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Biochemical characteristics of inconsistent free T_4 assays

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School of Medicine
in conjunction with the
Faculty of Graduate Studies

Biochemical Characteristics of Inconsistent Free T_4 Assays

By

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A Dissertation submitted in partial satisfaction
of the requirements for the degree of
Doctor of Philosophy in Biochemistry

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Trust in the Lord with all thine heart; and lean not unto thine own understanding.
In all thy ways acknowledge Him, and He shall direct thy paths. Proverbs 3:5-6

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TABLE OF CONTENTS

TABLES

FIGURES

CHAPTER 6

ABBREVIATIONS

RIA	Radioimmunoassay
TBG	Thyroxine Binding Globulin
ALB	Albumin
TTR	Transthyretin
TRH	Thyrotropin Releasing Hormone
TSH	Thyroid Stimulating Hormone
FT_4	Free Thyroxine
TT_4	Total Thyroxine
FT_3	Free Triiodothyronine
$^{125}I\text{-}T_4$	125-Iodine Radiolabeled Thyroxine
$^{125}I\text{-}T_3$	125-Iodine Radiolabeled Triiodothyronine
MWCO	Molecular Weight Cut-Off

ABSTRACT OF THE DISSERTATION

Biochemical Characteristics of Inconsistent Free T_4 Assays

by

Kristofer S. Fritz

Doctor of Philosophy, School of Medicine, Program in Biochemistry
Loma Linda University, May 2007
Dr. Bruce Wilcox, Chairperson

Serum levels of free thyroxine (T_4) are helpful in the clinical evaluation of patients who are suspected of having thyroid disease, since free T_4 (unbound) is known to closely relate to hyper, eu and hypothyroidism. There are documented inconsistencies among commercially available direct free thyroxine immunoassays. The biochemical basis for these inconsistencies is not understood and has not been characterized. Direct free T_4 measurements have been linked to both T_4-binding serum protein concentrations and protein-bound T_4 concentrations. A free T_4 reference method using direct equilibrium dialysis radioimmunoassay has been well characterized. New experiments have applied this equilibrium dialysis reference method and a well characterized total T_4 immunoassay to the characterization of eight commercially available direct free T_4 immunoassays. The data contained in this dissertation show that these direct free T_4 determinations correlate with protein-bound T_4 concentrations, total T_4 concentrations or both. These direct free T_4 immunoassays are non-specific, insensitive and inaccurate when used to measure normal levels of free thyroxine in serum.

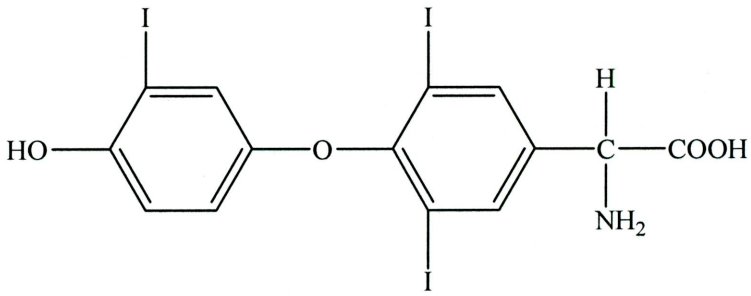
CHAPTER 1

INTRODUCTION

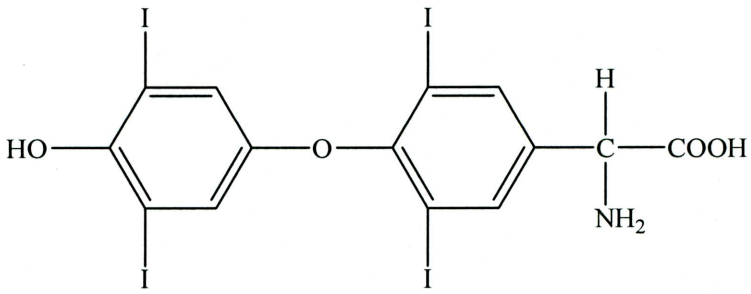
Approximately 27 million Americans are affected by thyroid disease (ie. hyperthyroidism, hypothyroidism…). The thyroid is the largest endocrine gland in the human body and is located just below the Adam's apple at the base of the neck (Braverman 1996). It produces and secretes the thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) (Figure 1.1) and is closely regulated by the hypothalamic-pituitary-thyroid axis (Figure 1.2). Thyroid hormones regulate the rate of metabolism and affect the function of many systems in the body. In humans, T_3 and T_4 are bound to and transported by three serum proteins; approximately 75%, 15% and 10% of T_4 is bound to thyroxine binding globulin (TBG), transthyretin (TTR) and albumin, respectively. Only a small fraction of T_3 (0.5%) and T_4 (0.02%) remain unbound or free in circulation. It is this free fraction of thyroid hormone that is metabolically active, exerting the hormone's action on cellular processes and correlating with clinical presentation and symptoms.

Over the past five decades the clinical relevance of free T_4 has led to the development of many different free T_4 immunoassays. These assays are classified into one of two distinct categories (Holm 2004). Methods calibrated using gravimetrically prepared mass standards dissolved in buffer solutions are absolute methods. Methods calibrated using serum standards containing free hormone concentrations that are determined using an absolute method are comparative methods (Ekins 1993).

Figure 1.1 Thyroid hormones.
Triiodothyronine (T_3) and Tetraiodothyronine (thyroxine, T_4) are secreted by the thyroid.

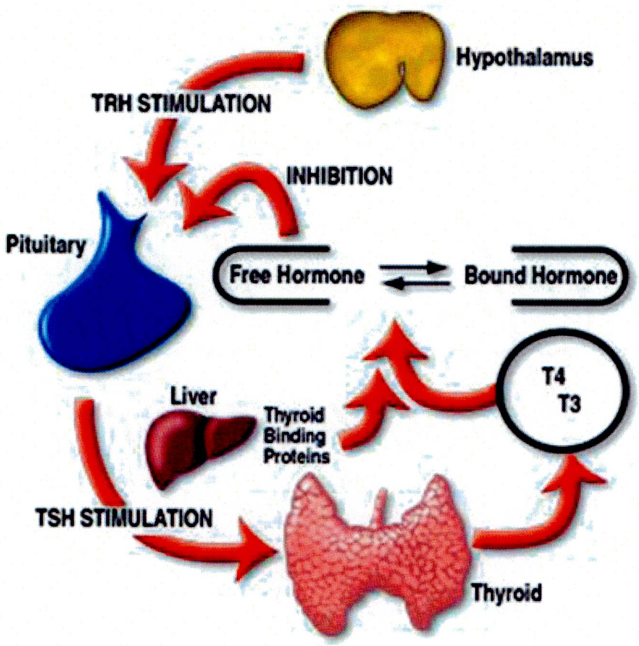


3,5,3',-Triiodothyronine (T_3)



3,5,3',5',-Tetraiodothyronine (T_4)

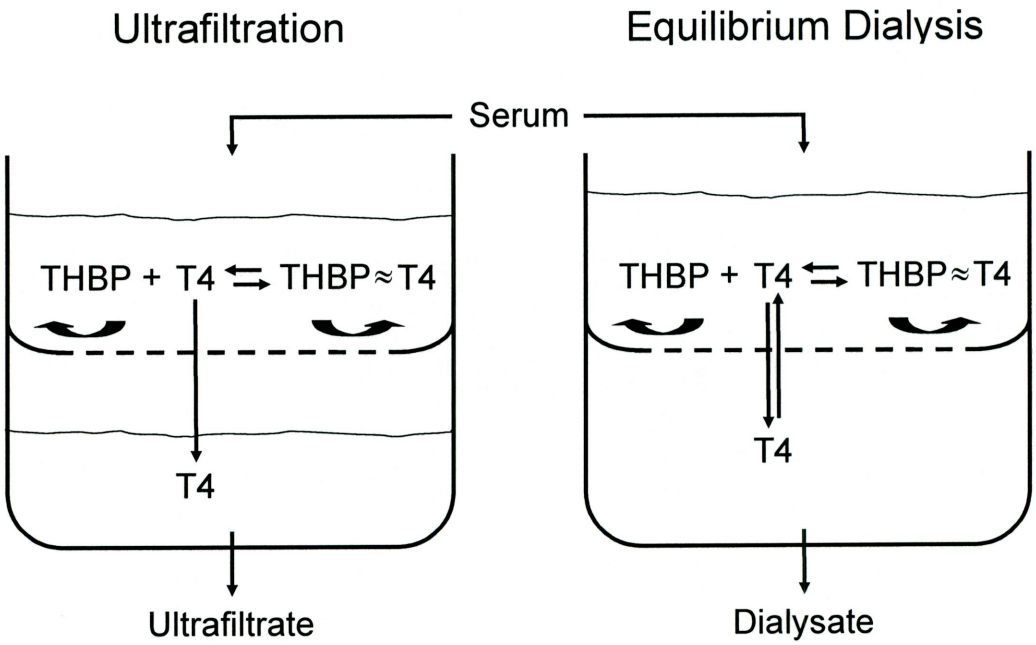
Figure 1.2 Hypothalamic-pituitary-thyroid axis.
Thyroid hormone levels are regulated by a feedback inhibition mechanism which operates along the hypothalamic-pituitary-thyroid axis. The hypothalamus secretes TRH which stimulates the pituitary to secrete TSH. TSH, in turn, stimulates the thyroid gland to produce and secrete thyroid hormones (T_3 and T_4) into circulation. Once levels of T_3 and T_4 are adequate, further production of TSH is suppressed according to the familiar negative feedback mechanism. There is, therefore, an inverse relationship between TSH and thyroid hormone levels. Because physiological activity is exerted by the free hormones (those not bound to binding proteins), this inverse relationship is actually between TSH and the free thyroid hormones. (Illustration taken from www.dpcweb.com)


Hypothalamus
TRH STIMULATION
INHIBITION
Pituitary
Free Hormone
Bound Hormone
T4
T3
Liver
Thyroid
Binding
Proteins
TSH STIMULATION
Thyroid

Absolute and comparative methods can be further classified as being indirect or direct assays. Indirect methods combine the measurements of total hormone concentrations and the free hormone fraction. Direct methods result in an estimate of free hormone concentration from a single measurement without determining the total hormone concentration. Today, most commercially available free T_4 immunoassays are direct comparative methods. Unfortunately, great inconsistencies exist among these assays. When the same serum is analyzed by various assays, reported T_4 determinations are often in disagreement (Helenius 1983, Hay 2002).

Current reference methods for the determination of free T_4 levels in serum incorporate equilibrium dialysis or ultrafiltration to separate free T_4 from serum proteins and are coupled with direct radioimmunoassays or direct mass spectrometry methods for the quantification of T_4 concentrations (Figure 1.3) (Soldin 2005, Van Uytfanghe 2006). These methods must minimize technical uncertainties such as adsorption, dilution, protein leakage and the alteration of protein-hormone binding equilibria. Equilibrium dialysis involves the dilution of serum small molecules, while ultrafiltration has a concentrating effect on serum proteins and protein bound molecules. When using either method, the effects of dilution and concentration must be well characterized. Also, the leakage of serum proteins into dialysates and ultrafiltrates is known to produce spurious free T_4 determinations. Both methods must be verified as retaining all T_4 binding serum proteins, thus eliminating the possibility of protein interference. Some or all of the aforementioned variables exist for every free T_4 assay, and must be dealt with in a systematic manner (Korsgaard 1961, Olsen 1979, Ekins 1990, Holm 2002).

Figure 1.3 Ultrafiltration and equilibrium dialysis.
Ultrafiltration and equilibrium dialysis are commonly used to obtain the free or unbound fraction of T_4 from serum samples. Serum proteins and protein bound T_4 are retained by these devices while free T_4 is collected. (THBP – Thyroid Hormone Binding Protein)



≈ denotes a complex

All clinical laboratory free T_4 assays are competitive binding immunoassays. Two groups of free T_4 immunoassays are currently used. In one group, free T_4 values are based on the binding and displacement of radiolabeled T_4 from T_4 antibody. As previously described, these assays use equilibrium dialysis or ultrafiltration to separate free T_4 from T_4 binding proteins and from protein bound T_4 prior to quantification of free T_4 by immunoassay (Figure 1.3). In the second group, free T_4 measurements are based on the binding and displacement of large molecular conjugates of T_4, called "analogs" of T_4 (Figure 1.4). Analogs selected for use in these free T_4 immunoassays should not bind to endogenous T_4 binding serum proteins. These assays were developed to avoid the need for "technically demanding" and time consuming equilibrium dialysis or ultrafiltration procedures. These analog-based direct free T_4 assays are applied directly to serum or plasma.

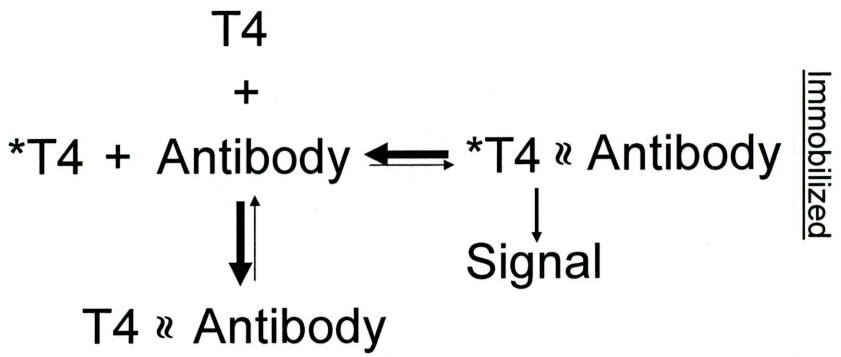
The vast majority of free T_4 assays used today are applied directly to human sera and are analog-based methods. The analytical chemistry of these methods is highly inconsistent. Their sensitivity is variable, and their specificity is uncertain (Stockigt 1981, Amino 1983, Csako 1987, Nelson 1994, Christofides 1999, Wang 2000, Nelson 2004). Nearly all are incompletely characterized.

The free T_4 immunoassays used in conjunction with equilibrium dialysis and ultrafiltration are non-analog methods (Figure 1.4). They detect and quantify serum free T_4 after it has been separated from proteins and protein bound T_4. Because of this separation, they are thought to be unaffected by serum proteins and protein bound T_4. They are more sensitive for detecting free T_4 than analog-based methods, by two or more log orders of magnitude.

Figure 1.4 Illustration of a non-analog free T4 immunoassay and an analog-based free T4 immunoassay.

The non-analog assay is applied to serum dialysate or ultrafiltrate, containing free T4 in the absence of serum proteins. The analog-based assay is applied to whole serum.

