A SIMPLIFIED METHOD FOR ESTIMATING FREE-THYROXINE FRACTION IN SERUM

Ralph R. Cavalieri, James N. Castle and Gilbert L. Searle

Veterans Administration Hospital, San Francisco, California

The level of free thyroxine (T_4) in the blood is an important correlate of an individual's thyroid status. Evidence for this concept has been recently reviewed (*1*). The association constants for the binding of the hormone to its specific carrier proteins in plasma are such that approximately 1 molecule of T_4 in 2,000 is in the free state (*1*). In recent years several methods have been developed to estimate the free-thyroxine fraction (F T_4 F), i.e., the percent of total serum T_4 that exists in the free form. All these methods involve some procedure to separate "free" and "bound" fractions of labeled T_4 added to the serum. In some of these techniques equilibrium dialysis is used (*2–4*); in others, ultrafiltration (*5*), charcoal particles (*6*) or columns of Dextran gel (Sephadex) (*7*) are used.

In an attempt to improve the existing methods of estimating F T_4 F we have developed a simple, inexpensive technique suitable for routine use in a diagnostic laboratory. The principle of our method depends on the competition for labeled T_4 between thyroxine-binding plasma proteins and Sephadex.

MATERIALS AND METHODS

Buffer. Potassium phosphate buffer, hereafter referred to as buffer, is made from 0.075 *M* K_2HPO_4 (405 ml) and 0.075 *M* KH_2PO_4 (95 ml) diluted to 1,000 ml with water. The final pH should be 7.40 ± .02.

"Working solution" of labeled thyroxine. ^{131}I-L-thyroxine is obtained every 2 weeks from a commercial supplier with a specific activity from 30 to 40 mCi/mg. A working solution was prepared as follows: approximately 0.5 ml of ^{131}I-T_4 solution is added to 2.5 ml of 2% serum albumin in phosphate buffer. In place of albumin, 1% serum (0.025 ml serum to 2.5 ml buffer) can be used here. The protein is necessary to prevent losses of T_4 by absorption to surfaces. The exact volume of ^{131}I-T_4 solution used in preparing this working solution is calculated from the concentration of carrier T_4 in the original shipment, so as to yield a concentration of approximately 1 μg T_4/ml. The working solution is kept at 4°C in the dark and should be made fresh every week. The ^{125}I-T_4 can also be used in this method.

Sephadex G-25, coarse grade, is obtained from a commercial source (Pharmacia Fine Chemicals, Piscathaway, N.J.). Exactly 0.20-gm portions are placed into dry, disposable plastic tubes, 16 × 125 mm. A precalibrated plastic spoon which delivers 0.20 gm Sephadex is recommended for this purpose. See Ref *17* for a description of the spoon which we have found satisfactory. This type of spoon delivers 0.2-gm Sephadex with 1% reproducibility.

Control serum. A pool of sera obtained from the Clinical Laboratory was used for quality control. One-milliliter aliquots of this pool were kept frozen until used.

PROCEDURE

To 1 ml of the serum to be tested add 20 μl of the working solution containing labeled T_4. This step results in the addition of 0.02 μg T_4/ml serum. Two 0.1-ml aliquots of the labeled T_4-serum mixture are diluted 32-fold by adding 3.1-ml phosphate buffer. An automatic diluting device is recommended at this step. The diluted sample is added quantitatively to 0.20 gm Sephadex, premeasured in plastic tubes. The tube is agitated on a Vortex mixer for at least 10 sec. The exact duration of mixing is not critical, but should be the same for each sample. The tube is set aside to allow the Sephadex to settle (about ½ min). Approximately ¾ of the supernatant is removed by aspiration and saved ("initial supernatant"). The sedimented Sephadex particles are washed with 3 ml of buffer, agitated for 5–10 sec and allowed to settle. About ¾ of the supernatant is removed by aspiration and discarded. The washing

Received Sept. 10, 1968; revision accepted March 28, 1969.

