), and the ratio of free to bound thyroxine (C) at each inhibitor concentration is expressed as a percentage of corresponding values at zero inhibitor concentration. Assay protein concentrations were 1/200th of normal serum concentrations: TTR (1.325 mg/L), and ALB (0.207 g/L). $[^{125}I]T_4$ was added in tracer quantity (1.5×10^{-2} mmol/L). Each point is the mean of triplicate determinations and error bars denote standard error of the mean. A * indicates significance (P=0.05) by ANOVA and Scheffe's Multiple Range Comparison.


TTR-ALB
Change in Free Thyroxine (Percent)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
0
.001
.01
.10
1.0
Furosemide Concentration (mol/L)
ALB
TTR
Change in Bound Thyroxine (Percent)
180
160
140
120
100
80
60
40
20
0
.001
.01
.10
1.0
Furosemide Concentration (mol/L)
Change in Bound / Free Thyroxine (Percent)
160
140
120
100
80
60
40
20
0
ALB
TTR
.001
.01
.10
1.0
Furosemide Concentration (mol/L)

A B C

the apparent affinity of TTR for T_4 , evidenced by the decreased ratio of bound/free T_4 in the TTR-albumin assay (Figure 4C). Furosemide produced no reduction in TTR bound T_4 in the TBG-TTR dose response (Figure 2B) despite reduction in apparent affinity of TTR for T_4 shown by a decrease in the bound/free ratio (Figure 2C). This decrease in apparent affinity was associated with an increase in FT_4 (Figure 2A). A statistically significant increase in the percent total $[^{125}I]T_4$ bound to TTR was observed in this assay at furosemide concentration of 0.01 mol/L (Figure 2B). This increase in binding to TTR represents a 24% increase in percent total $[^{125}I]T_4$ bound to TTR above the percent bound at 0.0 mol/L furosemide.

Effects of furosemide on $[^{125}I]T_4$ binding to Albumin

The percent total $[^{125}I]T_4$ bound to isolated albumin was significantly altered by furosemide in both the TBG-Albumin and TTR-Albumin assays. In the TBG-albumin assay a statistically significant decrease in albumin bound T_4 occurred at 1.0 mol/L furosemide concentration, decreasing albumin bound T_4 37% below binding at zero inhibitor concentration (Figure 3B). The opposite effect, however, was observed for T_4 binding to albumin in the TTR-albumin study. A statistically significant increase in the percent total $[^{125}I]T_4$ bound to albumin was observed in the TTR-Albumin study (Figure 4B) in response to a furosemide

concentration of 1.0 mol/L, producing an increase of 67% over the percent albumin bound tracer with no inhibitor present. This increase in albumin bound T_4 occurred despite the decrease in apparent affinity of albumin for thyroxine evidenced by the decrease in the bound/free T_4 ratio (Figure 4C) and was associated with an increase in FT_4 (Figure 4A).

Effects of furosemide on the percent free [^{125}I]T_4

The percent free [^{125}I]T_4 abruptly increased with furosemide dose in the TBG-TTR, TBG-albumin, and TTR-albumin experiments. In the TBG-TTR study, statistically significant increases in the percent free T_4 of 279%, 808%, and 1063% occurred at furosemide concentrations of 0.01, 0.10 and 1.0 mol/L, respectively, (Figure 2A) coincident with the decline of TBG bound T_4 at these furosemide concentrations (Figure 2B). Similarly, in the study using TBG and albumin as T_4 binding proteins, the % FT4 increased 42%, 116%, 200%, and 220% at 0.001, 0.01, 0.10, and 1.00 mol/L furosemide concentrations (Figure 3A). Furosemide concentrations of .10 and 1.0 mol/L produced FT_4 increases of 138% and 334% over FT_4 at zero inhibitor concentration in the TTR-albumin assay (Figure 4A), concurrent with a decrease in binding to TTR at these furosemide doses (Figure 4B).