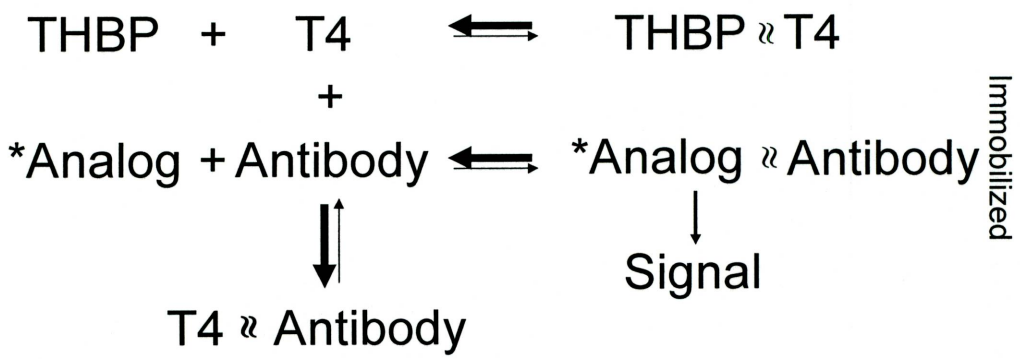
Non-Analog Free T4 Immunoassay



≈ Denotes a complex

* Denotes a ^{125}I labeled free T4

Labeled Analog Free T4 Immunoassay



≈ Denotes a complex

* Denotes a labeled compound

There are both numerical inconsistencies and categorical (high, normal or low) inconsistencies among analog-based free T_4 determinations. There are also quantitative and categorical inconsistencies between analog and non-analog methods. Most of these reports are observational studies that document the presence of inconsistencies (Stockigt 1981, Amino 1983, Csako 1987, Nelson 1994, Christofides 1999, Wang 2000, Nelson 2004). Few studies have reported controlled experiments using fixed or excluded variables. The clinical experience of senior endocrinologists has led to the conclusion that analog-based free T_4 immunoassays are variably inaccurate and unreliable. The biochemical conditions that account for all of this have not been reported.

Various hypothetical explanations exist regarding these inconsistencies. The most widely accepted explanation is that these different analog-based free T_4 methods are calibrated to different free T_4 standards. Efforts to standardize all direct free T_4 immunoassay calibrations are just underway by a working group of the International Federation of Clinical Chemistry, led by scientists at the University of Ghent in Belgium. A more likely explanation is that serum proteins and protein bound T_4 influence analog-based free T_4 determinations, and their influence varies from method to method. Our goal was to perform a systematic study of these effects to answer the question; what are the biochemical conditions under which direct free T_4 immunoassay methods report inconsistent free T_4 determinations?

The following focused unanswered questions have lead to specific experiments: (1) Are direct free T_4 determinations sometimes attributable to serum compounds other than thyroid hormones (i.e., "blank effects")? (2) Following equilibrium dialysis and ultrafiltration, which fraction of serum T_4 is most closely related to direct free T_4 determinations (ie. retentate, dialysate or ultrafiltrate)? (3) What concentrations of T_4 are most closely related to direct free T_4 determinations, when T_4 binding serum proteins and protein bound T_4 are not present? (4) What concentrations of T_4 are most closely related to direct free T_4 determinations, when T_4 binding serum proteins are held constant? (5) What T_4 is most closely related to direct free T_4 determinations, when free T_4 is held constant, while T_4 binding serum proteins, protein bound T_4 and total T_4 are varied? (6) What T_4 is most closely related to direct free T_4 determinations, when free T_4 replaces protein bound T_4 while total T_4 concentration is held constant? (7) How do analog-based free T_4 determinations relate to those obtained by direct equilibrium dialysis free T_4 assays and total T_4 assays?

Hypothesis

Our hypothesis is: Direct free T_4 immunoassays based on "analogs" of T_4 do not detect and quantify free T_4 but detect and quantify protein bound T_4, T_4 binding serum proteins and/or total T_4. These free T_4 methods and the reference methods they are standardized against must be further characterized.

Objectives and Specific Aims

Our objectives are: A) To investigate the factors involved in free T_4 determinations in analog-based free T_4 immunoassays. B) To investigate the application of equilibrium dialysis and ultrafiltration in the separation of free T_4 from T_4 binding proteins in human serum. C) To investigate the delayed diffusion of T_3 and T_4 when applied to equilibrium dialysis and ultrafiltration.

The specific aims of this dissertation are:

(1) To study dialyzed and ultrafiltered human serum in analog-based free T_4 immunoassays.

(2) To determine the contributions of serum proteins and protein bound T_4 to analog-based free T_4 determinations.

(3) To detect and verify the retention of thyroid hormone binding proteins during equilibrium dialysis and ultrafiltration.

(4) To characterize the delayed diffusion of thyroid hormones during equilibrium dialysis and ultrafiltration.

CHAPTER 2

A DIRECT FREE THYROXINE (T_4) IMMUNOASSAY WITH THE CHARACTERISTICS OF A TOTAL T_4 IMMUNOASSAY

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1. Graduate student who oversaw and completed all experiments and wrote the manuscript.
2. Mentor who provided research guidance and critically reviewed the manuscript.

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A Direct Free Thyroxine (T_4) Immunoassay with the Characteristics of a Total T_4 Immunoassay

Running Title: Free T_4 Assay Tracks Total T_4

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Abstract

Background: Direct free thyroxine (T_4) measurements have been linked to both T_4-binding serum protein concentrations and protein-bound T_4 concentrations. Whether this is evidence of a relationship to total T_4 concentrations has not been reported.

Methods: We compared an analog-based direct free T_4 immunoassay and a total T_4 immunoassay. Each assay was applied to the fractions of serum T_4 obtained by ultrafiltration and equilibrium dialysis. Both were applied to serum-based solutions in which free T_4, T_4-binding proteins, protein-bound T_4, and total T_4 were systematically varied, held constant, or excluded.

Results: Neither the free T_4 assay nor the total T_4 assay detected dialyzable or ultrafilterable serum T_4. Both assays detected and reported the T_4 retained with serum proteins. Both free and total T_4 results were related to the same total T_4 concentrations in the presence and absence of T_4-binding proteins. Both results were similarly related to total T_4 concentrations when free T_4 was held constant while total T_4 was varied. Both were similarly related to a total T_4 concentration that was held constant while free T_4 progressively replaced protein-bound T_4. These free T_4 results, like total T_4 results, were unresponsive to a 500-fold variation in dialyzable T_4 concentrations.

Conclusion: New experiments extend the characterization of a longstanding and incompletely characterized analog-based free T_4 immunoassay. These free T_4 measurements relate to total T_4 concentrations in the same way that total T_4 measurements do.

Introduction

Interrelationships among serum free thyroxine (T_4), the proteins that bind T_4, protein-bound T_4, and total T_4 are variable (1-6). Protein-bound and total T_4 concentrations vary (correlate) directly with free T_4 concentrations when serum T_4-binding proteins are constant. When free T_4 is constant, protein-bound T_4 and total T_4 concentrations vary directly with concentrations of T_4-binding proteins. There are reports of direct analog-based free T_4 results that vary directly with serum concentrations of T_4-binding protein (7-18) or vary as total T_4 concentrations vary (while free T_4 is held constant) (19, 20). These relationships imply that these analog-based direct free T_4 assays detect total T_4 concentrations.

This possibility led us to ask the following questions about the direct analog-based free T_4 immunoassay. What form of T_4 does the assay detect, and what concentrations of T_4 does it measure? Which form of T_4 does it detect after equilibrium dialysis and ultrafiltration? Do the free T_4 values correlate with total T_4 concentrations when total T_4 is varied (while free T_4 is constant) or when total T_4 is constant (while free T_4 is varied)?

Materials and methods

T_4 immunoassays

We studied a manual analog-based free T_4 immunoassay (Coat-A-Count, Diagnostic Products Corp.) that uses a radiolabeled T_4 analog, immobilized T_4 antibody, a single incubation, and 21-fold dilution with a reagent solution. The total T_4 immunoassay (Coat-A-Count, Diagnostic Products Corp.) uses radiolabeled free T_4 (nonanalog), immobilized T_4 antibody, and a single incubation; it is a manual method that dilutes the solutions it analyzes 41-fold with a reagent solution. Both assays were applied to the same experimental T_4 solutions. The nonanalog free T_4 immunoassay (Nichols Institute Diagnostics) applied to equilibrium dialysates and ultrafiltrates (21-30) uses radiolabeled free T_4, immobilized T_4 antibody, and a single incubation; it is a manual method that dilutes the solutions it analyzes 1.0625-fold with a reagent solution.

We performed each assay according to its manufacturer's instructions. Each T_4 result reported was a mean of triplicates, and each experiment was repeated for confirmation. We detected and quantified gamma radiation by use of a Gamma 4000 multiwell automated gamma counter (Beckman-Coulter).

Normal human serum

We obtained normal human serum from 16 healthy male volunteers, ages 21 to 55 years. These sera were pooled. Serum collection was approved by the institutional review board, and serum samples were given anonymous identifiers. In this pool, serum thyroid-stimulating hormone (TSH), total T_4, free T_4, thyroxin-binding globulin (TBG),

transthyretin (TTR), and albumin were within their respective reference intervals, and test results for anti-T_4, anti-T_3, and anti-IgG antibodies and for salicylates were negative (testing performed at Quest Diagnostics) (data not presented).

T_4-depleted normal human serum

We stripped an aliquot of the serum pool of T_4 using Amberlite IRA-410 anion exchange resin (Alfa Aesar) (31). No residual total T_4 was detected by the total T_4 radioimmunoassay, and no dialyzable T_4 was detected by the nonanalog free T_4 immunoassay. TBG, TTR, and albumin concentrations remained normal after T_4 depletion. We estimated the affinity of serum proteins for T_4 as the free fraction of serum T_4 (the ratio of dialyzable free T_4 to total T_4). To measure the free fraction after T_4 depletion, T_4 was restored to its original concentration. There was no significant change in affinity after T_4 depletion (data not presented).

Sodium levothyroxine

We obtained sodium levothyroxine, for injection, in 500-μg vials (Bedford Labs). It was dissolved at room temperature in 5 mL of 9 mL/L NaCl, USP grade (Abbott Labs). This produced a stock solution containing 125 μmol/L (10 000 μg/dL) sodium levothyroxine.

Equilibrium dialysis

We obtained dialysis devices and dialysate buffer from Nichols Institute Diagnostics. The chemical composition of this buffer has been reported (27). Serum

samples were dialyzed for 18 h at 37°C in an Isotemp Incubator, model 630D (Fisher Scientific). A moisture-saturated atmosphere was maintained during dialysis by enclosing dialysis cells in containers with open water reservoirs.

Ultrafiltration

We ultrafiltered large volumes of serum (up to 50 mL) under 20 psi nitrogen gas pressure at 37°C using a stirred ultrafiltration device with a regenerated cellulose membrane that has a nominal molecular weight cutoff of 12 to 14 kDa (Millipore). We ultrafiltered small volumes of serum (up to 2 mL) using a Centricon YM-10 ultrafiltration device with a regenerated cellulose membrane that has a 10-kDa molecular weight cutoff (Fisher Scientific). Centrifugation at 3000*g* was carried out at 37°C in a temperature-controlled Eppendorf 5702RH centrifuge (Fisher Scientific). All ultrafiltration devices were prewashed twice with deionized water.

Serum pH control

When dialysis was applied, the pH of serum dialysate and retentate was controlled to 7.4 (± 0.1) during equilibrium dialysis at 37°C by the HEPES acid in dialysate buffer (27). At equilibrium, the final HEPES ion concentration was calculated to be 54 mmol/L.

When ultrafiltration was applied, whole serum pH was controlled to 7.4 (± 0.1) at 37°C, before ultrafiltration, by adding 40 µL of 1200 mmol/L HEPES acid (Fisher Biotech) per mL serum. Serum pH stability was obtained during 15 min of vortexing while passing a continuous stream of moist air across the serum. The final HEPES ion concentration was 54 mmol/L.

Free T_4 adsorption to container surfaces

Free T_4 can adsorb onto solid surfaces from aqueous solutions. We tested the borosilicate glass vials and test tubes used (Fisher Scientific) for free T_4 adsorption by a modification of the procedure reported by Holm et al. (32). Radiolabeled free T_4 (^{125}I-T_4; Perkin Elmer Life Sciences) was freshly repurified by column chromatography before use, using Sephadex G-25 (Sigma-Aldrich) (33, 34). Columns were equilibrated to 10 mmol/L PBS (1 PBS tablet dissolved in 200 ml of water to obtain: 10 mmol/L phosphate buffer, 2.7 mmol/L potassium chloride and 137 mmol/L sodium chloride) (Sigma-Aldrich) at pH 5.4 and room temperature. Stock ^{125}I-T_4 was added to the column and eluted with 100 mmol/L sodium hydroxide (Sigma-Aldrich). We collected fractions in 13 × 100-mm glass test tubes using an automated fraction collector (LKB) and quantified gamma radiation. The test tubes adsorbed less than 0.4% of free ^{125}I-T_4 in the absence of serum proteins.

We also tested the 2-mL screwcapped glass vials used to store test samples. They too adsorbed less than 0.4% of free ^{125}I-T_4 in the absence of serum proteins. Samples were stored at –80°C before assay.

Experimental strategies

We applied the direct free T_4 immunoassay and the total T_4 immunoassay to the following solutions:

• Fractions of serum T_4 obtained by equilibrium dialysis and ultrafiltration.

• Varied concentrations of sodium levothyroxine added to normal human serum dialysate (see *Materials and Methods*) at concentrations of 39 to 309 nmol/L (3 to 24 μg/dL).

• Varied concentrations of sodium levothyroxine added to T_4-depleted normal human serum at concentrations of 39 to 309 nmol/L (3 to 24 μg/dL). The dialyzable T_4 in these solutions (direct equilibrium dialysis) was 7.7 to 125 pmol/L (0.6 to 9.7 ng/dL).

• Varied concentrations of serum proteins, protein-bound T_4, and total T_4 while free T_4 concentration remained constant. Ultrafiltration was applied to normal serum. One aliquot of retentate was undiluted. Other aliquots were diluted 2-fold, 4-fold, and 8-fold with the corresponding ultrafiltrate. Thus, serum proteins, protein-bound T_4, and total T_4 were varied from a low of 25% of native serum concentrations to a high of 200% of native serum concentrations, while free T_4 was constant. We confirmed the variation in total T_4 concentrations by measuring total T_4, the variation in protein concentrations by measuring total protein and transthyretin (Quest Diagnostics) (data not presented), and the constancy of free T_4 concentration by measuring dialyzable T_4 (data not presented). T_4-depleted serum proteins were varied in the same way.

• Varied concentrations of serum proteins and protein-bound T_4 progressively replaced with free T_4 while total T_4 was held constant. We applied equilibrium dialysis to normal

serum. We measured the total T_4 in retentate using the total T_4 immunoassay (Table 2.1) and added sodium levothyroxine solution to the dialysate to match the concentration of total T_4 in the retentate. The retentate was progressively diluted with T_4-enriched dialysate until dialyzable T_4 concentrations reached a plateau. We measured dialyzable T_4 by use of nonanalog free T_4 immunoassay (see *Methods and Materials*). High dialyzable T_4 concentrations were diluted with immunoassay zero calibrator, as needed, to bring them into the interval quantified by the nonanalog free T_4 immunoassay (2.6 to 129 pmol/L). These dialyzable T_4 measurements varied from 16.7 to 8940 pmol/L (1.3 to 693 ng/dL) (Fig. 2A).

Results

Neither the analog-based direct free T_4 immunoassay nor the total T_4 immunoassay detected ultrafilterable or dialyzable serum T_4. Both assays detected and quantified the T_4 retained with serum proteins during dialysis and ultrafiltration. The nonanalog free T_4 immunoassay was the only assay that detected and quantified ultrafilterable and dialyzable serum T_4 (Table 2.1).

When T_4 was added to normal human serum dialysate at concentrations of 39 to 309 nmol/L (3 to 24 μg/dL), the direct free T_4 values were 9 to 81 pmol/L (0.7 to 6.3 ng/dL) and the total T_4 values were 30 to 257 nmol/L (2.3 to 20 μg/dL) (Table 2.2).

When T_4 was added to T_4-depleted normal human serum at concentrations of 39 to 309 nmol/L (3 to 24 μg/dL), the direct free T_4 values were 6 to 71 pmol/L (0.5 to 5.5 ng/dL) and the total T_4 values were 40 to 257 nmol/L (3.1 to 20 μg/dL) (Table 2.3).