For reprints contact: Ralph R. Cavalieri, Veterans Administration Hospital, 4150 Clement St., San Francisco, Calif. 94121.

procedure is repeated four times (total of five washes). Following the final wash, the particles adhering to the side of the tube are washed to the bottom by addition of 1 ml buffer. Both the washed Sephadex and exactly 1 ml of the "initial supernatant" are counted in a well counter.

In a typical run using normal serum, the counting rate in the Sephadex is approximately 3% of the counting rate in 1 ml of the initial supernatant. In a typical case, the Sephadex counting rate was 292 cpm (net), and 1 ml of the supernate yielded 8,488 cpm (net). Duplicates should agree within ±5% of the mean. To maintain uniformity, the results are expressed as a percent of the value obtained in the control serum. Thus

$$\frac{\text{Patient: cpm Sephadex/cpm 1 ml Supernatant}}{\text{Control: cpm Sephadex/cpm 1 ml Supernatant}} \times 100 = \text{F T}_4\text{ F.}$$

The normalized free T_4 fraction (F T_4 F) in percent of the control value is used to calculate the free T_4 concentration index: F T_4 F (% of control) × T_4 iodine (μg/100 ml) = Free T_4 Conc. Index where the T_4 iodine is determined by chemical analysis following separation on an ion-exchange column (*8*). The serum PBI can also be used at this point as an approximation of total T_4 iodine. No significance should be attached to the units in which free T_4 concentration is expressed (hence an "index").

RESULTS

The effects of several variables were tested. Increasing the quantity of Sephadex from 0.07 to 0.20 gm resulted in a linear increase in the proportion of labeled T_4 taken up (Fig. 1A). Quantities of Sephadex greater than 0.2 gm gave proportionately higher uptake values. Although the absolute quantity of Sephadex added is important, it is more essential to add exactly the same amount to each tube because the final results for F T_4 F are expressed in percent of the control value.

The effect of varying the number of washes is shown in Fig. 1B. The first two or three washes removes most of the protein-bound radioactivity. Increasing the number of washes beyond five results in only a slight decrease in the percent of label associated with Sephadex. For routine use, five washes proved to be adequate.

The duration of exposure to Sephadex was found not to be critical. As shown in Fig. 2A, only a