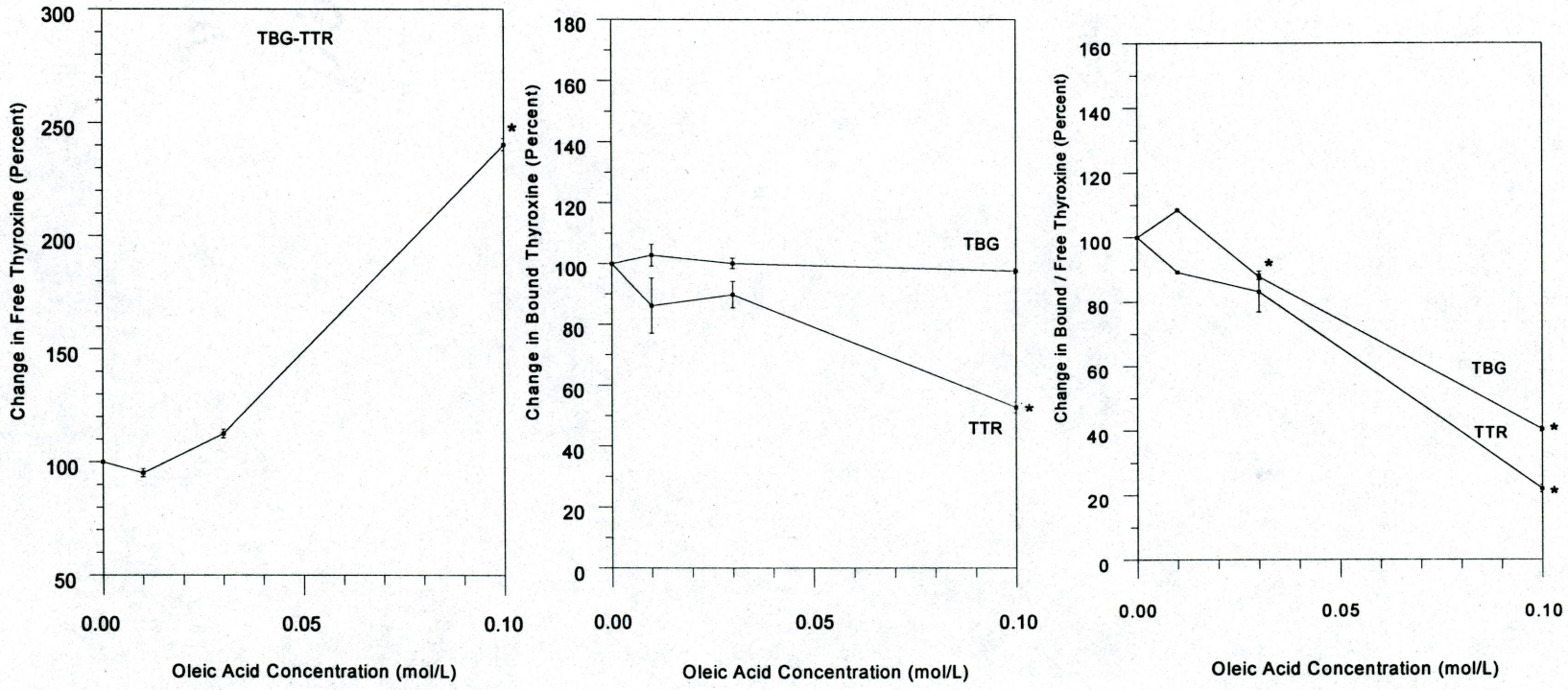
Effects of oleic acid on [^{125}I]T_4 binding to TBG

In the TBG-TTR study, the percent total [^{125}I]T_4 bound to TBG was not affected by oleic acid in the range of concentrations studied (0.0-0.10 mol/L)(Figure 5B), but a decrease in apparent affinity of TBG for T_4 was suggested by the statistically significant decrease in the bound/free T_4 ratio (Figure 5C). In the TBG-albumin assay, a statistically significant 3% decline in percent total [^{125}I]T_4 bound to TBG was noted between 0.00 and 0.10 mol/L oleic acid concentrations (Figure 6B). This decrease in TBG bound T_4 was associated with a decrease in the bound/free T_4 ratio (Figure 6C) which was not statistically significant.