When serum protein concentrations, protein bound T_4 concentrations, and total T_4 concentrations were varied from 25% to 200% of whole serum concentrations while free T_4 was constant, the direct free T_4 values varied from 2.6 to 34.8 pmol/L (0.2 to 2.7 ng/dL) and the total values varied from 21.9 to 139 nmol/L (1.7 to 10.8 μg/dL) (Fig. 1) (Standard deviations reported by error bars).

When free T_4 progressively replaced protein bound T_4 while total T_4 was constant, both analog-based direct free T_4 measurements and total T_4 measurements were closely related to total T_4 concentration (Fig. 2B and C). Neither the analog-based direct free T_4 assay nor the total T_4 assay detected or followed the variation in dialyzable T_4 concentrations when they varied from 16.7 to 8940 pmol/L (1.3 to 693 ng/dL) (Fig. 2A). The mean total T_4 result was 77.5 nmol/L (6.0 μg/dL) (Fig. 2C). The mean free T_4 result was 18.6 pmol/L (1.4 ng/dL) (Fig. 2B).

Table 2.1 Serum T_4 fractions obtained by equilibrium dialysis and ultrafiltration applied to three T_4 assays.

The four fractions obtained by equilibrium dialysis and ultrafiltration were applied to one total T_4 assay, one direct analog-based free T_4 assay and one direct free T_4 radioimmunoassay. The direct analog-based free T_4 assay detects the same fractions of serum T_4 that the total T_4 assay detects

Table 2.1 Serum fractions and T_4 values.

	Ultrafiltration		Equilibrium Dialysis	
Immunoassays	**Retentate**	**Ultrafiltrate**	**Retentate**	**Dialysate**
Total T_4	139 nmol/L	ND	77 nmol/L	ND
Analog Free T_4	34.7 pmol/L	ND	17 pmol/L	ND
Non Analog Free T_4	>129 pmol/L	20.6 pmol/L	>129 pmol/L	19 pmol/L

ND = not detected: total T_4 <12.87 nmol/L or free T_4 <1.29 pmol/L

Table 2.2 T_4 added to serum dialysate and applied to a total T_4 assay and a direct analog-based free T_4 assay.

An analog-based free T_4 immunoassay parallels a total T_4 immunoassay in the absence of T_4 binding serum proteins

Table 2.2 Results when T_4 was added to serum dialysate.		
T_4 Added (nmol/L)	**Analog-based Free T_4 (pmol/L)**	**Total T_4 (nmol/L)**
39	9	30
77	21	60
116	33	104
154	44	130
193	54	176
232	66	196
270	79	229
309	81	257
Mean	48	148

T_4: 1 μg/dL = 12.87 nmol/L

Table 2.3 T_4 added to T_4-depleted serum and applied to a total T_4 assay and a direct analog-based free T_4 assay.

An analog-based free T_4 immunoassay parallels a total T_4 immunoassay in the presence of T_4 binding serum proteins.

Table 2.3 Results when T_4 was added to T_4 depleted serum.

T_4 Added (nmol/L)	Analog-based Free T_4 (pmol/L)	Total T_4 (nmol/L)
39	6	40
77	14	76
116	23	112
154	32	139
193	39	169
232	51	199
270	58	241
309	71	257
Mean	37	154

T_4: 1 μg/dL = 12.87 nmol/L

Figure 2.1 Free T_4 is constant: proteins, protein bound T_4 and total T_4 vary. Comparison of analog-based free T_4 estimates to total T_4 determinations. Free T_4 concentration was held constant at 20 pmol/L (1.6 ng/dL). The concentrations of serum proteins, protein bound T_4 and total T_4 were varied from 25%, 50%, 100% and 200% of those in whole serum.

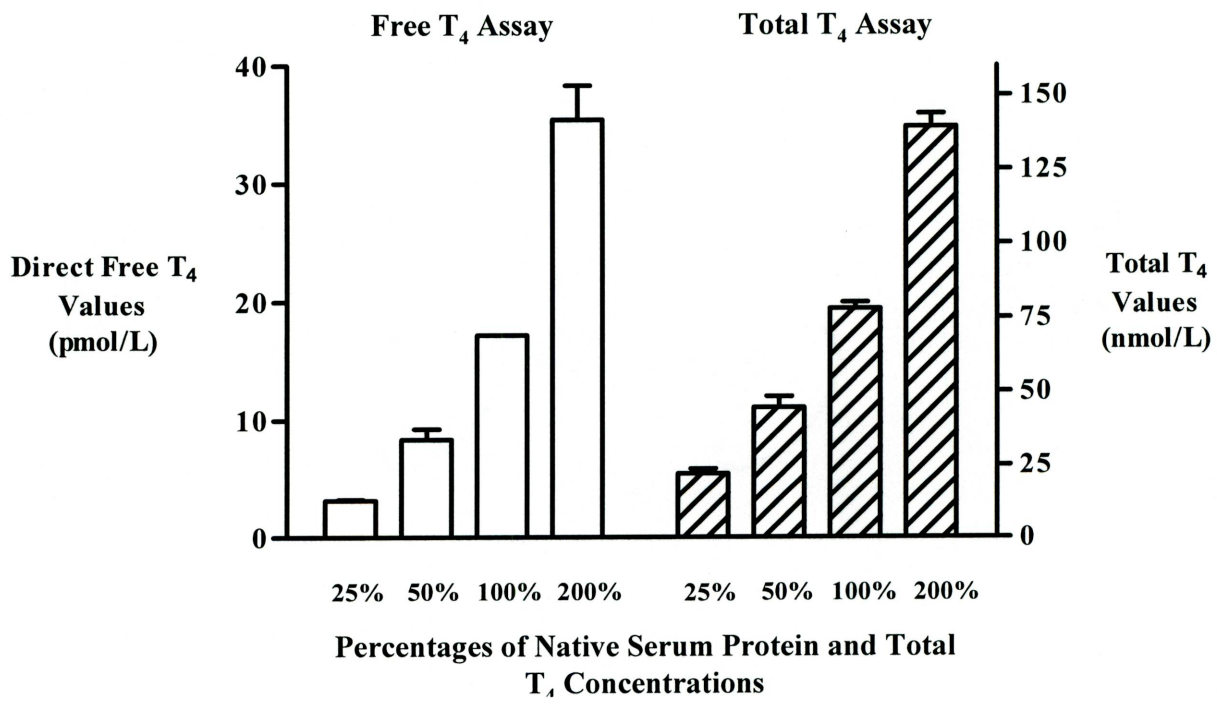
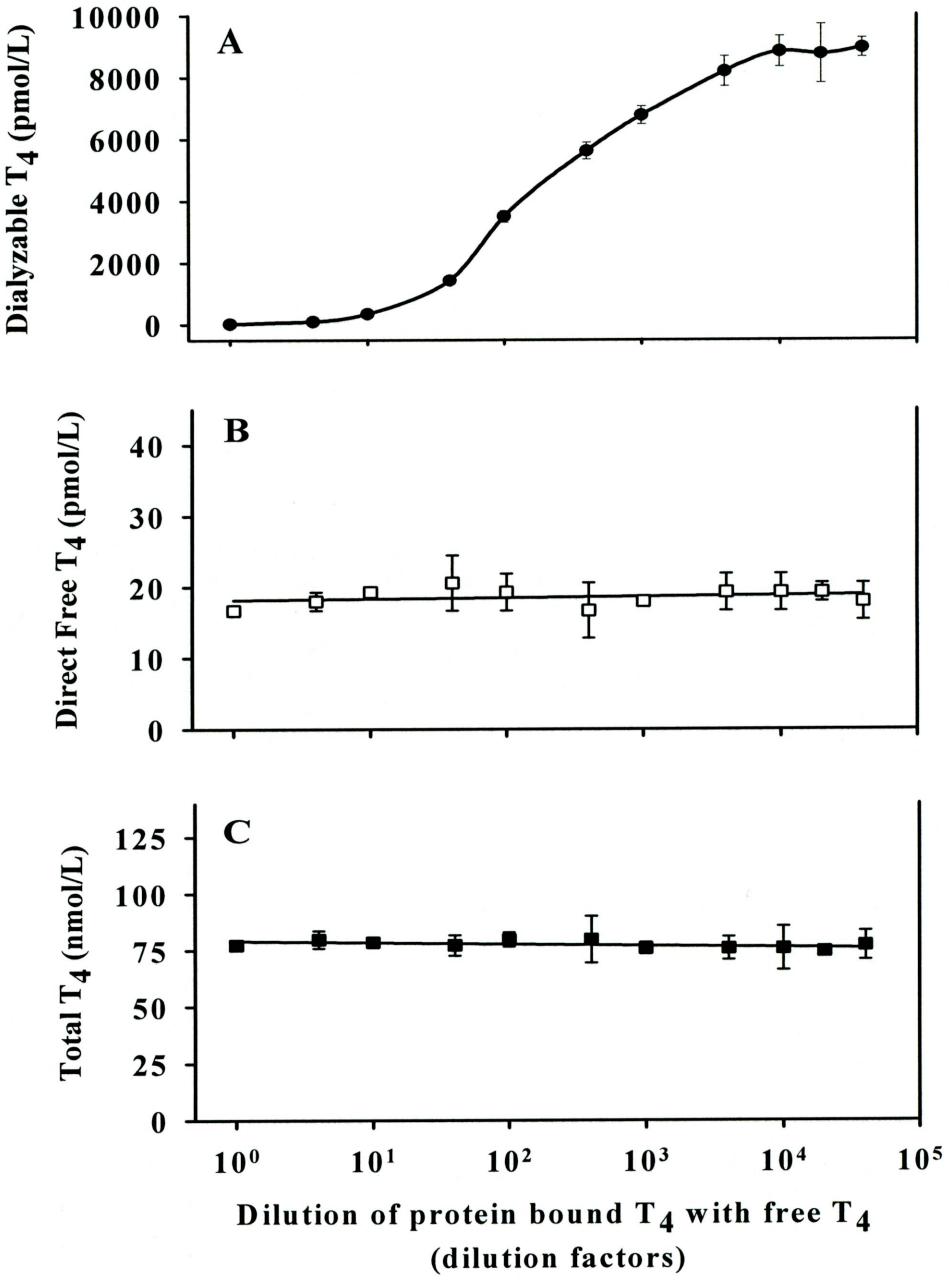

Free T_4 Assay
Total T_4 Assay
Direct Free T_4 Values (pmol/L)
Total T_4 Values (nmol/L)
40
30
20
10
0
150
125
100
75
50
25
0
25% 50% 100% 200% 25% 50% 100% 200%
Percentages of Native Serum Protein and Total T_4 Concentrations

Figure 2.2 Total T_4 constant: proteins, protein bound T_4 and free T_4 vary. Assay responses to replacement of protein bound T_4 by free T_4, while holding total T_4 constant. (*A*) Dialyzable T_4 values by nonanalog free T_4 RIA, (*B*) direct free T_4 values by analog-based free T_4 RIA, and (*C*) total T_4 values.


A
Dialyzable T_4 (pmol/L)
10000
8000
6000
4000
2000
0
B
Direct Free T_4 (pmol/L)
40
30
20
10
0
C
Total T_4 (nmol/L)
125
100
75
50
25
0
10^0
10^1
10^2
10^3
10^4
10^5
Dilution of protein bound T_4 with free T_4
(dilution factors)

Discussion

The primary reason for carrying out free T_4 measurements is to differentiate patients with unusual or abnormal serum free T_4 concentrations from patients with unusual or abnormal T_4 binding to serum proteins. When T_4 binding to serum proteins is normal (or constant), total T_4 measurements will be proportional to free T_4 concentrations, and both will be categorically similar (low, normal, or high) (see Table 2.3). This is not the situation when free T_4 concentrations are constant (or similar) and T_4 binding serum protein concentrations (or affinities) are unusual or abnormal. Under these conditions, dialyzable T_4 concentrations will have a different relationship to total T_4 concentrations (16, 19, 35, 36).

The data obtained with these experiments document a direct analog-based free T_4 immunoassay that reports free T_4 values with the characteristics of total T_4 values, but each assay's calibration is strikingly different. The direct free T_4 immunoassay did not follow normal free T_4 concentrations in the presence of varied total T_4 concentrations (Figure 2.1) or abnormal free T_4 concentrations when total T_4 was normal (Figure 2.2). The information provided by this direct free T_4 immunoassay is qualitatively similar to the information provided by total T_4 immunoassays. There is no evidence that this free T_4 immunoassay will be more useful than a total T_4 immunoassay.

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CHAPTER 3

QUANTIFYING SPURIOUS FREE T_4 RESULTS ATTRIBUTABLE TO THYROXINE-BINDING PROTEINS IN SERUM DIALYSATES AND ULTRAFILTRATES

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1. Graduate student who oversaw and performed most experiments and wrote the manuscript.
2. Mentor who provided research guidance and critically reviewed the manuscript.

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Quantifying Spurious Free T_4 Results Attributable to Thyroxine-Binding Proteins in Serum Dialysates and Ultrafiltrates

Running Title: T_4 Binding Serum Proteins and Spurious Free T_4 Values

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Key Terms: Spurious free T_4 results, T_4 binding proteins

Abbreviations: thyroxine binding globulin (TBG), thyroxine binding serum proteins (T_4BSPs).

Abstract

Background: Direct equilibrium dialysis and direct ultrafiltration-free thyroxine (T_4) assays rely on semipermeable membranes to exclude T_4-binding serum proteins from dialysates and ultrafiltrates. The presence of these proteins in dialysates or ultrafiltrates will yield spuriously high free T_4 values when free T_4 is quantified by RIA.

Methods: We used a nonanalog free T_4 RIA that detects and quantifies dialyzable and ultrafilterable serum free T_4 to detect T_4-binding serum proteins. Two equilibrium dialysis devices and 3 ultrafiltration devices were used to illustrate this application. Displacements of ^{125}I-T_4 from anti-T_4 by various concentrations of T_4-depleted TBG, albumin, and serum total protein were compared to displacements by various concentrations of free T_4.

Results: Both dialysis devices excluded detectable T_4-binding serum proteins from dialysates. Two of 3 ultrafiltration devices excluded detectable T_4-binding serum proteins from ultrafiltrates. One did not. This ultrafiltrate yielded spurious free T_4 values that correlated directly with serum protein concentrations.

Conclusion: The presence or absence of T_4-binding proteins in dialysates and ultrafiltrates, and the spurious free T_4 values that these proteins cause, can be documented using a nonanalog free T_4 RIA.

Introduction

Direct equilibrium dialysis free T_4 assays and direct ultrafiltration free T_4 assays use semi-permeable membranes to separate free T_4 from T_4 binding serum proteins (1-12). Non-analog free T_4 immunoassays are widely used to quantify this T_4. These free T_4 assays are radioimmunoassays (RIAs) based on the binding of ^{125}I-T_4 to anti-T_4, and displacements of ^{125}I-T_4 from anti-T_4 by unlabeled T_4 (13-15). This is illustrated in Figure 3.1 A.

When T_4 binding serum proteins (T_4BSPs) are present in dialysates or ultrafiltrates they spontaneously bind ^{125}I-T_4 and displace it from anti-T_4. These assays do not distinguish displacements by protein from displacements by T_4. As a consequence, spurious free T_4 determinations are reported by the assay in the absence of T_4, and spurious overestimates of T_4 are reported when T_4 is present. These interactions are illustrated in Figure 3.1 B.