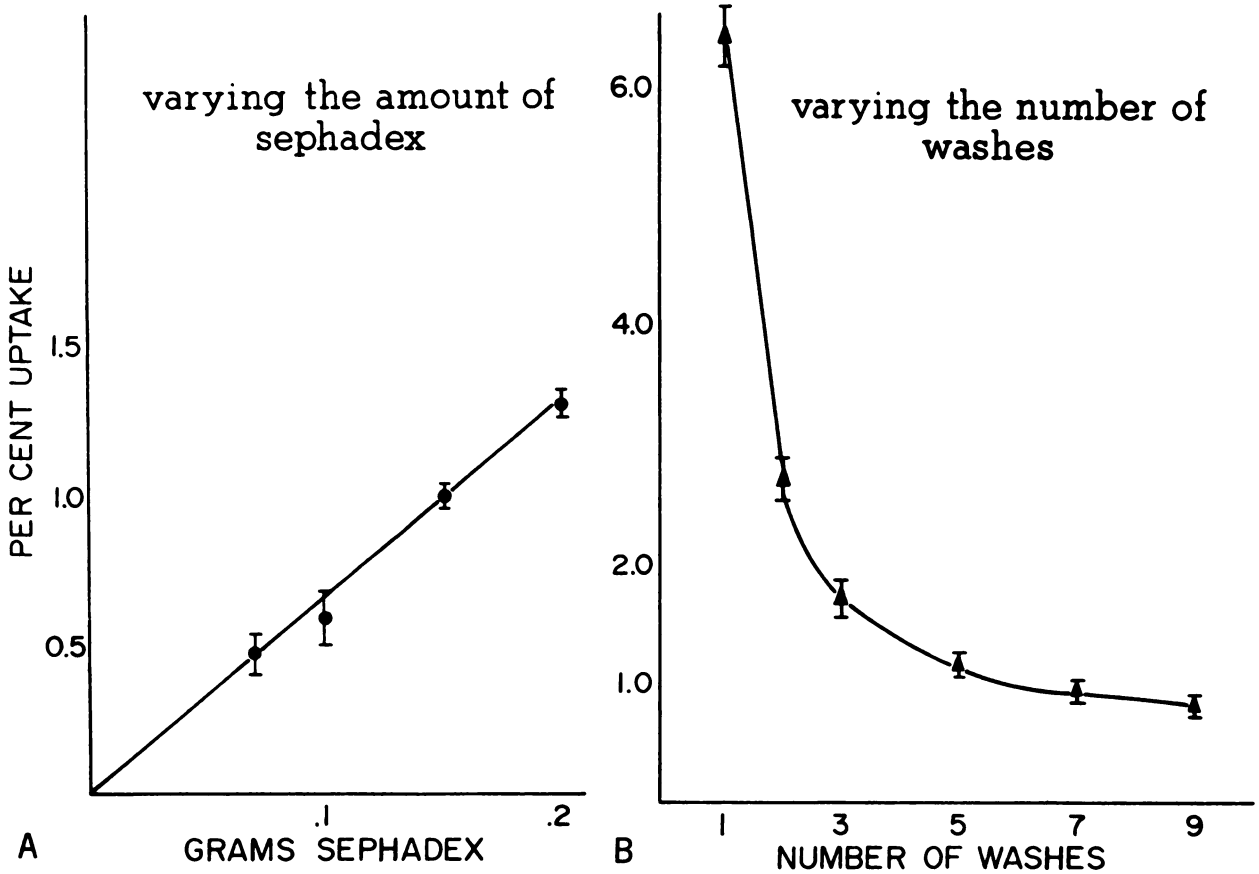

FIG. 1. A gives proportion of labeled T_4 (^{131}I) taken up by Sephadex as function of quantity of Sephadex added. Normal serum was diluted 1:32. Sephadex was washed five times as described in Methods. All other variables were kept constant. In this and all other figures data points indicate mean and brackets show ±1 s.d. of five determinations. B gives "uptake" of ^{131}I-T_4 by Sephadex (0.20 gm) as function of number of washes following 10-sec exposure. Normal serum (1:32) was used.