Effects of oleic acid on [^{125}I]T4 binding to TTR

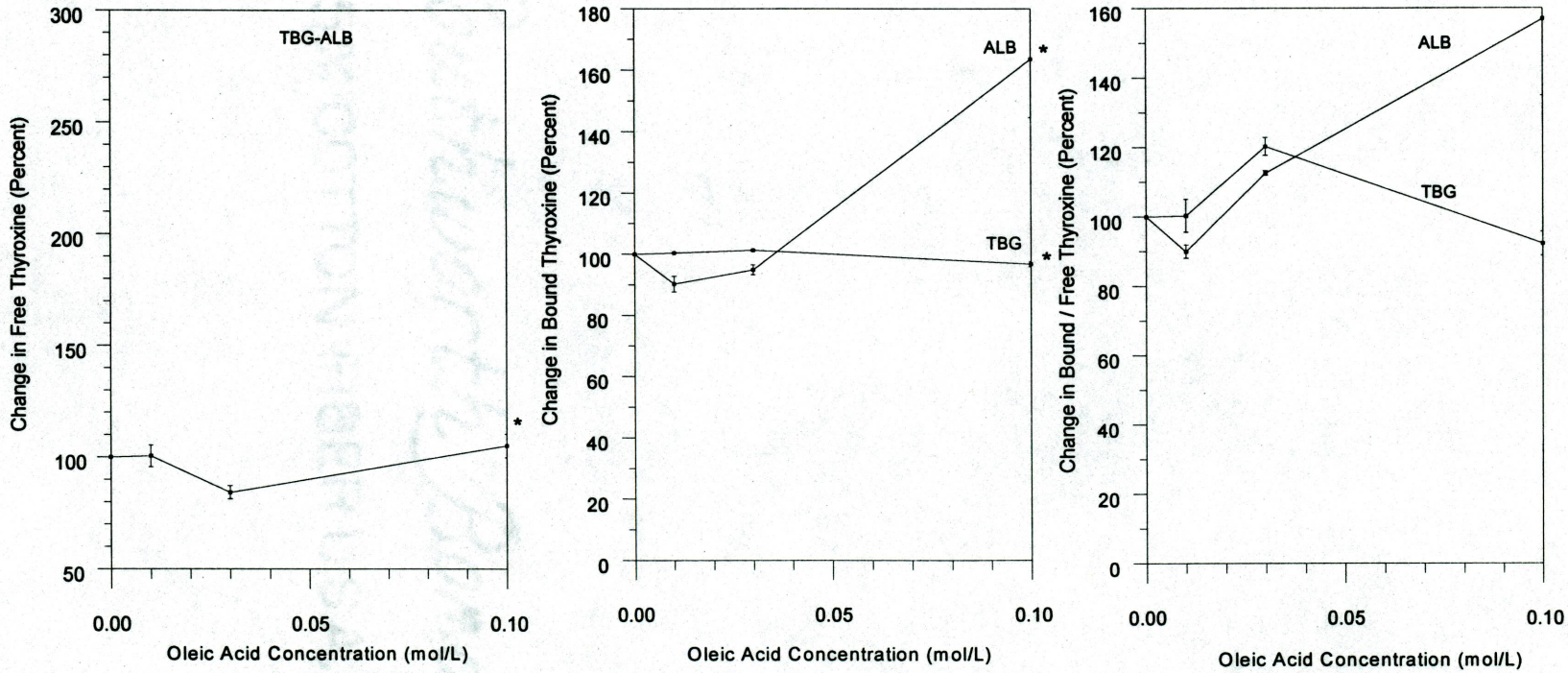
Significant inhibition of [^{125}I]T4 binding to TTR was observed in TBG-TTR and TTR-albumin partitioning assays. There was a 47% TTR binding reduction between 0.0 and 0.10 mol/L oleic acid doses in the TBG-TTR study (Figure 5B) and a 59% reduction in the same inhibitor concentration range in the TTR-Albumin assay (Figure 7B). In both assays the reduction in binding of T_4 to TTR was associated with a decrease in the bound/free T_4 ratio (Figure 5C)(Figure 7C), indicating a decrease in the apparent affinity of TTR for T_4.