The spurious free T_4 determinations attributable to T_4BSP, and the concentrations of T_4BSP that account for them, have not been reported. The present study reports the experiments that provide these data.

Materials and methods

Non-analog free T_4 RIA

The same non-analog free T_4 immunoassay (Nichols Institute Diagnostics) was used to detect and quantify free T_4 in the absence of T_4BSP, and to detect T_4BSP in the absence of T_4. This assay has been reported previously (1).

The human serum albumin and human serum TBG to which the free T_4 RIA was applied.

Highly purified human serum albumin (> 99%, Sigma-Aldrich) and TBG (> 95%, Cortex Biochem) were each dissolved in T_4 depleted normal human serum dialysate (see below) at room temperature. Albumin (200 g/L) was diluted with T_4 depleted human serum dialysate to concentrations ranging from $2x10^{-2}$ to 200 g/L. TBG (60 mg/L) was diluted with T_4 depleted human serum dialysate to concentrations ranging from $6x10^{-5}$ to 60 mg/L. T_4 was not detected in these protein preparations when a total T_4 RIA (Diagnostic Products Corporation) was applied, or in the dialysates of these solutions, when the non-analog free T_4 RIA was applied.

The T_4 depleted serum total protein to which the free T_4 RIA was applied

A pool of normal human serum was obtained (Equitech-Bio) and characterized. Serum TBG (17 mg/L), transthyretin (0.27 g/L), albumin (42 g/L), total protein (86 g/L), total T_4 (94 nmol/L, 7.3 ug/dL), free T_4 (12.9 pmol/L, 1.0 ng/dL) and TSH (2.15 MIU/L) were within their respective reference ranges. Anti-T_4, anti-T_3, anti-IgG and salicylates were not detected (Quest Diagnostics).

An aliquot of this serum was stripped of T_4 using the method of Grundy, et al (16). T_4 depleted serum total protein concentrations were varied from $5x10^{-7}$ to 0.05 g/L by diluting T_4 depleted serum total protein (86 g/L) with T_4 depleted human serum dialysate.

The T_4 depleted serum total protein to which equilibrium dialysis and ultrafiltration were applied.

T_4 depleted serum total protein (86 g/L) was concentrated to 160 g/L by ultrafiltration, under 25 psi nitrogen gas pressure at 37 °C, using a 50 mL stirred ultrafiltration cell, with a regenerated cellulose membrane that has a nominal molecular weight cut-off (MWCO) of 12-14 kDa (Millipore). (No T_4BSPs were detected in this serum ultrafiltrate, data not presented). Retentate and ultrafiltrate were then mixed to obtain additional total protein concentrations of 40, 80 and 120 g/L. In this way, total protein concentrations were varied from 40-160 g/L.

Dialysis

Two dialysis devices were studied; a vertical membrane dialysis device (Fisher Scientific) using 10 mL of retentate and 10 mL of dialysate, and a horizontal membrane dialysis device using 200 µL of retentate and 2400 µL of dialysate (Nichols Institute Diagnostics). The dialysis buffer (Nichols Institute Diagnostics) has been reported previously (1). Both devices used a regenerated cellulose membrane (Spectra/Por, Fisher Scientific) with a MWCO of 12-14 kDa. T_4 depleted serum total protein was dialyzed for 18 hours at 37 °C in an Isotemp incubator (model 630D, Fisher Scientific). During

dialysis, pH was controlled to 7.4 (±0.1) at 37 °C (in both retentates and dialysates) by the HEPES ion contained in the dialysis buffer (see above). The final HEPES concentration was 54 mM (1).

Ultrafiltration

Three ultrafiltration devices were tested; Centricon YM-10, Centricon YM-30 and Amicon Ultra-4 (Millipore). These devices have reported MWCOs of 10 kDa, 30 kDa and 10 kDa, respectively. A fourth device, Centricon YM-100 (Millipore) with a reported MWCO of 100 kDa, was used as a positive control, since T_4BSPs have molecular weights of approximately 54-66 kDa. The semi-permeable membrane in each device was made of regenerated cellulose.

Centrifugation was carried out in a temperature controlled, fixed-angle rotor ultracentrifuge (5702 RH, Eppendorf AG) at 37 °C. The pH was controlled prior to ultrafiltration to 7.4 (±0.1) at 37 °C by adding 40 µL of 1200 mM HEPES acid (Fisher Biotech) per mL of serum. The pH was stabilized during 15 minutes of vortexing while passing a continuous stream of moist air across the serum. The final HEPES ion concentration was 54 mM (1).

Sodium levothyroxine

Sodium levothyroxine (for injection) was obtained in 500 µg vials (Bedford Labs) and dissolved in 5 mL of 0.9% sodium chloride, USP grade (Abbott Labs), at room temperature. This provided a T_4 stock solution containing 125 µmol/L (10 mg/dL)

sodium levothyroxine. Aliquots were diluted with T_4 depleted human serum dialysate to concentrations of 2.6 x 10^{-5} to 26 nmol/L (2x10^{-3} to 2000 ng/dL).

The possibility of free T_4 losses due to adsorption

Free T_4 is often adsorbed from aqueous solutions onto solid surfaces. Borosilicate glass test tubes and borosilicate glass screw-capped vials (Fisher Scientific, Pittsburgh, PA) were tested for ^{125}I-T_4 adsorption by a modification of the procedure reported by Holm, et al (17). ^{125}I-T_4 (^{125}I-T_4, Perkin Elmer Life Sciences) was purified using Sephadex G-25 (Sigma-Aldrich) column chromatography (18, 19). The Sephadex columns were equilibrated to 0.01 M PBS (Sigma-Aldrich), at pH 5.4 and room temperature. Stock ^{125}I-T_4 was added to the column and eluted with 100 mM sodium hydroxide (Sigma-Aldrich). Fractions were collected in 13x100 mm glass test tubes using an automated fraction collector (LKB). Gamma radiation was quantified using a multi-well, automated gamma counter (Gamma 4000, Beckman-Coulter). These test tubes, and the 2 mL screw-capped glass vials used for storage, adsorbed less than 0.4% of free ^{125}I-T_4 dissolved in human serum dialysate. T_4 solutions were stored at -80°C.

Results

The displacements of ^{125}I-T_4 from anti-T_4 by varied concentrations of T_4-depleted TBG, albumin, and serum total protein are compared to the displacements by varied concentrations of free T_4 (in the absence of T_4BP) (Figure 3.2). A dialysate of T_4 depleted human serum was used as the negative control. A theoretical lower limit for the detection of displacements was calculated as the mean minus two standard deviations of the negative control. These values were 0.001 mg/L for T_4 depleted TBG, 0.025 g/L for T_4 depleted albumin and 0.0015 g/L for T_4 depleted total protein. This compares to 0.8 pmol/L for free T_4 (Table 3.1). Fifty percent displacements of ^{125}I-T_4 from anti-T_4 were obtained with T_4 depleted TBG at 0.028 mg/L, T_4 depleted albumin at 0.76 g/L and T_4 depleted serum total protein at 0.038 g/L. This compares to free T_4 at 34 pmol/L (Table 3.2).

When T_4 depleted TBG concentrations were 0.01%, 0.1% and 1.0% of whole serum levels (0.0017, 0.017 and 0.17 mg/L), labeled T_4 displacements were equivalent to free T_4 concentrations of 3, 30 and 300 pmol/L (0.23, 2.3 and 23 ng/dL). When T_4 depleted albumin concentrations were 0.01%, 0.1% and 1.0% of whole serum levels (0.0042, 0.042 and 0.42 g/L), labeled T_4 displacements were equivalent to free T_4 concentrations of 0.3, 3 and 30 pmol/L (0.023, 0.23 and 2.3 ng/dL). When T_4 depleted serum total protein concentrations were 0.01%, 0.1% and 1.0% of whole serum levels (0.86, 8.6 and 86 g/L), labeled T_4 displacements were equivalent to unlabeled T_4 concentrations of 3.9, 39 and 390 pmol/L (0.3, 3 and 30 ng/dL).

As a percent of whole serum concentrations, T_4 depleted serum total proteins were as effective or marginally more effective in displacing ^{125}I-T_4 than T_4 depleted TBG concentrations, and were more effective than T_4 depleted albumin concentrations (Tables 3.1 and 3.2). We chose T_4 depleted serum total protein to test for spurious free T_4 determinations in dialysates and ultrafiltrates because it contains all T_4BSP.

No spurious free T_4 determinations were obtained with either dialysis device. Spurious free T_4 determinations were obtained with one of three ultrafiltration devices (Figure 3.3). These free T_4 determinations correlated with the concentrations of serum total protein in the solutions to which ultrafiltration was applied ($r^2 = 0.97$, $p<0.02$). These data are striking, because the designated molecular weight cut-off for this device was 10 kDa, well below the molecular weights of T_4BSPs. Spurious free T_4 determinations, due to protein leakage, were obtained with the positive control (Figure 3.3) and none were obtained with a negative control (see results).

Table 3.1 Minimum displacements of ^{125}I-T_4 from anti-T_4.
The minimum displacements, determined as the mean minus two standard deviations from the negative control, were determined for T_4 Depleted TBG, albumin, total serum proteins and free thyroxine.

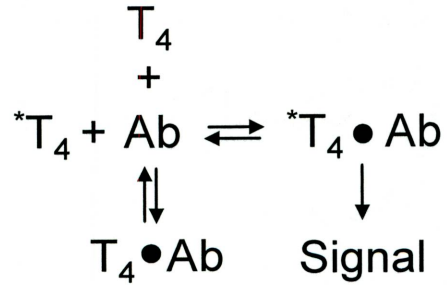
Table 3.1 Minimum displacements of ^{125}I-T_4 from anti-T_4.		
	T_4BSP concentrations	**T_4BSP as portion of normal concentration**
T_4 Depleted TBG	0.001 g/L	0.005
T_4 Depleted Albumin	0.025 g/L	0.05
T_4 Depleted Total Protein	0.0015 g/L	0.002
Free T_4	0.8 pmol/L	0.001

Table 3.2 Fifty percent displacements of ^{125}I-T_4 from anti-T_4.
The fifty percent displacements were determined for T_4 Depleted TBG, albumin, total serum proteins and free thyroxine.

Table 3.2 Fifty percent displacements of ^{125}I-T_4 from anti-T_4.		
	Concentrations	**Percent of normal serum concentrations**
T_4 Depleted TBG	0.028 mg/L	0.14
T_4 Depleted Albumin	0.76 g/L	1.65
T_4 Depleted Total Protein	0.038 g/L	0.05
Free T_4	34 pmol/L	0.05

Figure 3.1 A direct free T_4 RIA
Interactions of unlabeled T_4 (A) and T_4 binding serum proteins (B) in a non-analog free T_4 RIA

A.



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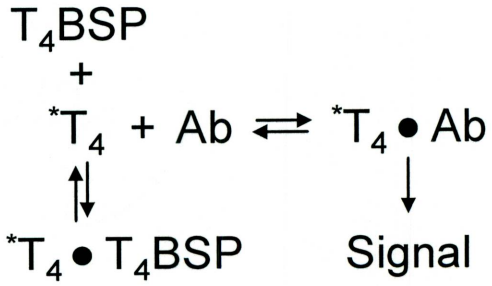


Figure 3.2 Comparison of displacements in a free T_4 RIA.

^{125}I-T_4 displacements from anti-T_4 in a non-analog free T_4 RIA obtained with; ♦ free T_4, □ T_4 depleted TBG, Δ T_4 depleted serum total proteins and, ○ T_4 depleted albumin. The upper horizontal solid line represents maximum binding obtained with the negative control. The dash-dot line represents the lower 95% confidence limit of the negative control data.

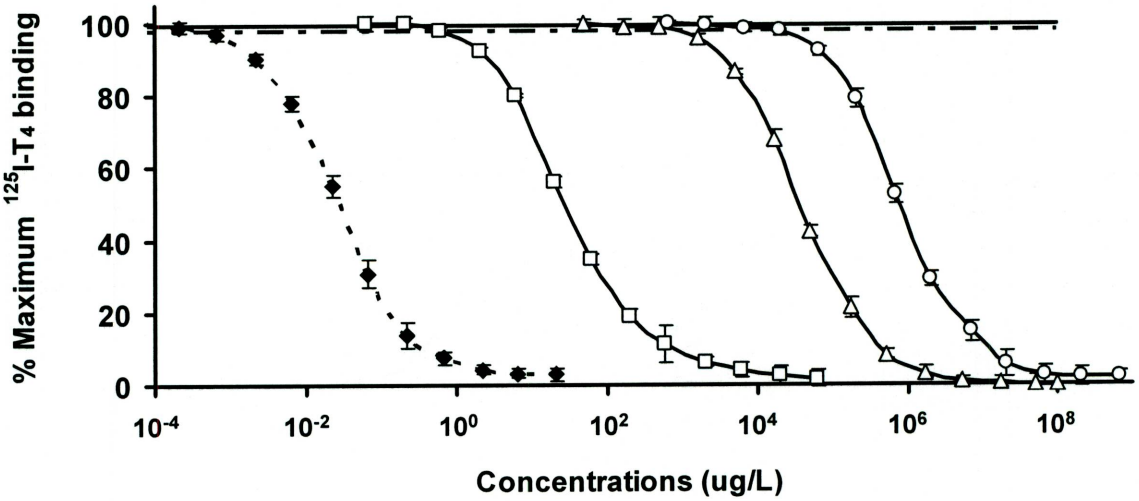
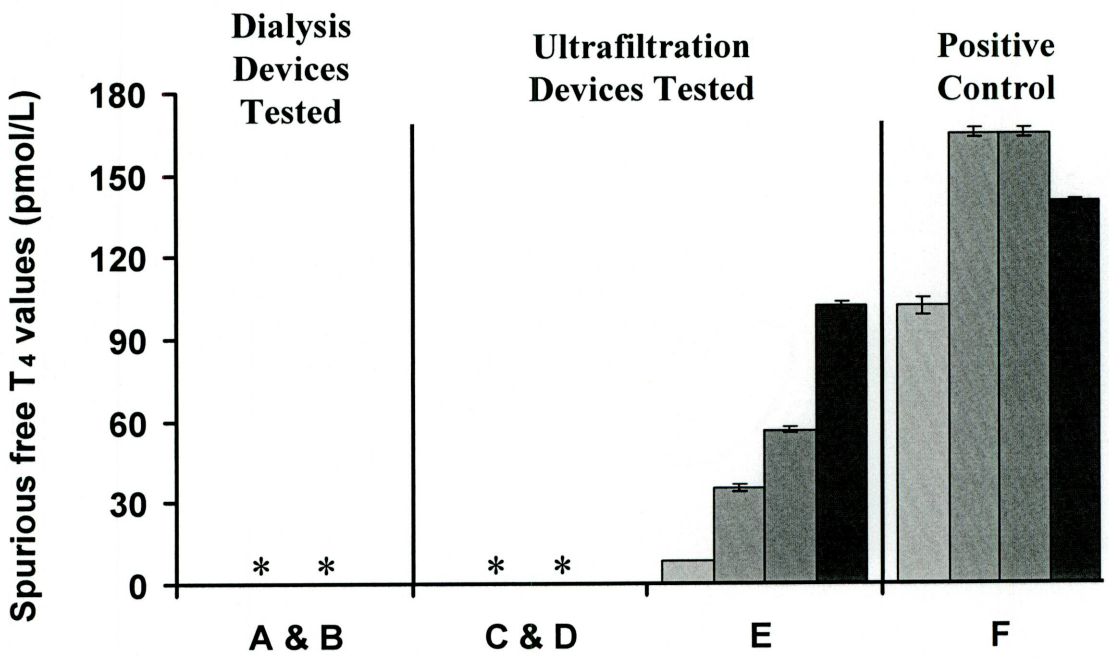

% Maximum ^{125}I-T$_4$ binding
100
80
60
40
20
0
10^{-4}
10^{-2}
10^{0}
10^{2}
10^{4}
10^{6}
10^{8}
Concentrations (ug/L)

Figure 3.3 Protein retention in equilibrium dialysis and ultrafiltration. Dialysis and ultrafiltration devices applied to 40, 80, 120 and 160 g/L of T_4-depleted serum total proteins. No spurious free T_4 determinations were obtained with 2 dialysis devices (A & B). None were obtained with 2 ultrafiltration devices (C & D). Spurious free T_4 determinations of 9 to 102 pmol/L (7 to 79 ng/L) were obtained using a 3rd ultrafiltration device with a designated MWCO of 10 kDa (E). Spurious free T_4 determinations of 102 to 165 pmol/L (79 to 128 ng/L) were obtained with the positive control, an ultrafiltration device with a designated MWCO of 100 kDa (F). (* denotes not detected) Ultrafiltration devices: (C) Centricon YM-10, (D) YM-30, (E) Amicon Ultra-4 and (F) Centricon YM-100.