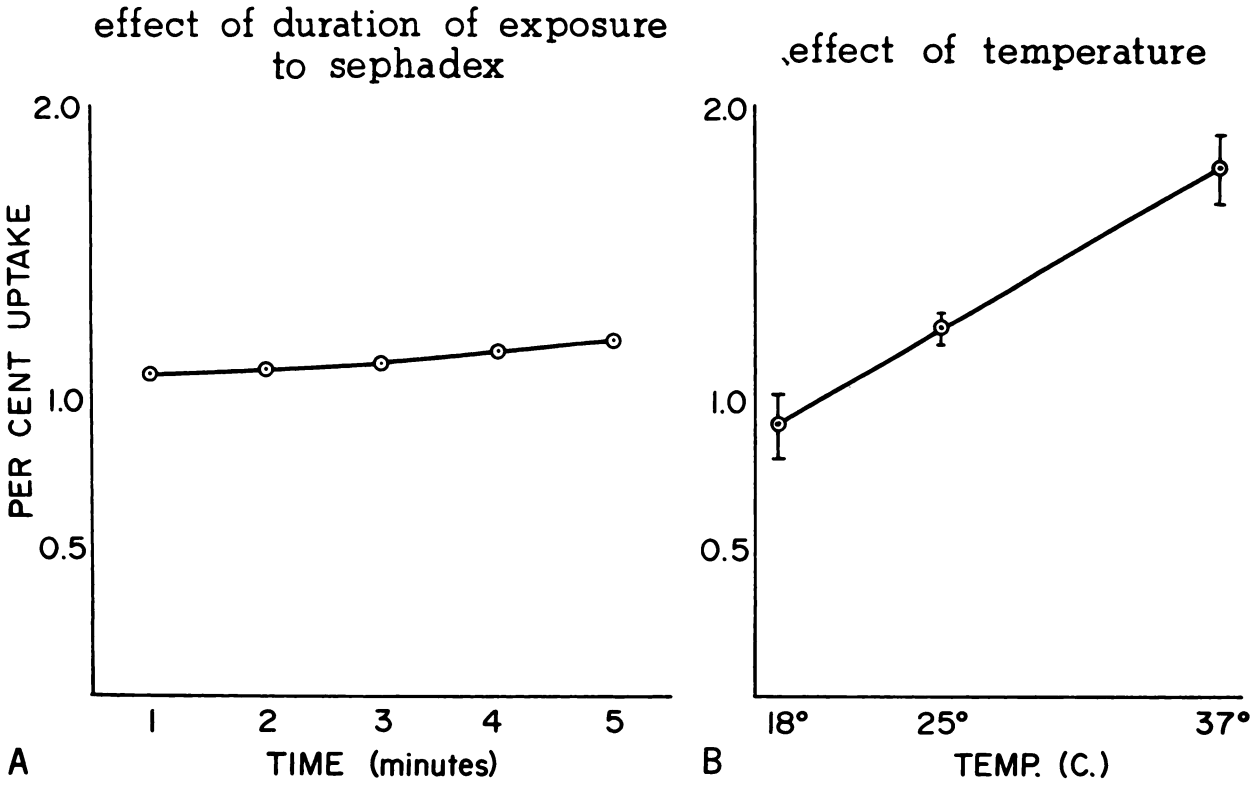

FIG. 2. A gives "uptake" of ^{131}I-T_4 by Sephadex (0.20 gm) as function of duration of exposure before washing. Tubes containing mixture of Sephadex, labeled T_4 and dilute serum were agitated continuously for period indicated. Normal serum (1:32), five washes of Sephadex. B shows effect of temperature on Sephadex "uptake" of ^{131}I-T_4 using normal serum (1:32) with 10-sec exposure and five washes. Uptake increased linearly by 0.05% for each degree rise in ambient temperature.

negligible increase in Sephadex uptake occurred when the duration of exposure before the initial wash was increased from 1 to 5 min. In other experiments it was determined that mixing for 10 sec yielded the same value as mixing for 1 min. It is apparent, therefore, that equilibration of distribution of labeled T_4 between protein and Sephadex is attained rapidly and is virtually complete within 10 sec.

Temperature affects the uptake of T_4 by Sephadex (Fig. 2B). In the temperature range between 18° and 37°C, uptake increased linearly by 0.05% for each degree rise in ambient temperature. Therefore wide fluctuations in temperature during the test are to be avoided. The inclusion of a reference control serum with each run helps to avoid errors due to day-to-day variations in room temperature.

The reproducibility of the method was evaluated by periodic testing of aliquots of a pool of normal sera stored frozen. In 17 determinations over a period of 5 weeks using three lots of ^{131}I-T_4, uptake by Sephadex as a percent of the counting rate in 1 ml of supernate ranged from 2.82 to 3.62%. (Mean = 3.28 ± 0.24 s.d.)

The effect of radioiodide which is usually present as a contaminant in ^{131}I-T_4 or ^{125}I-T_4 was tested. In three separate experiments, in which radioiodide was added to diluted serum and the procedure was carried through five washes, the radioiodide uptake by Sephadex averaged only one-fifth of the uptake of labeled T_4. Since in all lots of labeled T_4 tested within 2 weeks after arrival, the content of radioiodide never exceeded 10% of total radioactivity (usually 3–8%), then the error from the source will be less than 2%.