Figure 5. Effect of serial increments of oleic acid on change in $[^{125}I]T_4$ distribution between isolated human thyroxine binding serum proteins thyroxine binding globulin (TBG) and transthyretin (TTR). Change in percent free thyroxine (A), percent bound to TBG and TTR (B), and the ratio of free to bound thyroxine (C) at each inhibitor concentration is expressed as a percentage of corresponding values at zero inhibitor concentration. Assay protein concentrations were 1/200th of normal serum concentrations: TBG (0.108 mg/L) and TTR (1.325 mg/L). $[^{125}I]T_4$ was added in tracer quantity (8.2×10^{-3} mmol/L). Each point is the mean of triplicate determinations and error bars denote standard error of the mean. A * indicates significance (P=0.05) by ANOVA and Scheffe's Multiple Range Comparison.


TBG-TTR
Change in Free Thyroxine (Percent)
300
250
200
150
100
50
0.00
0.05
0.10
Oleic Acid Concentration (mol/L)
*
Change in Bound Thyroxine (Percent)
180
160
140
120
100
80
60
40
20
0
TBG
TTR
0.00
0.05
0.10
Oleic Acid Concentration (mol/L)
*
*
Change in Bound / Free Thyroxine (Percent)
160
140
120
100
80
60
40
20
0
TBG
TTR
0.00
0.05
0.10
Oleic Acid Concentration (mol/L)
*
*
*