(A & B) Vertical and horizontal membrane dialysis cells. (C& D) Centricon YM-10 and YM-30. (E) Amicon Ultra-4. (F) Centricon YM-100.

Discussion

Previous studies have measured albumin in serum dialysates or ultrafiltrates. Weeke et al. measured albumin in serum ultrafiltrates using a microalbuminuria RIA with a sensitivity of 0.001 g/L (20, 21). They concluded that an albumin leakage of 0.0015% of undiluted serum albumin would induce an error of approximately 5% in free T_4 determinations. Using our assay, the presence of 0.05% (0.025 g/L) of serum albumin in ultrafiltrate resulted in spurious free T_4 values of approximately 3% (0.5 pmol/L).

Tikanoja, et al. measured albumin in serum ultrafiltrates using an albumin RIA with a sensitivity of 8.0×10^{-4} g/L (22). The study used a cutoff value for protein leakage of 0.005% of serum proteins as reported by Weeke, et al (20). Ultrafiltration devices that allowed less than this 0.005% (0.002 g/L) of albumin into ultrafiltrates were regarded as acceptable. Only one device out of four met this criterion. Again, this compares to our assay where 0.05% (0.025 g/L) of serum albumin in ultrafiltrate resulted in spurious free T_4 values of approx. 3% (0.5 pmol/L).

Holm, et al. measured albumin in serum dialysates and serum ultrafiltrates using a double antibody sandwich ELISA with a sensitivity of 2.8×10^{-6} g/L (23). They found no detectable albumin in serum dialysates, and detectable albumin in the ultrafiltrates obtained with each of five ultrafiltration devices.

These previous studies used albumin assays that were more sensitive for the detection of albumin than the free T_4 RIA used in the present study (0.001 g/L, 8.0×10^{-4} g/L and 2.8×10^{-6} g/L, compared to 0.025 g/L). However, the free T_4 RIA detected and

quantified the interference by albumin, and therefore provides the most relevant data regarding protein interference in free T_4 determinations.

Intralot and interlot variations in the retentive characteristics of the same ultrafiltration devices are another issue (22, 23). This was evident in our positive control (Figure 3.3).

There are no previous studies reporting the spurious free T_4 determinations that result from the presence of T_4BSPs in serum dialysates or ultrafiltrates. Any laboratory with a free T_4 RIA that detects and quantifies T_4 in serum dialysates or serum ultrafiltrates has the ability to detect spurious free T_4 values due to T_4BSP in dialysates and ultrafiltrates. Testing for T_4BSP interferences could easily be a routine part of quality control in any published data reporting dialyzable or ultrafilterable free T_4 determinations. In our opinion, it should always be included in a study reporting inconsistencies between dialysis based free T_4 determinations and ultrafiltration based free T_4 determinations (24). Without this information, it will be uncertain whether disparities in free T_4 determinations represent a serum problem or an assay problem.

Acknowledgments

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CHAPTER 4

UNEQUAL CONCENTRATIONS OF FREE T_3 AND FREE T_4 FOLLOWING ULTRAFILTRATION

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1. Graduate student who oversaw and performed most experiments and wrote the manuscript.
2. Researcher who contributed experimental procedures.
3. Mentor who provided research guidance and critically reviewed the manuscript.

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Unequal Concentrations of Free T_3 and Free T_4 Following Ultrafiltration

Running Title: Impeded free T_3 and free T_4 diffusions

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Number of Tables: 1

Number of Figures: 1

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Key Terms: Thyroxine, Equilibrium Dialysis, Ultrafiltration

Abbreviations: thyroxine (T_4), thyroxine binding globulin (TBG), thyroid stimulating hormone (TSH).

Abstract

Background: Ultrafiltration has been regarded as a "standard" method for the separation of free thyroid hormones from serum proteins and protein bound thyroid hormones. This application of ultrafiltration is understood to yield equal concentrations of free hormones in aqueous solutions on opposite sides of a semi-permeable membrane. The present study was undertaken to document this equality. We found unequal concentrations instead of equal concentrations.

Design: $^{3}H_2O$, ^{125}I-T_3 and ^{125}I-T_4 were dissolved in separate aliquots of pH controlled normal human serum ultrafiltrate. Four ultrafiltration devices were applied to each solution. The movement of labeled water across semi-permeable membranes was compared to the movement of labeled free hormones in this biologically relevant protein free solution.

Results: There was wide variability in the movement of free T_3 and free T_4 across semi-permeable membranes. Variability was dependent upon the device used and the progress of ultrafiltration. There was also variability in the recovery of free hormones from ultrafiltration devices. The loss of T_3 was up to 75% ±0.6 and the loss of T_4 was up to 56% ±1.7.

Conclusion: These variabilities complicate the interpretation of free thyroid hormone measurements involving ultrafiltration.

Introduction

Ultrafiltration has been regarded as a "standard" method for separating free T_3 and free T_4 from serum proteins and protein bound hormone (1-6). It is expected that free T_3 and free T_4 in aqueous solutions will accompany water as water moves across semi-permeable membranes. This would result in equal concentrations of free T_3 and free T_4 in the two aqueous solutions on opposite sides of semi-permeable membranes following ultrafiltration. The movements of free thyroid hormones through semi-permeable membranes have not been compared to the movement of water, nor have the concentrations of free hormones on opposite sides been determined in the absence of hormone binding proteins. This is a report of experiments undertaken to determine the movements of $^{3}H_2O$, ^{125}I-T_3 and ^{125}I-T_4 across semi-permeable membranes in four different types of ultrafiltration devices using a biologically relevant aqueous solution after excluding serum proteins.

Materials and methods

Serum ultrafiltrate

A pool of normal human serum was obtained from Equitech-Bio. Serum thyroxine binding globulin (TBG), transthyretin (TTR), albumin, total protein, total T_4, dialyzable (free) T_4 and thyroid stimulating hormone (TSH) were within their respective reference intervals. Test results for anti-T_4, anti-T_3, anti-IgG and for salicylates were negative (testing performed at Quest Diagnostics). Serum pH was controlled at 7.4 by adding HEPES acid to a final concentration of 54 mM (7).

Ultrafiltrate was obtained from 50 mL of this serum by applying 25 psi of nitrogen gas pressure at 37°C to a stirred ultrafiltration device with a regenerated cellulose membrane that has a 10-kDa molecular weight cutoff (Millipore). Ultrafiltration was continued until the volume of retentate and ultrafiltrate were equal. Serum proteins were not detected in this ultrafiltrate (data not presented).

Solutions of isotope labeled water, T_3 and T_4

Radiolabeled water (^3H_2O), triiodothyronine (^{125}I-T_3) and thyroxine (^{125}I-T_4) were obtained from PerkinElmer Life Sciences. ^3H_2O was quantified using an automated liquid scintillation counter (Beckman LS 7500, Beckman Coulter). ^{125}I-T_3 and ^{125}I-T_4 were quantified using an automated gamma counter (Gamma 4000, Beckman Coulter). Routine checks were performed to maintain precision, accuracy and counting efficiency.

Prior to each experiment, ^{125}I-T_3 and ^{125}I-T_4 were purified using Sephadex G-25 (Sigma-Aldrich) column chromatography as previously described (8). These freshly

purified radiolabeled analytes (^{125}I-T_3 and ^{125}I-T_4) and the ^3H_2O were added to different aliquots of the same serum ultrafiltrate at room temperature.

T_3 and T_4 adsorption to glassware

T_3 and T_4 can be adsorbed onto solid surfaces from aqueous solutions. The borosilicate glassware used in this study (Fisher Scientific) was tested for T_3 and T_4 adsorption by a modification of the procedure reported by Holm, et al (9). ^{125}I-T_3 and ^{125}I-T_4 were purified (see above) and used within an hour of purification. Less than 0.7% of ^{125}I-T_3 and less than 0.4% of ^{125}I-T_4 was adsorbed in the absence of serum proteins.

Water, T_3 and T_4 diffusion during ultrafiltration

The four ultrafiltration devices studied (Millipore) were: Centricon YM-10, YM-30 and YM-100 (10-kDa, 30-kDa and 100-kDa MWCO) and Amicon Ultra-4 (10-kDa MWCO). All devices used regenerated cellulose membranes. Devices of each type were applied to aliquots of serum ultrafiltrate containing ^3H_2O, ^{125}I-T_3 or ^{125}I-T_4 and ultrafiltered at 37°C in a temperature controlled centrifuge at 3000 x g (5702RH, Eppendorf). Samples containing one of the radiolabeled compounds were ultrafiltered until 25%, 50% or 75% of the aqueous solution had passed through the membrane, using a separate device for each percentage. Each experiment was performed twice in triplicate. Ultrafiltrates were transferred to 12 x 75 borosilicate glass test tubes (Fisher Scientific). The radioactivity (cpm/200 µL) was determined for each retentate and ultrafiltrate solution. The radioactive stock solutions were used as controls.

Determining the progress of water across semi-permeable membranes

In each experiment, the progress of ultrafiltration to 25%, 50% and 75% was determined gravimetrically. Each ultrafiltration device was rinsed with deionized water, shaken to remove excess water and weighed. The sample was placed into the retentate chamber and the device compartments were weighed again. Ultrafiltration was performed. The progress of ultrafiltration was determined by weighing each retentate and ultrafiltrate compartment. Ultrafiltration to 25%, 50% and 75% was defined as the percent of water mass that crossed the membrane. The time necessary to accomplish this was determined in pilot studies.

Determining the loss of radiolabeled compounds during ultrafiltration

After ultrafiltration was discontinued, the loss of radiolabeled compounds (cpm/200 μL) was determined for each retentate and ultrafiltrate. Retentate and ultrafiltrate radioactivities were averaged together and divided by the radioactivities of controls to calculate the percentage of radiolabeled compound lost during ultrafiltration.

Results

The concentration of $^{3}H_2O$ was evenly distributed between retentates and ultrafiltrates in all four ultrafiltration devices (Figure 4.1 A-D). The concentrations of ^{125}I-T_3 and ^{125}I-T_4 were unevenly distributed between retentates and ultrafiltrates in all four ultrafiltration devices (Figure 4.1 A-D).

The losses of radiolabeled compounds during ultrafiltration are reported (Table 4.1). The losses of $^{3}H_2O$ were trivial. The losses of ^{125}I-T_3 declined as ultrafiltration progressed. The losses of ^{125}I-T_4 declined as ultrafiltration progressed. It is important to note that $^{3}H_2O$, ^{125}I-T_3 and ^{125}I-T_4 were dissolved in normal human serum ultrafiltrate containing normal levels of serum electrolytes and free thyroid hormones.

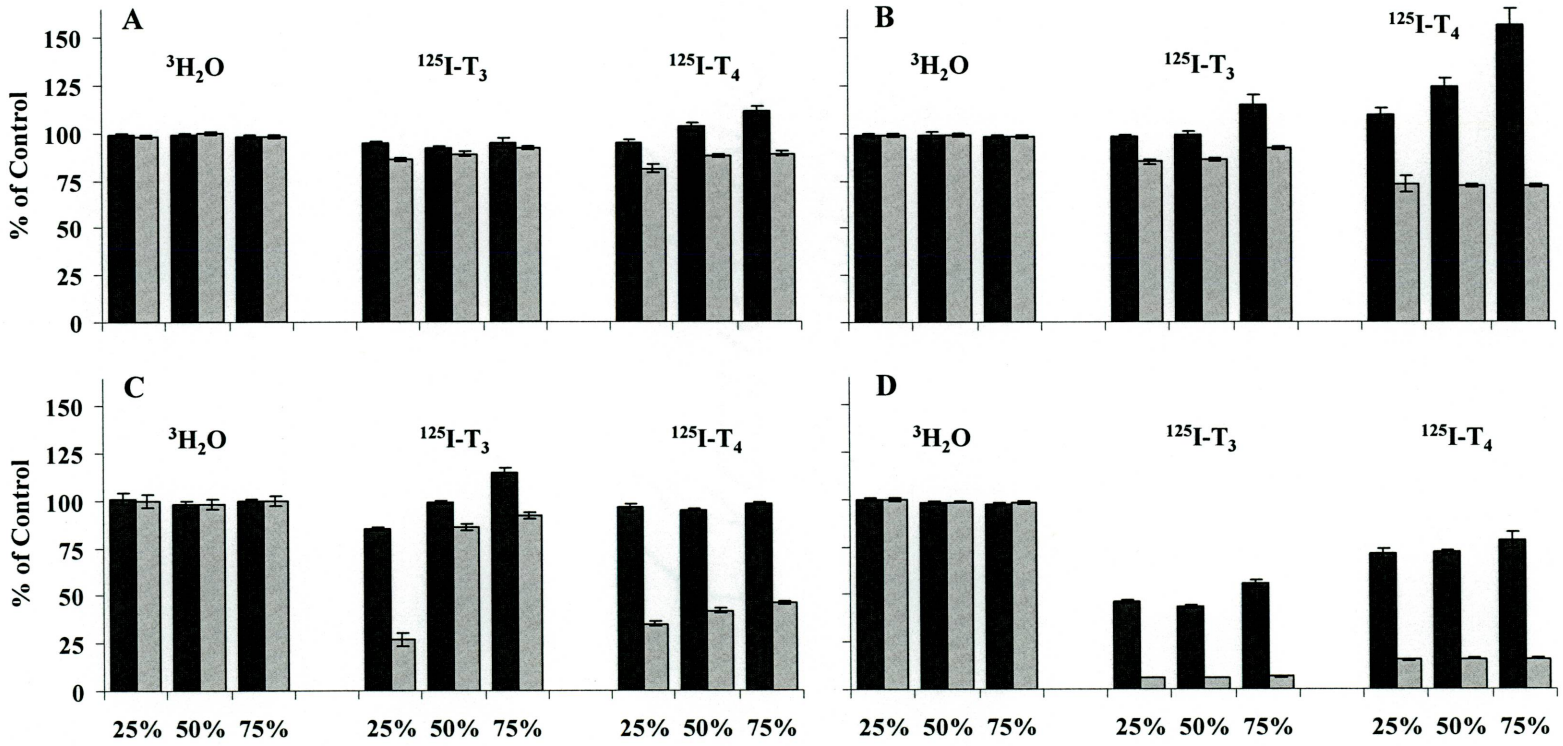
Tables 4.1 A-C. Losses of radiolabeled compounds related to the progress of ultrafiltration among ultrafiltration devices as a percent of control (cpm/200 µl).