Organic radioiodinated impurities which have been detected in commercial preparations of ^{131}I-T_4 can be removed by dialysis in the presence of serum (*5,9*). We tested three lots of ^{131}I-T_4 by determining the Sephadex uptake from normal (pooled) serum before and after dialysis of ^{131}I-T_4 as recommended by Schussler and Plager (*5*). The results were not significantly affected by this dialysis. Therefore, preliminary purification of ^{131}I-T_4 appears unnecessary provided the preparation is used within 2 weeks after it is received.

The sensitivity of the method was tested by adding unlabeled T_4 to normal serum. As the concentration of total (labeled plus unlabeled) T_4 increases, more T_4-binding sites on serum proteins are occupied and an increasing proportion of T_4 is free and available for uptake by Sephadex. The results, given in Fig. 3, show that Sephadex uptake does in fact

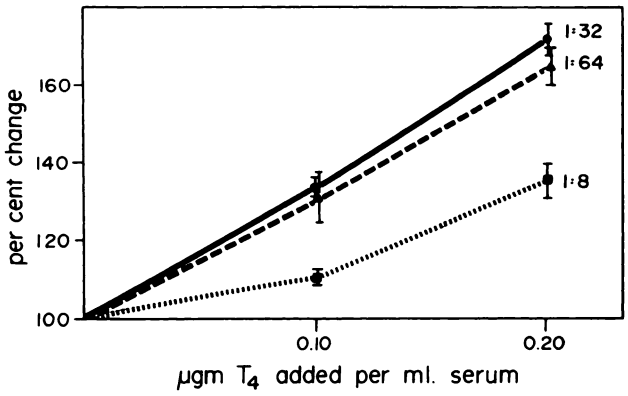

FIG. 3. Effect of added unlabeled L-T_4 on Sephadex uptake of ^{131}I-T_4 at various dilutions of serum (normal pool). L-T_4 was added to serum before dilution with buffer. Ordinate gives value obtained as percent of control value obtained with ^{131}I-T_4 but no additional unlabeled T_4.

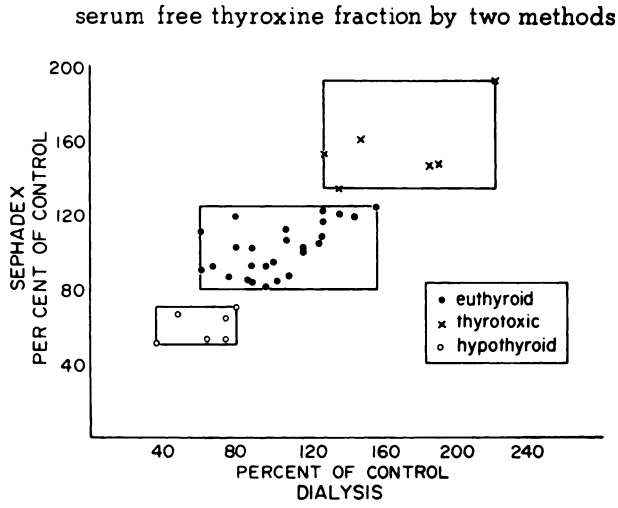

FIG. 4. Comparison of results obtained using two methods of estimating serum free thyroxine fraction (F T_4 F). On vertical axis is shown value by present Sephadex method. In both methods results are expressed as percent value obtained in control pooled serum. On horizontal axis is value obtained by equilibrium dialysis method of Sterling and Brenner (*4*). Both tests were performed on serum obtained at same time from each patient. Boxes enclose ranges in each group of patients.

increase with addition of unlabeled T_4 to serum. The highest sensitivity to loading occurred when the serum was diluted 1:32 with buffer. It was decided therefore to use this dilution routinely.