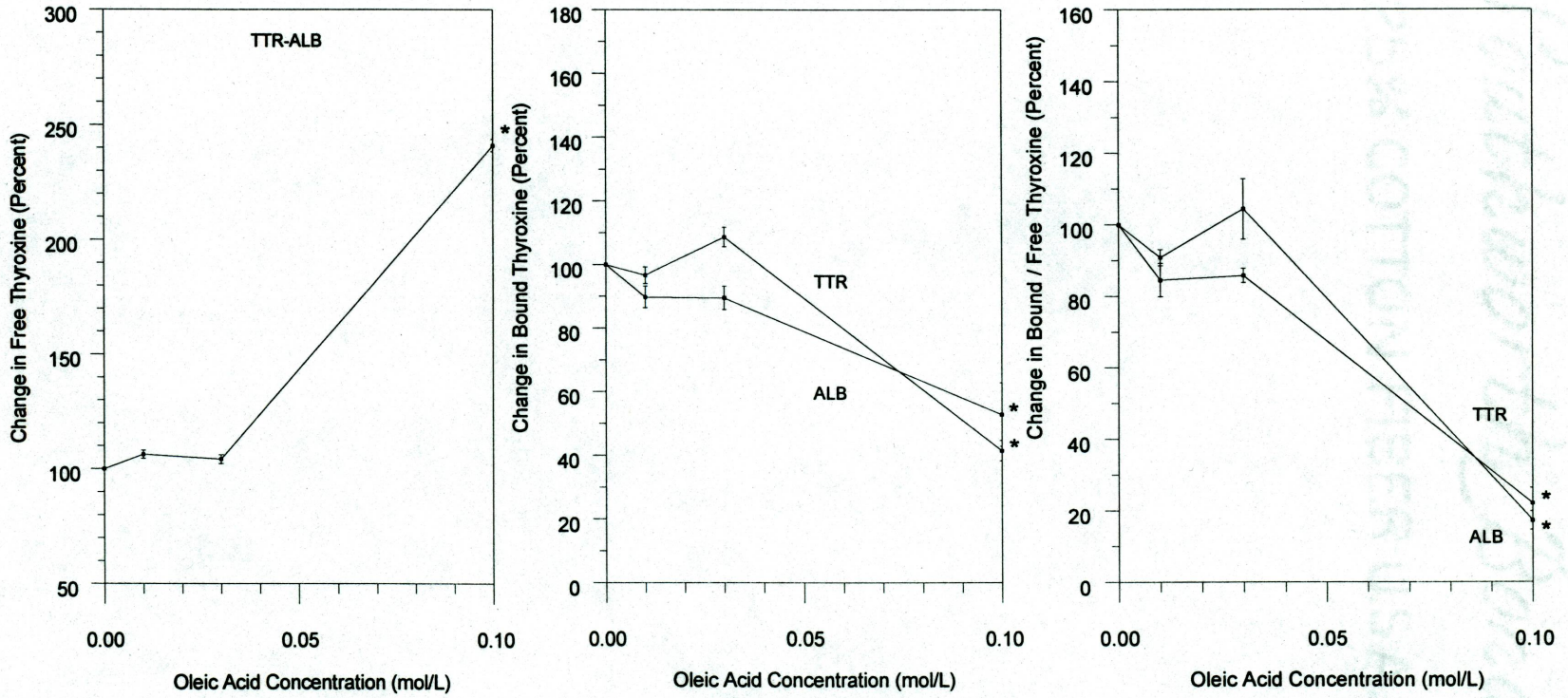
A B C

Figure 6. Effect of serial increments of oleic acid on change in $[^{125}I]T_4$ distribution between isolated human thyroxine binding serum proteins thyroxine binding globulin (TBG) and human serum albumin (ALB). Change in percent free thyroxine (A), percent bound to TBG and ALB (B), and the ratio of free to bound thyroxine (C) at each inhibitor concentration is expressed as a percentage of corresponding values at zero inhibitor concentration. Assay protein concentrations were 1/200th of normal serum concentrations: TBG (0.108 mg/L) and ALB (0.207 g/L). $[^{125}I]T_4$ was added in tracer quantity (1.3×10^{-3} mmol/L). Each point is the mean of triplicate determinations and error bars denote standard error of the mean. A * indicates significance (P=0.05) by ANOVA and Scheffe's Multiple Range Comparison.


TBG-ALB
Change in Free Thyroxine (Percent)
300
250
200
150
100
50
0.00
0.05
0.10
Oleic Acid Concentration (mol/L)
*
ALB
TBG
Change in Bound Thyroxine (Percent)
180
160
140
120
100
80
60
40
20
0
0.00
0.05
0.10
Oleic Acid Concentration (mol/L)
*
*
ALB
TBG
Change in Bound / Free Thyroxine (Percent)
160
140
120
100
80
60
40
20
0
0.00
0.05
0.10
Oleic Acid Concentration (mol/L)

A B C

Figure 7. Effect of serial increments of oleic acid on change in $[^{125}I]T_4$ distribution between isolated human thyroxine binding serum proteins transthyretin (TTR) and human serum albumin (ALB). Change in percent free thyroxine (A), percent bound to TTR and ALB (B), and the ratio of free to bound thyroxine (C) at each inhibitor concentration is expressed as a percentage of corresponding values at zero inhibitor concentration. Assay protein concentrations were 1/200th of normal serum concentrations: TTR (1.325 mg/L), and ALB (0.207 g/L). $[^{125}I]T_4$ was added in tracer quantity (1.7×10^{-2} mmol/L). Each point is the mean of triplicate determinations and error bars denote standard error of the mean. A * indicates significance (P=0.05) by ANOVA and Scheffe's Multiple Range Comparison.


TTR-ALB
Change in Free Thyroxine (Percent)
300
250
200
150
100
50
0.00
0.05
0.10
Oleic Acid Concentration (mol/L)
*
A
Change in Bound Thyroxine (Percent)
180
160
140
120
100
80
60
40
20
0
TTR
ALB
*
*
0.00
0.05
0.10
Oleic Acid Concentration (mol/L)
B
Change in Bound / Free Thyroxine (Percent)
160
140
120
100
80
60
40
20
0
TTR
ALB
*
*
0.00
0.05
0.10
Oleic Acid Concentration (mol/L)
C

Effects of oleic acid on [^{125}I]T_4 binding to albumin

In the TBG-albumin study a 35% increase in percent total 125I]T4 bound to albumin between 0.0 and 0.10 mol/L oleic acid was noted (Figure 6B), associated with an increase in apparent affinity of albumin for T4 evidenced by the increase in the bound/free T4 ratio (Figure 6C). This increase was not statistically significant. In the TTR-albumin study, an 66% decrease in albumin bound T4 was noted in the same concentration range (Figure 7B). This decrease was accompanied by a statistically significant decrease in the bound/free T4 ratio, and indicates a reduction in apparent affinity of T4 for albumin.