Table 1A. Percent $^{3}H_2O$ lost			
	25%	50%	75%
YM-10	1.6 ±1.2	0.3 ±0.8	2.0 ±0.9
YM-30	1.0 ±1.1	0.6 ±1.4	2.1 ±1.1
YM-100	-0.7 ±3.5	2.1 ±2.2	0 ±1.6
Ultra-4	0.2 ±1.1	1.7 ±0.7	2.5 ±1.0

Table 1B. Percent ^{125}I-T_3 lost			
	25%	50%	75%
YM-10	9.6 ±0.9	9.6 ±1.5	6.6 ±1.9
YM-30	8.6 ±1.3	7.7 ±1.2	-3.7 ±3.1
YM-100	44 ±2.2	40 ±1.4	36 ±2.2
Ultra-4	74 ±0.8	75 ±0.6	69 ±1.2

Table 1C. Percent ^{125}I-T_4 lost			
	25%	50%	75%
YM-10	12 ±1.8	4.7 ±1.6	0 ±2.2
YM-30	9.0 ±4.6	1.8 ±2.9	-8.4 ±5.1
YM-100	34 ±1.8	31 ±0.9	27 ±1.1
Ultra-4	56 ±1.7	56 ±0.9	53 ±2.4

Figure 4.1 $^{3}H_2O$, ^{125}I-T_3 and ^{125}I-T_4 applied to four ultrafiltration devices in the absence of serum proteins. Unequal concentrations of radiolabeled T_3 and T_4, shown as a percent of control (y-axis), were related to the mass of water that moved across semi-permeable membranes during the progress of ultrafiltration (x-axis). The radiolabeled compounds were dissolved separately in pH controlled normal human serum ultrafiltrate, before the experimental ultrafiltration. The devices were: (A) YM-10, (B) YM-30, (C) YM-100 and (D) Ultra-4. (■Retentate and ■Ultrafiltrate)


A
B
C
D
^3H_2O
^{125}I-T_3
^{125}I-T_4
% of Control
150
125
100
75
50
25
0
25% 50% 75%

Discussion

The application of various ultrafiltration devices to radiolabeled water, T_3 and T_4 yielded striking inconsistencies (Fig. 1 A-D). The differences in hormone concentrations on opposite sides of semi-permeable membranes and the magnitudes of hormone losses during ultrafiltration were unexpected.

A recent study by the authors documented inconsistency in the retention of serum proteins by these same devices (10). The present study confirms the findings of the previous study; ultrafiltration is complex, poorly characterized and incompletely understood. The movement of water, T_3 and T_4 across semi-permeable membranes could not be predicted based on the reported molecular weight cutoff. These inconsistencies complicate the interpretation of previous free thyroid hormone measurements involving uncharacterized ultrafiltration.

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10. Fritz KS, Wilcox RB, Nelson JC. Quantifying Spurious Free T4 Results Attributable to Thyroxine-Binding Proteins in Serum Dialysates and Ultrafiltrates. Clin Chem. 2007;53:985-8.

CHAPTER 5

UNPUBLISHED DATA

THE DIFFUSION OF FREE THYROID HORMONES IN EQUILIBRIUM DIALYSIS

KRISTOFER S. FRITZ [1], R BRUCE WILCOX [2] and JERALD C. NELSON [2]

1. Graduate student who oversaw and performed most experiments.
2. Mentor who provided research guidance.

Introduction

The thyroid hormones in serum, triiodothyronine (T_3) and thyroxine (T_4), exist in two forms: protein-bound and unbound (free). For decades, widely accepted reference methods for the quantification of free thyroxine have been direct equilibrium dialysis radioimmunoassays (RIA) (Nelson 1988, Helenius 1983, Weeke 1978). These methods are based on the separation of free T_4 from serum proteins and protein bound T_4 using equilibrium dialysis. A lack of scientific evidence characterizing the diffusion of free thyroid hormones during equilibrium dialysis has led us to ask the following questions: Do free T_3 and free T_4 diffuse across semi-permeable membranes at the same rate? How do the diffusion rates of free T_3 and free T_4 compare to the diffusion rate of water? Does the volumetric ratio of retentate to dialysate have an effect on the time-to-equilibrium of water, free T_3 and free T_4? And, will the presence of normal serum proteins in the sample have an effect on the time-to-equilibrium of water, free T_3 and free T_4?

Materials and methods

Serum dialysate

A pool of normal human serum was obtained (Equitech-Bio) and characterized. Serum TBG, transthyretin, albumin, total protein, total T_4, free T_4 and TSH were within their respective reference ranges, and anti-T_4, anti-T_3, anti-IgG and salicylates were negative (Quest Diagnostics).

Normal serum dialysate was obtained by dialyzing 200 µL of this serum against 2400 µL of dialysis buffer. Dialysis cells and dialysate buffer were obtained (Nichols Institute Diagnostics) and have been previously described (Nelson 1988). Dialysis was performed in a moisture saturated atmosphere (greater than 90% humidity) at 37°C for 18 hours using a regenerated cellulose membrane of 12-14 kDa molecular weight cutoff (MWCO) (Millipore).

Isotope labeled Water, T_3 and T_4

Radiolabeled water (^3H_2O), triiodothyronine (^{125}I-T_3) and thyroxine (^{125}I-T_4) were obtained (PerkinElmer Life Sciences). ^3H_2O was quantified using an automated liquid scintillation counter (Beckman LS 7500, Beckman Coulter). ^{125}I-T_3 and ^{125}I-T_4 were quantified using an automated gamma counter (Gamma 4000, Beckman Coulter). Routine background checks were performed on these instruments to maintain precision, accuracy and counting efficiency.

Radiolabeled T_3 and T_4 are known to undergo radiolytic degradation, forming radiolabeled impurities. Prior to each experiment, radiolabeled T_3 and T_4 were purified as

previously described (Fritz 2007). Each of the three, high purity, radiolabeled analytes ($^{3}H_2O$, ^{125}I-T_3 and ^{125}I-T_4) was added, individually, to normal serum dialysate.

Water, T_3 and T_4 Diffusion in Dialysis

The solutions of $^{3}H_2O$, ^{125}I-T_3 and ^{125}I-T_4 were each dialyzed against normal serum dialysate. Three dialysis devices were studied: A vertical membrane dialysis device applied to 10 mL of sample retentate and 10 mL of dialysate (Fisher Scientific), a horizontal membrane dialysis device applied to 200 uL or 600 uL of sample retentate and 2400 µL of dialysate (Nichols Institute Diagnostics) and a vertical membrane dialysis device applied to 200 uL of sample retentate and 1400 µL of dialysate (Quest Diagnostics Reference Labs). Spectra/Por regenerated cellulose membrane (Fisher Scientific) with a 12-14 kDa MWCO, was used in all devices. Samples were dialyzed at 37°C in an Isotemp Incubator, Model 630D (Fisher Scientific). Each of the 4 selected dialysis methods (3 devices) were applied separately to the solutions of $^{3}H_2O$, ^{125}I-T_3 and ^{125}I-T_4.

In one method, the three analytes were added, individually, to an aliquot of the normal serum and were dialyzed against normal serum dialysate.

T_3 and T_4 adsorption to glassware

T_3 and T_4 can be adsorbed onto solid surfaces from aqueous solutions. The borosilicate glassware used in this study (Fisher Scientific) was tested for T_3 and T_4 adsorption as previously described (Fritz 2007). Less than 0.7% of ^{125}I-T_3 and less than 0.4% of ^{125}I-T_4 was adsorbed in the absence of serum proteins.

Results

The diffusion of $^{3}H_2O$ was 5 to 8 times more rapid than the diffusions of free T_3 and free T_4, varying with the volumes of aqueous solutions (Figure 5.1). In the absence of serum proteins, the diffusions of $^{3}H_2O$, ^{125}I-T_3 and ^{125}I-T_4 during equilibrium dialysis were determined, in triplicate, for each of the four dialysis methods. Dialysis was monitored until equilibrium was reached (Table 5.1). In all four of the dialysis methods, no apparent differences exist between the delayed diffusion of T_3 and of T_4.

In one dialysis method, 200 uL retentate and 2400 uL dialysate, the three analytes were applied in the absence of serum proteins as well as in the presence of serum proteins. Normal levels of serum proteins in the retentate had no affect on the diffusion rate of $^{3}H_2O$ (Figure 5.2). However, the presence of serum proteins delayed the diffusion of thyroid hormones from 12 hours to 18 hours, resulting in a 50% increase in the time required to reach equilibrium (Figure 5.2).

Table 5.1 Time (in hours, at 37° C) before equilibrium is reached for H_2O, free T_3 and free T_4 when retentate and/or dialysate volumes differ. Determined in the absence[a] and presence[b] of normal serum proteins.

Retentate Volume	Dialysate Volume	H_2O	T_3	T_4
200 µL[a]	1400 µL	1	6	6
600 µL[a]	2400 µL	5	24	24
10 mL[a]	10 mL	5	32	32
200 µL[a]	2400 µL	1.5	12	12
200 µL[b]	2400 µL	1.5	18	18

Figure 5.1 Retentate and dialysate volume affects rates of diffusion.

Diffusion of ○ $^{3}H_2O$, □ ^{125}I-T_3 and Δ ^{125}I-T_4 when retentate and/or dialysate volumes differ. Top Left (10 mL : 10 mL), Top Right (200 μL : 1400 μL), Bottom Left (600 μL : 2400 μL), Bottom Right (200 μL : 2400 μL).

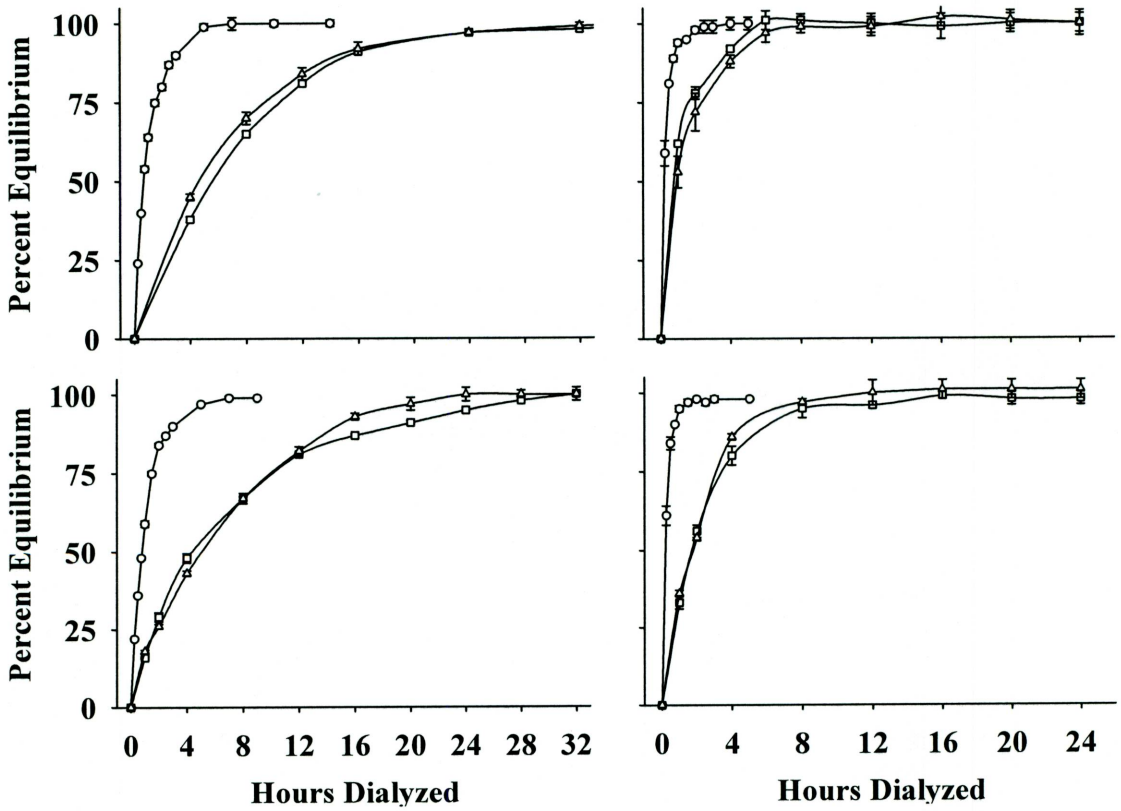
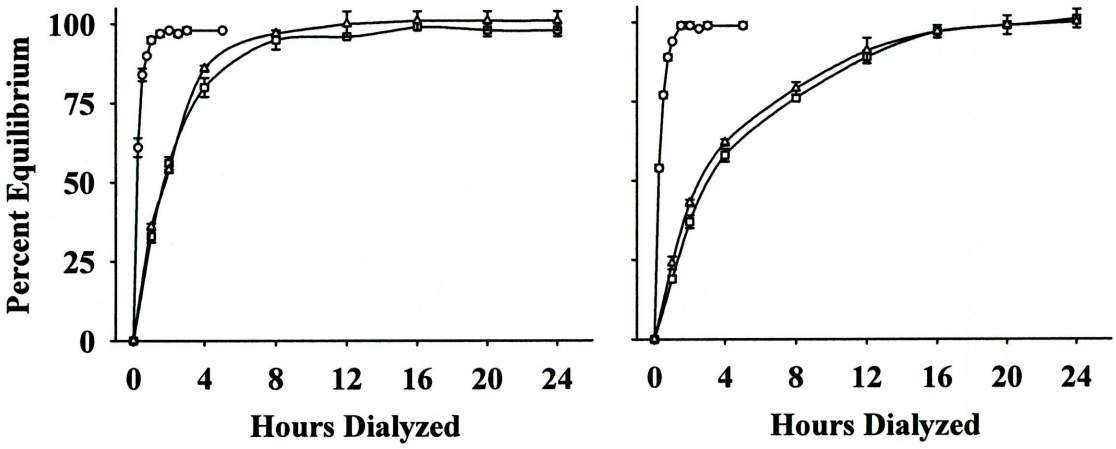

Percent Equilibrium
100
75
50
25
0
Percent Equilibrium
100
75
50
25
0
0 4 8 12 16 20 24 28 32
Hours Dialyzed
0 4 8 12 16 20 24
Hours Dialyzed

Figure 5.2 Normal serum proteins affect the rate of diffusion.
The diffusions of ○ $^{3}H_2O$, □ ^{125}I-T_3 and Δ ^{125}I-T_4 at 37°C in the absence (left) and presence (right) of normal serum proteins. (200 μL retentate and 2400 μL dialysate volumes were used in both.)


Percent Equilibrium
100
75
50
25
0
0
4
8
12
16
20
24
Hours Dialyzed
0
4
8
12
16
20
24
Hours Dialyzed

Discussion

The application of four equilibrium dialysis methods to $^{3}H_2O$, ^{125}I-T_3 and ^{125}I-T_4 provides previously unreported evidence on the diffusion of thyroid hormones during dialysis (Figure 1 & 2). Free thyroid hormones do not diffuse through semi-permeable dialysis membranes at the same rate as water. The presence of serum proteins has no affect on the diffusion rate of water while having a 50% decrease in the diffusion rate for both thyroid hormones. These data show the significant role that thyroid hormone binding serum proteins have on the rate of dialysis of thyroid hormones. Based on the findings of this study, equilibrium dialysis methods used for the measurement of free T_3 or free T_4 in aqueous solutions should be characterized for rates of hormone diffusion related to protein binding affects as well as to retentate and dialysate volumes.