Comparison with equilibrium dialysis method: To determine the efficacy of this method over a wide range of values, sera from patients with hypothyroidism, thyrotoxicosis and from euthryroid individuals were tested using both the present method and the equilibrium dialysis technique of Sterling and Brenner (*4*). The results are shown in Fig. 4. In general, the values obtained by the two methods correlated well (correl. coeff. = +0.72). Furthermore, the range within each group was narrower by the present method, indicating improved precision.

Free T_4 fraction and free T_4 concentration index: Table 1 lists the results of determining the F T_4 F by the Sephadex method, total serum T_4 iodine by a column chromatographic method and the free T_4 concentration index calculated from these values in hyperthyroid and hypothyroid patients and in several clinically euthyroid individuals selected for abnormalities in serum T_4-binding proteins. In the hyperthyroid group (diagnosis made by clinical examination and by ^{131}I thyroid uptake) the F T_4 F ranged from 121 to 159% of control, the total T_4 iodine values were above 9 μg/100 ml and the calculated free T_4 concentration index ranged from 13.3 to 19.5. In hypothyroid patients (clinical diagnosis) the F T_4 F was below 75% in five of the six patients, total T_4 iodine was 3.0 or lower in all and calculated free T_4 concentration index was 1.8 or less. The euthyroid group showed extreme variations in F T_4 F and total T_4 iodine ranging from low to very high values. In all of these patients the free T_4 conc. index ranged from 3.7 to 6.8, within the normal range and consistent with the clinically euthyroid state of these individuals.

TABLE 1. FREE T_4 FRACTION (F T_4 F), TOTAL T_4 IODINE AND FREE T_4 CONCENTRATION INDEX IN PATIENTS WITH THYROID DISEASES AND IN EUTHYROID PATIENTS WITH VARIOUS ABNORMALITIES IN T_4-BINDING PROTEINS

Clinical Status	F T_4 F (% of control)	Total T_4 iodine (μg/100 ml)	Free T_4 conc. index
Normal range	80–120	3.0–7.0	3.0–7.0
Hyperthyroid			
AA	145	9.2	13.3
RT	121	16.1	19.5
RC	126	12.2	15.4
JR	121	12.6	15.2
BH	142	10.0	14.2
BC	159	11.8	18.8
Hypothyroid			
RG	75	2.0	1.5
CD	60	3.0	1.8
BMcG	64	1.0	0.6
NB	71	2.2	1.5
HS	93	1.0	0.9
BF	70	1.5	1.0
Euthyroid			
BB Oral contraceptive (high TBG*)	58	10.2	5.9
DR Viral hepatitis (high TBG)	55	7.2	4.0
VC Idiopathic (low TBG)	169	2.2	3.7
MZ Cirrhosis (low TBG)	203	1.9	3.9

* TBG = Serum thyroxine-binding globulin (capacity).

DISCUSSION

Each of the various methods already available for determination of the F T_4 F in serum has disadvantages limiting its usefulness as a routine diagnostic test. The techniques involving equilibrium dialysis (*2–4*), ultrafiltration (*5*) or charcoal adsorption (*6*) require prolonged incubation or repeated centrifugation. The present method was specifically designed to avoid such drawbacks without sacrificing sensitivity.

In principle the method involves the competitive binding of labeled hormone between specific sites on serum proteins and Dextran gel. A technique based on the same principle using columns of Sephadex has already been described (*7*) but has not received widespread application. In the present method the disadvantages of the column technique have been avoided. Our results indicate that within the range tested the "uptake" of labeled T_4 by Sephadex particles is proportional to the F T_4 F and that distribution equilibrium of the hormone between protein and Sephadex is achieved within a few seconds. Thus two important requirements, sensitivity and speed, have been met.