Effects of oleic acid on the percent free [125I]-T4

Statistically significant increases in the percent FT4 were observed at 0.10 mol/L oleic acid concentration in the TBG-TTR (Figure 5A), TBG-albumin (Figure 6A) and TTR-albumin (Figure 7A) assays. The percent FT4 at 0.10 mol/L was elevated 140% above the percent FT4 at zero inhibitor concentration in both the TBG-TTR (Figure 5A) and TTR-albumin (Figure 7A) assays, consequent to inhibition of T4 binding to TTR at 0.10 mol/L oleic acid dose. In the TBG-albumin oleic acid assay, a slight increase (5%) in FT4 at 0.10 mol/L above percent FT4 at 0.0 mol/L was evident

(Figure 6A), concurrent with a statistically significant binding reduction to TBG (Figure 6B).

Inhibitor effects on [125I]T4 partitioning between protein binding moieties

Redistribution of T4 between serum thyroxine binding proteins by furosemide is evidenced by the corresponding decrease in TBG bound [125I]T_4, and increase in TTR bound [^{125}I]T_4 at 0.01 mol/L furosemide in the TBG-TTR furosemide assay (Figure 2B). Similarly, a tracer partitioning shift between TTR and albumin occurred in the furosemide TTR-albumin assay: a statistically significant increase in albumin bound T_4 occurs at inhibitor concentration of 1.0 mol/L, simultaneous with a significant decrease in TTR bound T_4 at this inhibitor concentration (Figure 4B).

In the oleic acid TBG-Albumin assay, inhibitor concentration of 0.10 mol/L produced a statistically significant 3% decline in percent tracer bound to TBG and a 64% increase in that bound to albumin compared to percent of tracer bound to TBG and albumin at zero inhibitor concentration (Figure 6B).

[^{125}I]T_4 Recovery

[^{125}I]T_4 loss, presumably by adsorption to plastic dialysis cell components, was evidenced by failure to

recover comparable amounts of tracer at the lowest and highest inhibitor concentrations. Tracer loss at the highest inhibitor concentration for the TBG-TTR, TBG-albumin, and TTR-albumin assays was 28%, 16% and 18%, respectively, for furosemide assays, and 8%, 4% and 15%, respectively, for oleic acid assays.

DISCUSSION

The displacement of [^{125}I]T_4 from TBG, TTR and albumin by furosemide in our study of tracer partitioning between dual binders is consistent with the previous finding that furosemide displaces T_4 from isolated preparations of TBG, TTR and albumin *in vitro* (14). The finding that furosemide interacts with the T_4 binding site of TTR (14) was later revised (28) with the explanation that what had been considered isolated TTR contained both TBG and albumin. New data in the later study led to the conclusion that furosemide was in fact not interactive with the T_4 binding site of TTR (28). TBG and TTR are very different proteins both in molecular structure and in the order of affinities of drug inhibitors for binding with them (28) so our finding of T_4 displacement from both TBG and TTR with increasing furosemide dosage was unexpected. Our observation that T_4 binding to TBG was decreased by greater than 90% at 1.0 mol/L furosemide concentration was expected, since furosemide and T_4 share a common binding site on TBG for which they directly compete (14). Scatchard analysis of serum binding to furosemide identified albumin as the dominant binding site (27). At furosemide concentrations above the molar concentration of albumin, there is an abrupt increase in the unbound fraction of furosemide in serum (27). In hypoproteinemic serum with decreased albumin and TBG concentrations, T_4 displacement by furosemide occurs at

a lower furosemide concentration (14) suggesting that the increase in free furosemide with decreased albumin concentration makes the interaction of furosemide with TBG and TTR sites for T_4 displacement more likely in situations where albumin concentration is low. Previous studies on furosemide effects on serum binding of thyroxine show dose dependent binding inhibition *in vivo* in patients on high dose furosemide treatment and *in vitro* with addition of furosemide to normal serum (31,32). In one of these studies, serum furosemide levels of 10 μg/mL following high dosage furosemide were shown to elevate dialyzed FT_4 by 26%, but did not affect serum TSH levels. It was therefore concluded that therapeutic concentrations of furosemide are unlikely to decrease serum T_4 (14). Our results are consistent with this conclusion, since significant elevations in free T_4 were observed in this study only at furosemide levels of 0.01 mol/L in the TBG-TTR (Figure 2A) and TTR-albumin assays (Figure 4A), a concentration which is 300 times higher than therapeutic serum levels of furosemide (10 μg/mL)(14). The result of the TBG-albumin study is inconclusive, since increase in FT_4 is observed at our lowest furosemide concentration (.001 mol/L)(Figure 3A).