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We thank Nichols Institute Diagnostics, San Clemente, CA, for providing some of the devices and reagents used in this study. Jerald C. Nelson was formerly Senior Medical Director of Quest Diagnostics Nichols Institute, San Juan Capistrano. He has no current affiliation with Quest Diagnostics. He is a consultant to Antech Diagnostics. The authors wish to thank the Department of Biochemistry at Loma Linda University School of Medicine for funding.

CHAPTER 6

UNPUBLISHED DATA AND CONCLUSIONS

A DOCUMENTED LACK OF SENSITIVITY AND SPECIFICITY AMONG SEVEN DIRECT FREE T4 IMMUNOASSAYS

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1. Graduate student who oversaw and completed all experiments and analyzed the data.
2. Mentor who provided research guidance and critically reviewed the project.

Introduction

The experiments and data described in this chapter were performed to fulfill the specific aims of the study and are currently being incorporated into manuscripts for publication. The results of these experiments provide additional insight into the first two specific aims of the dissertation: (1) To study dialyzed and ultrafiltered human serum in analog-based free T_4 immunoassays. (2) To determine the contributions of serum proteins and protein bound T_4 to analog-based free T_4 determinations.

Materials and Methods

Normal human serum

We obtained normal human serum from 16 healthy male volunteers, ages 21 to 55 years. These sera were pooled. Serum collection was approved by the institutional review board, and serum samples were given anonymous identifiers. In this pool, serum thyroid-stimulating hormone, total T_4, free T_4, thyroxin-binding globulin, transthyretin, and albumin were within their respective reference intervals, and test results for anti-T_4, anti-T_3, and anti-IgG antibodies and for salicylates were negative (testing performed at Quest Diagnostics) (data not presented).

T_4 immunoassays

Seven direct analog-based free T_4 immunoassays were studied: Bayer Centaur, Roche Elecsys, TOSOH 600 II, Ortho Vitros, Beckman Access, Abbott AxSym and DPC Immulite 2000. One direct dialysis free T_4 radioimmunoassay (Antech Diagnostics) and

one direct total T_4 immunoassay (Diagnostic Products Corp) were applied to the same solutions.

Each assay was performed according to its manufacturer's instructions. Each T_4 result reported was a mean of triplicate. We detected and quantified gamma radiation by use of a Gamma 4000 multiwell automated gamma counter (Beckman-Coulter).

T_4-depleted normal human serum

We stripped an aliquot of the serum pool of T_4 using Amberlite IRA-410 anion exchange resin as previously described (Chapter 2). No residual total T_4 was detected by the total T_4 radioimmunoassay, and no dialyzable T_4 was detected by the nonanalog free T_4 immunoassay.

Sodium levothyroxine

We obtained sodium levothyroxine, for injection, in 500-μg vials (Bedford Labs). It was dissolved at room temperature in 5 mL of 9 mL/L NaCl, USP grade (Abbott Labs). This produced a stock solution containing 125 μmol/L (10 000 μg/dL) sodium levothyroxine.

Equilibrium dialysis

We obtained dialysis devices and dialysate buffer from Antech Diagnostics. The chemical composition of this buffer has been reported (Chapter 2). Serum samples were dialyzed for 18 h at 37°C in an Isotemp Incubator, model 630D (Fisher Scientific). A

moisture-saturated atmosphere was maintained during dialysis by enclosing dialysis cells in containers with open water reservoirs.

Serum pH control

When dialysis was applied, the pH of serum dialysate and retentate was controlled to 7.4 (± 0.1) during equilibrium dialysis at 37°C by the HEPES acid in dialysate buffer (Chapter 2). At equilibrium, the final HEPES ion concentration was calculated to be 54 mmol/L.

Free T_4 adsorption to container surfaces

Free T_4 can adsorb onto solid surfaces from aqueous solutions. We tested the borosilicate glass vials and test tubes used (Fisher Scientific) for free T_4 adsorption as described in Chapter 2.

Experimental strategies

The previously described analog-based free T_4 immunoassays, direct dialysis free T_4 immunoassay and direct non-analog total T_4 immunoassay were applied to the following normal human serum based solutions:

- The fractions of serum T_4 obtained by equilibrium dialysis.
- Varied concentrations of sodium levothyroxine added to T_4 depleted normal human serum at concentrations ranging from 3 to 24 μg/dL. The dialyzable T_4 in these solutions (direct equilibrium dialysis) was (0.6 to 9.7 ng/dL).

- Varied concentrations of sodium levothyroxine added to normal human serum dialysate at concentrations ranging from 0.05 to 21 μg/dL.
- Varied concentrations of serum proteins, protein bound T_4 and total T_4 were concentrated, while free T_4 concentration remained constant. Equilibrium dialysis was applied to normal serum. One aliquot of retentate was undiluted. Other aliquots of the normal serum retentate were diluted from 90% to 30% using the normal serum dialysate as the diluent. Thus, serum proteins, protein bound T_4 and total T_4 were varied from a low of 30% of native serum levels to a high of 100% of native serum levels, while free T_4 remained constant. The variation in total T_4 concentrations was confirmed by measuring total T_4. The constancy of free T_4 concentration was confirmed by measuring dialyzable T_4 (Figure 6.5).
- Varied concentrations of serum proteins and protein-bound T_4 were progressively replaced with free T_4 while total T_4 was held constant. We applied equilibrium dialysis to normal serum. We measured the total T_4 in retentate using the total T_4 immunoassay and added sodium levothyroxine solution to the dialysate to match the concentration of total T_4 in the retentate. The retentate was progressively diluted with T_4-enriched dialysate to a dilution of 1 to 4000. We measured dialyzable T_4 by use of nonanalog free T_4 immunoassay. High dialyzable T_4 concentrations were diluted with immunoassay zero calibrator, as needed, to bring them into the interval quantified by the nonanalog free T_4 immunoassay. These dialyzable T_4 measurements varied from 1.3 to 634 ng/dL (Table 6.2). Total T_4 concentrations were constant and unchanged as measured by direct total T_4 RIA (6.0 ug/dL).

Results

Following equilibrium dialysis, the protein bound fraction of normal serum yielded test results from all seven free T_4 immunoassays (Table 6.1). The free fraction of normal serum T_4 did not account for the test results from any of the seven immunoassays (Table 6.1).

Co-varied concentrations of free T_4, protein bound T_4 and total T_4 yielded test results from all assays. The lowest test result, 0.4 ng/dL, was associated with a total T_4 concentration of 3000 ng/dL, and a dialyzable T_4 concentration of 0.6 ng/dL. The highest test result, 5.8 ng/dL, was associated with a total T_4 concentration of 21,000 ng/dL, and a dialyzable T_4 concentration of >10 ng/dL. These T_4 concentrations overlap the concentrations found in human sera (Figures 6.1 & 6.2).

Varied concentrations of free T_4 also yielded test results when serum proteins and protein bound T_4 were not present. The lowest test result, 0.1 ng/dL, was associated with a free T_4 concentration of 250 ng/dL. The highest test result, 11.5 ng/dL, was associated with a free T_4 concentration of 12,000 ng/dL (Figure 6.3). These free T_4 concentrations did not overlap the concentrations of free T_4 found in human sera. They exceed the free T_4 concentrations and overlap the concentrations of total T_4 found in human sera.

Comparing the responses of these seven analog-based free T_4 immunoassays to T_4 in the presence and absence of serum proteins yields striking inconsistencies. While both sets of solutions applied to the seven assays result in reported mean T_4 values of 3.5 ng/dL, they are clearly responding to two very different forms of T_4 (Figure 6.4).

When serum protein concentrations, protein bound T_4 concentrations, and total T_4 concentrations were varied from 100% to 30% of whole serum concentrations while free T_4 was constant, the seven analog-based free T_4 test results varied wildly (Figure 6.5). At 100% of normal serum protein and total T_4, the reported free T_4 values ranged from 0.9 to 1.3 ng/dL. While at 30% of normal serum protein and total T_4 concentrations, the reported free T_4 values ranged from 0.6 to 3.9 ng/dL. Dialyzable free T_4 was constant and unchanged across the same sample range (Figure 6.6).

When free T_4 progressively replaced protein bound T_4 while total T_4 was constant, most of the analog-based free T_4 assays reported measurements that were not related to free T_4 concentrations, total T_4 concentrations, protein bound T_4 concentrations or serum protein concentrations (Table 6.2).

Table 6.1 Normal human serum was applied to equilibrium dialysis. Both fractions obtained after dialysis were applied to seven analog-based free T_4 immunoassays. These assays did not detect the free T_4 in serum dialysate. These assays reported test results for retentates, the fraction containing serum proteins, protein bound T_4 and free T_4.

Table 6.1. The fractions of normal serum T_4 that account for test results (ng/dL).

	Centaur	Elecsys	Tosoh	Access	Vitros	AxSYM	Immulite
Dialysate	ND	ND	0.4*	ND	0.3*	ND	ND
Retentate	1.1	1.3	1.0	1.0	1.1	0.9	1.3

* Denotes a false T_4 value (see Results)

Table 6.2 Seven analog-based free T_4 immunoassays were applied to solutions where total T_4 was held constant and protein bound T_4 was progressively replaced with free T_4.

Table 6.2. Seven analog-based free T_4 assays were applied to solutions where total T_4 was held constant while serum protein, protein bound T_4 and free T_4 were varied by dilution. (ng/dL)

Protein (g/L)	Dialyzable T_4	Centaur	Elecsys	Vitros	AxSym	Tosoh	Access	Immulite
86.000	1.3	1.3	1.3	1.1	0.9	1.0	1.0	1.3
21.500	6.9	3.8	3.9	5.7	2.9	> 10	4.2	2.8
8.600	26	6.2	6.1	> 7	4.3	> 10	5.4	3.8
2.150	110	8.2	7.5	> 7	5.3	> 10	> 6	4.4
0.860	306	8.0	7.7	> 7	5.3	> 10	> 6	4.0
0.215	435	7.3	6.8	> 7	5.2	> 10	> 6	4.3
0.086	524	6.8	5.9	> 7	4.9	> 10	> 6	3.8
0.022	634	6.8	5.9	> 7	4.8	> 10	> 6	3.9

Figure 6.1 Comparison of analog-based free T_4 measurements in the presence of normal serum proteins. Analog free T_4 test results vs. dialyzable free T_4 measurements.

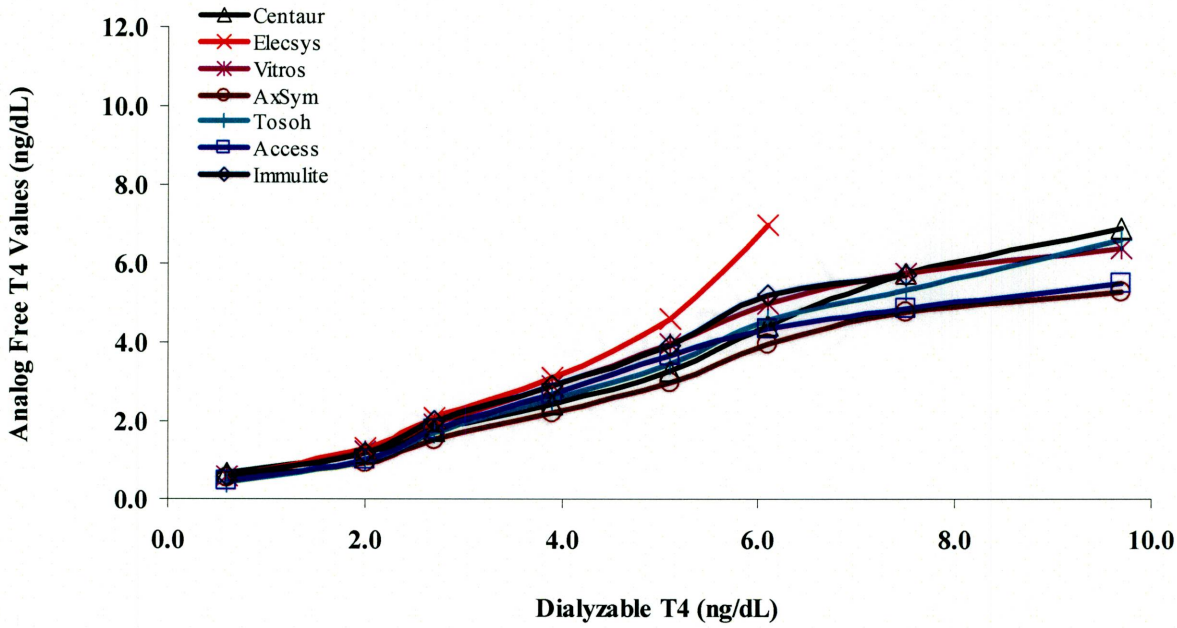

Centaur
Elecsys
Vitros
AxSym
Tosoh
Access
Immulite
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0.0
2.0
4.0
6.0
8.0
10.0
Analog Free T4 Values (ng/dL)
Dialyzable T4 (ng/dL)

Figure 6.2 Comparison of analog-based free T_4 measurements in the presence of normal serum proteins. Analog free T_4 test results vs. total T_4 added to serum (gravimetrically added).

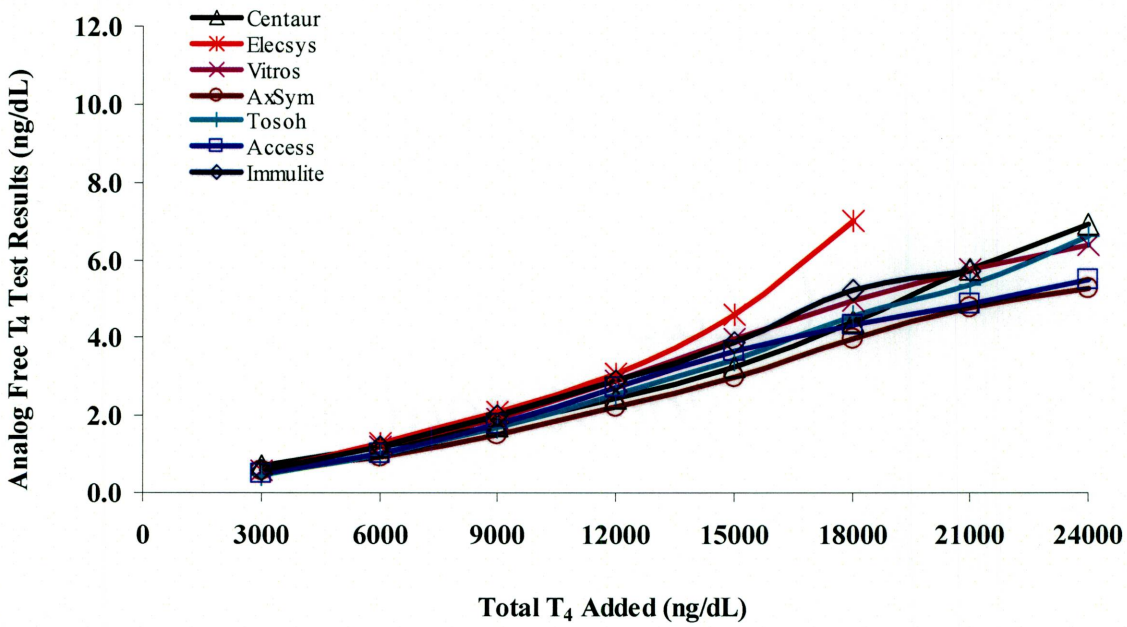

Centaur
Elecsys
Vitros
AxSym
Tosoh
Access
Immulite
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
3000
6000
9000
12000
15000
18000
21000
24000
Analog Free T4 Test Results (ng/dL)
Total T4 Added (ng/dL)

Figure 6.3 Comparison of analog-based free T_4 measurements in the absence of serum proteins. Analog free T_4 test results vs. free T_4 added to serum dialysate (gravimetrically added).