It must be emphasized that the present method provides a relative, not an absolute, estimate of F T_4 F. The "uptake" of labeled T_4 by Sephadex depends on several factors, including the amount of Sephadex added, the number of washes, the ambient temperatures, etc. We do not consider it a drawback of the method that it does not give an absolute value for F T_4 F because, as has been pointed out by Oppenheimer (*10*), it is doubtful that any available *in vitro* method yields a truly reliable and absolute estimate of F T_4 F in the circulation. The clinical usefulness of the test depends on its ability to reflect relative changes in F T_4 F under various physiologic or pathologic conditions. The results show that our method meets this requirement.

The question arises, what advantage if any does the determination of F T_4 F offer over the older tests involving T_3 uptake by red cells or resin? From *a priori* considerations one would expect F T_3 F (or T_3-uptake) to be proportional to F T_4 F. However, since TBPA binds T_4 but not T_3 (*11*), the T_3 uptake only indirectly reflects overall binding of T_4 by plasma proteins. Now with a simple, rapid technique for F T_4 F, it is no longer necessary to depend on the use of T_3 tests to estimate F T_4 F for routine diagnostic purposes.

The importance of the free-T_4 concentration as a correlate of thyroid status has already been mentioned. Our experience and that of others (*3,4,12*) provide ample evidence that the free T_4 conc. index is superior to either F T_4 F or total T_4 values alone in differentiating hyper- and hypothyroid from euthyroid individuals and especially in determining thyroid status in patients with alterations in binding proteins. The free T_4 conc. index is calculated from the F T_4 F and the level of total T_4. We routinely estimate the latter from the chemical determination of T_4 iodine (T_4 by column) or the PBI. Alternatively, the technique of displacement analysis developed by Murphy and Pattee (*13,14*) can be used to measure total T_4.

In the case of many other tests of thyroid hormone binding, results must be interpreted with caution in patients taking drugs known to affect T_4-binding. Diphenylhydantoin (Dilantin—Parke Davis and Co., Detroit, Mich.) and salicylates, the latter in doses greater than 3 gm/day, competitively inhibit the binding of T_4 to plasma proteins (*15*). As shown previously by Chin and Schussler (*16*) using an ultrafiltration method, F T_4 F tends to be elevated by the addition of diphenylhydantoin when undiluted serum is tested but remains relatively unchanged in a system containing dilute serum. Using our present Sephadex method (serum diluted 1:32) we have also found that F T_4 F is insensitive to addition of diphenylhydantoin at therapeutic levels of the drug. We have not yet tested the effects of salicylates in our system, but on theoretical grounds the results should be relatively unaffected by the presence of this drug.

The test is useful in evaluation of patients taking thyroid hormones as replacement therapy, depending on the preparation of hormone. In patients taking desiccated thyroid, the F T_4 F, total T_4 and free T_4 concentration index are usually within the normal range if the patient is euthyroid. In the case of individuals on adequate L-thyroxine replacement therapy, all three parameters tend to be high-normal. In patients taking L-triiodothyronine, however, all three values are below normal and these tests are therefore not diagnostically useful.

Further clinical experience is needed before all of the advantages and limitations of this test are known.

SUMMARY

A method has been developed for estimating relative changes in free T_4 fraction (F T_4 F) in serum. The principle involves the partition of added labeled T_4 between binding sites on specific proteins and sites on Dextran gel (Sephadex). By using dilute serum and rapidly sedimenting particles of Sephadex, the method avoids the necessity for prolonged incubation and centrifugation. The Sephadex "uptake" of labeled T_4 from patient's serum is expressed as a percent of the uptake from a control serum. The results correlate well with those obtained by equilibrium dialysis over a wide range of thyroid states.

The normalized F T_4 F, obtained by this method, is used together with the total T_4 value to calculate an index of free T_4 concentration. The latter is considered to be the parameter which best reflects the level of thyroid function.

ACKNOWLEDGMENTS

The authors are grateful to Paul R. Jensen and Miss Patricia Lazarus for their cooperation in obtaining samples of serum and the results of chemical thyroxine analyses. The expert technical assistance of Mrs. Suzanne Emberton is greatly appreciated.