The pronounced displacement by oleic acid of $[^{125}I]T_4$ from TTR and albumin, but not from TBG suggests oleic acid interaction with the binding of T_4 to TTR and albumin. This

result supports the finding of Mendel et al (22) that TTR bound T_4 in serum is more readily displaced by supraphysiologic oleic acid doses than is TBG bound T_4. It has been proposed that an inhibitor which interacts with secondary T_4 binding sites, such as TTR and albumin, is unlikely to elevate levels of serum free T_4 (28). Many studies report that physiological NEFA concentrations (normal serum = 0.52 mmol/L; NTI serum = 0.82 mmol/L) (23) have a negligible effect on T_4 binding in serum from normal and non-thyroidally ill humans (12,15,17,22,33). Our result that oleic acid interacts only with T_4 bound to TTR and albumin but not with TBG bound T_4 is consistent with this conclusion, since only inhibitors which decrease binding to TBG are considered able to lower serum T_4 levels to the extent observed in NTI (28). Evidence exists that oleic acid inhibits binding of T_4 to isolated TBG in equilibrium dialysis studies (34) and in the competitive ligand binding assay (CLBA) (24,35), but in our assay system we observed no evidence of this inhibition. Evidence for the idea that T_4 displaced from TTR and albumin will "equilibrate rapidly" with sites on TBG (28) was not found in the results of this study. While oleic acid did displace T_4 from TTR in the TBG-TTR assay, the displaced T_4 did not contribute to an increase in the percent total $[^{125}]T_4$ bound to TBG, but rather contributed to an increase in the free T_4 fraction.

Munro et al (28) have suggested that inhibition of T_4 binding to TTR may produce important effects in FT_4 in situations of high occupancy of TTR with T_4, such as in TBG deficiency or the situation of high TBG occupancy seen in NTI.

The metabolic importance of TTR bound T_4 is suggested by various findings: normal thyroid function in humans with genetic absence of TBG (36); the identification of a cell membrane receptor specific for the uptake of TTR in a TTR-T_4 dose dependent manner (37); structural complementarity of TTR and DNA T_4 binding sites(38); homology between TTR and the T_4 binding site on 5' deiodinase suggested by common flavenoid T_4 binding inhibition (39) and by similar binding requirements and binding substrate affinities of TTR and Type I 5' deiodinase (40). T_4 kinetic data of NTI patients show decreased binding of T4 to vascular and extravascular sites, a result which could be explained by a common inhibitor of T_4 binding to TTR and 5' deiodinase (41). These findings suggest that inhibitors which affect T_4 binding to TTR, such as oleic acid in our study, may result in abnormal thyroid hormone levels in tissue and in serum. Receptor mediated cellular uptake of TTR-T_4 is likely to be decreased in NTI due to such inhibition.

The role of albumin as a modulator of inhibitor binding to higher affinity proteins has been suggested for both

oleic acid (4,10,22) and furosemide (14). However, in our study even when albumin was not included, as in the TBG-TTR assays, inhibition of T_4 binding TTR and TBG was not evidenced at lower inhibitor concentration in either the furosemide or the oleic acid assays.

It is evident both from the present study and from previous studies that furosemide and oleic acid act as inhibitors of T_4 binding to serum proteins and may therefore contribute to the anomalous thyroid hormone levels observed in NTI. However, the evidence suggests that in physiologic concentrations neither inhibitor is capable of generating the magnitude of the effects observed in this disorder. The recent suggestion of common binding sites on albumin for oleic acid and furosemide based on the observation of dose dependent increase in free furosemide with increasing oleic acid concentration (13) and the finding that the effects of oleic acid and furosemide are synergistic in displacement of T_4 from serum binding (13) indicate that though the effect of neither inhibitor may by itself be able to fully account for the thyroxine hormone abnormalities in NTI, the combined effect of the two together may together be sufficient to generate them.

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