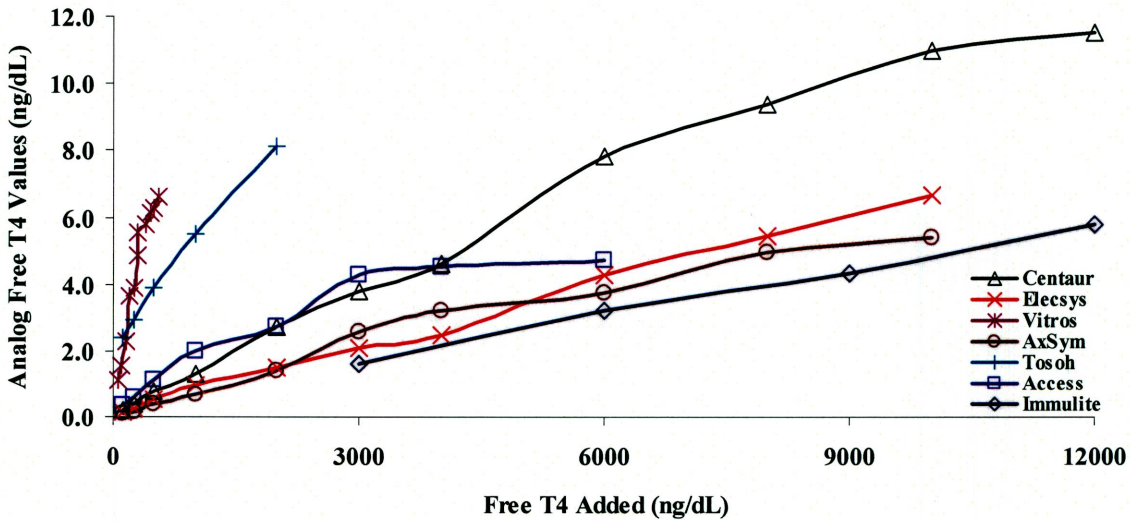
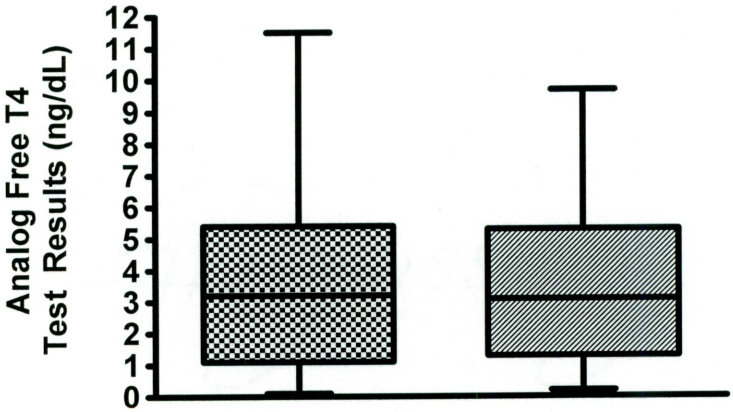

12.0
10.0
8.0
6.0
4.0
2.0
0.0
Analog Free T4 Values (ng/dL)
0
3000
6000
9000
12000
Free T4 Added (ng/dL)
Centaur
Elecsys
Vitros
AxSym
Tosoh
Access
Immulite

Figure 6.4 Comparison of analog-based free T_4 measurements, from seven immunoassays, in the presence of serum proteins and in the absence of serum proteins. The difference between analog free T_4 test results when free T_4 concentration changed from 0.6 to 9.7 ug/dL (normal serum proteins present and constant) to 0.05 to 15 ug/dL (when serum proteins are absent).

The Influence of Serum Proteins on Analog-based Free T4 Measurements



Proteins Absent	Proteins Present
n = 59	n = 59
Median = 3.2	Median = 3.1
Mean = 3.5	Mean = 3.5
StDev = 2.7	StDev = 2.3
StErr = 0.4	StErr = 0.3

Figure 6.5 Seven analog-based free T_4 immunoassays were applied to progressive dilutions of serum protein and total T_4, while free T_4 was held constant.

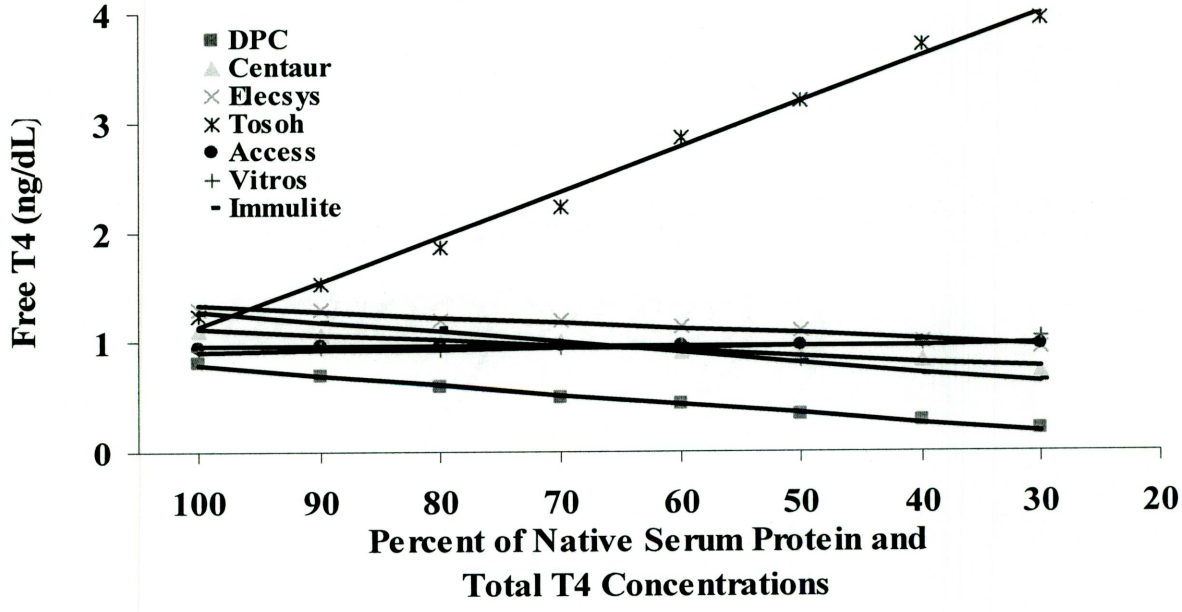
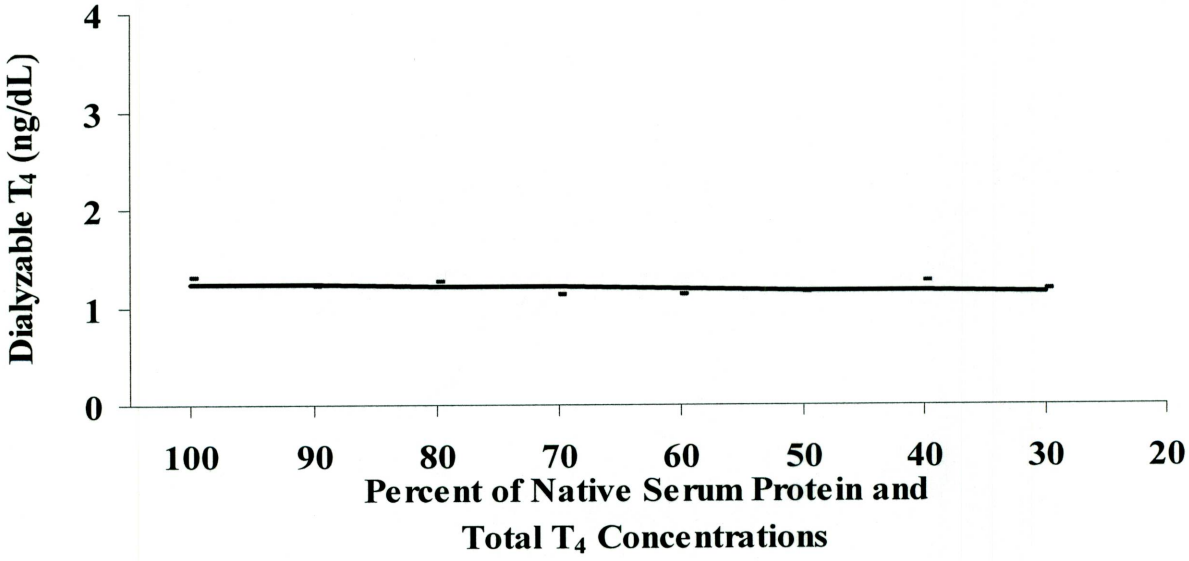

Free T4 (ng/dL)
4
3
2
1
0
DPC
Centaur
Elecsys
Tosoh
Access
Vitros
Immulite
100
90
80
70
60
50
40
30
20
Percent of Native Serum Protein and
Total T4 Concentrations

Figure 6.6 A direct equilibrium dialysis free T_4 assay was applied to progressive dilutions of serum protein and total T_4, while free T_4 was held constant.


Dialyzable T_4 (ng/dL)
4
3
2
1
0
100
90
80
70
60
50
40
30
20
Percent of Native Serum Protein and
Total T_4 Concentrations

Discussion

The data published in Chapter 2 and the unpublished data contained in this chapter present clear and valid experiments that were applied to the characterization of analog-based free T_4 immunoassays. Equilibrium dialysis is a well characterized procedure that can retain T_4 binding serum proteins and protein bound T_4 while water and free T_4 move across semi-permeable membranes. Free T_4 concentrations should be equal on both sides of dialysis membranes when equilibrium is reached. Dialyzable T_4, at physiologic concentrations, is measurable by non-analog free T_4 immunoassays (Nelson 1988, Wilkinson 1991). Applying serum-based solutions obtained from the two fractions of serum dialysis to the characterization of analog-based free T_4 immunoassays is a new and novel concept that has yielded striking results.

In the absence of serum proteins, increasing serum total T_4 concentrations to 250 ng/dL lead to a concentration of dialyzable T_4 that was measured by analog free T_4 immunoassays. This is a clinically irrelevant concentration of free T_4. It is a clinically relevant concentration of total T_4. Increasing concentrations of T_4 in normal serum dialysate (no proteins present) yielded a range of free T_4 concentrations that were measured by analog free T_4 immunoassays. These measurements were 0.1-11.5 ng/dL (Figure 6.3). The concentrations of T_4 added ranged from 0.05 to 12.0 μg/dL (50 to 12,000 ng/dL). These are clinically irrelevant free T_4 concentrations. They are clinically relevant concentrations of total T_4, overlapping both the hypothyroid and euthyroid ranges. These data provide direct evidence that analog-based free T_4 immunoassays respond to and measure clinically relevant total T_4 concentrations.

In the presence of clinically relevant serum protein concentrations, analog-based free T_4 measurements were obtained with clinically relevant total T_4 concentrations (3 to 21 μg/dL). The corresponding dialyzable T_4 concentrations were 0.6 to 7.5 ng/dL. These are concentrations not detected by analog free T_4 assays (Figure 6.3). The data contained in Figures 6.2 and 6.3 document striking evidence of insensitivity and nonspecificity among analog-based free T_4 immunoassays. It is important to note that analog-based free T_4 measurements correlated closely with dialyzable T_4 measurements (Figure 6.1), despite the evidence of insensitivity and nonspecificty. This can be attributed to the close correlation between serum total T_4 concentrations and free (dialyzable) T_4 concentrations, in the presence of normal serum proteins.

Further experiments characterizing the lack of specificity among these assays were used to determine if these analog-based assays would track normal and constant free T_4 concentrations while serum proteins and total T_4 were progressively diluted. Under these conditions, analog free T_4 estimates did not correlate with dialyzable free T_4 concentrations (Figures 6.5 and 6.6).

A final experiment was introduced to determine if these analog-based assays would track total T_4 concentrations while protein bound T_4 was progressively replaced with free T_4. The results again confirmed a lack of specificity and sensitivity for free T_4 (Table 6.2).

These new data are inconsistent with a widely accepted concept of analog-based free T_4 immunoassays (Christofides 1992, Midgley 1993, Midgley 2001). These assays are based on the displacement of a conjugated form of T_4 (analog) from T_4 antibodies by the T_4 in serum. The current concept assumes that analog-based free T_4 measurements

will be obtained with free T_4 concentrations and the free form of T_4 only. That assumption is inconsistent with the experimental data presented in Chapter 2 and this chapter (Figures 6.2, 6.3, 6.5, 6.6 and Tables 1 & 2).

There are two simple hypotheses that could explain the present data. One postulates that serum protein T_4 complexes, as well as free T_4, are capable of displacing T_4 analogs from T_4 antibodies. This hypothesis is attractive because it is simple and untested.

Another simple hypothesis is that T_4 binding inhibitors are present, releasing protein bound T_4 from serum protein T_4 complexes and raising free T_4 concentrations during the assay procedure. Suppliers of assay reagents could easily refute or confirm this hypothesis by fully disclosing the compounds in their reagent solutions.

Previous studies have found correlations between analog free T_4 measurements and dialyzable T_4 concentrations and are confirmed by our data (Figure 6.1). Unlike earlier studies, our experiments also looked for correlations between analog free T_4 measurements and total T_4 concentrations. Correlations were found (Figures 2.1, 2.2 and Table 6.2). This leads to the question of which is the direct correlation and which is the indirect correlation. The data presented in Chapter 2 and this chapter led us to conclude that the direct correlation is with total T_4 concentrations.

A question to be answered is whether the insensitivity and nonspecificity found in these analog free T_4 immunoassays will be present in other, as yet untested, analog-based free T_4 immunoassays. It is time for project directors and journal editors to question the sensitivity and specificity of direct free T_4 immunoassays. They have been used for decades, without fully understanding their characteristics.

Conclusions

The progressive development of the following focused and previously unanswered questions led to the specific experiments described in this dissertation: (1) Are direct free T_4 determinations sometimes attributable to serum compounds other than thyroid hormones (i.e., "blank effects")? (2) What portion of serum T_4 is most closely related to direct free T_4 determinations, following equilibrium dialysis and ultrafiltration? (3) What concentrations of T_4 are most closely related to direct free T_4 determinations, when T_4 binding serum proteins and protein bound T_4 are not present? (4) What concentrations of T_4 are most closely related to direct free T_4 determinations, when T_4 binding serum proteins are held constant? (5) What T_4 is most closely related to direct free T_4 determinations, when free T_4 is held constant, while T_4 binding serum proteins, protein bound T_4 and total T_4 are varied? (6) What T_4 is most closely related to direct free T_4 determinations, when free T_4 replaces protein bound T_4 while total T_4 concentration is held constant? (7) How do analog-based free T_4 determinations relate to those obtained by direct equilibrium dialysis free T_4 assays and total T_4 assays?

The data presented in Chapters 3-5 illustrate the technical deficiencies of ultrafiltration as a method for separating free T_4 from serum proteins; that it is unreliable and should not be used in free hormone assays. Equilibrium dialysis, on the other hand, is shown to be a well characterized and reliable method. More importantly, the evidence presented in this dissertation (Ch. 2 & 6) supports our hypothesis: Direct free thyroxine immunoassays do not detect and quantify free T_4 but detect and quantify protein bound thyroxine and/or total thyroxine. These assays are insensitive and nonspecific for free T_4.

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