REFERENCES

1. ROBBINS, J. AND RALL, J. E.: The iodine-containing hormones. In *Hormones in Blood,* 2nd ed., vol. 1, Gray, C. H. and Bacharach, A. L., eds., Academic Press, New York, 1967, p. 383.

2. OPPENHEIMER, J. H. AND SURKS, M. I.: Determination of free thyroxine in human serum: A theoretical and experimental analysis. *J. Clin. Endocrinol.* **24**:785, 1964.

3. INGBAR, S. H., BRAVERMAN, L. E., DAWBER, N. A. AND LEE, G. Y.: A new method for measuring the free thyroid hormone in human serum and an analysis of the factors that influence its concentration. *J. Clin. Invest.* **44**:1,679, 1965.

4. STERLING, K. AND BRENNER, M. A.: Free thyroxine in human serum; Simplified measurement with the aid of magnesium precipitation. *J. Clin. Invest.* **45**:153, 1966.

5. SCHUSSLER, G. C. AND PLAGER, J. E.: Effect of preliminary purification of ^{131}I-thyroxine on the determination of free thyroxine in serum. *J. Clin. Endocrinol.* **27**:242, 1967.

6. KUMAGAI, L. F., JUBIZ, W. AND JESSOP, L. D.: A simple and rapid method for determining free thyroxine by charcoal absorption. *Clin. Research* **15**:124, 1967.

7. LEE, N. D., HENRY, R. J. AND GOLUB, O. J.: Determination of free thyroxine content in serum. *J. Clin. Endocrinol.* **24**:486, 1964.

8. PELLEGI, V. J., LEE, N. D., GOLUB, O. J. AND HENRY, R. J.: Determination of iodine compounds in serum. I. Serum thyroxine in the presence of some iodine contaminants. *J. Clin. Endocrinol.* **21**:1,272, 1961.

9. VOLPERT, E. M., MARTINEZ, M. AND OPPENHEIMER, J. H.: Radioiodinated impurities in commercial preparations of ^{131}I-thyroxine and their effect on measurement of free thyroxine in human serum by equilibrium dialysis. *J. Clin. Endocrinol.* **27**:421, 1967.

10. OPPENHEIMER, J. H.: Role of plasma proteins in the binding, distribution and metabolism of the thyroid hormones. *New Engl. J. Med.* **278**:1,153, 1968.

11. INGBAR, S. H. AND FREINKEL, N.: Regulation of the peripheral metabolism of the thyroid hormones. *Recent Progr. Hormone Res.* **16**:353, 1960.

12. ARANGO, G., MAYBERRY, W. E., HOCKERT, T. J. AND ELVEBACK, L. R.: Total and free human serum thyroxine in normal and abnormal thyroid states. *Mayo Clin. Proc.* **43**:503, 1968.

13. MURPHY, B. E. P. AND PATTEE, C. J.: Determination of thyroxine utilizing the property of protein-binding. *J. Clin. Endocrinol.* **24**:187, 1964.

14. MURPHY, B. E. P.: The determination of thyroxine by competitive protein-binding analysis employing an anion-exchange resin and radiothyroxine. *J. Lab. Clin. Med.* **66**:161, 1965.

15. WOLFF, J., STANDAERT, M. E. AND RALL, J. E.: Thyroxine displacement from serum proteins and depression of serum protein-bound iodine by certain drugs. *J. Clin. Invest.* **40**:1,373, 1961.

16. CHIN, W. AND SCHUSSLER, G. C.: Decreased serum free thyroxine concentration in patients treated with diphenylhydantoin. *J. Clin. Endocrinol.* **28**:181, 1968.

17. MURPHY, B. E. P.: Some studies of the protein-binding of steroids and their application to the routine micro and ultra micro measurement of various steroids in body fluids by competitive protein-binding radioassay. *J. Clin. Endocrinol.* **27**:973, 1967.