SERUM TRIIODOTHYRONINE UPTAKE USING ALBUMIN MICROSPHERES IN THE ASSESSMENT OF THYROID FUNCTION

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As it has been reported in a previous paper (*1*), microspheres of serum albumin (MSA) show adsorbing properties which make them a potentially useful material for studying protein-small molecule interactions. MSA binds reversibly some steroids and the thyroid-circulating hormones with an energy of the same order of that of native serum albumin. From this previous work, an indication can be derived of the suitability of this material for the rapid and convenient determination of some binding parameters or its potential interest as a competitive adsorbent for the setting up of reliable methods of competition analysis.

The present paper describes a study leading to the development of a T_3 test based on the use of MSA instead of ion-exchange resins (*2–4*) or coated charcoal (*5–7*) as adsorbent for free triiodothyronine (T_3). As will be reported herewith, the measurement of the labeled T_3 uptake by MSA in competition with serum proteins occurs in conditions of true equilibrium. Therefore the dependence on incubation time, after equilibration, is obviously eliminated and, in addition, the dependence on temperature is reduced. In this way the main experimental sources of error, met when using irreversible adsorbents; are avoided. In order to establish the reference ranges and to check the diagnostic accuracy of the MSA-T_3 test, 241 fresh human sera were analyzed. The results are reported and discussed in the present paper.

MATERIALS AND METHODS

Reagents. Microspheres of bovine serum albumin were prepared according to the procedure described by Pasqualini, et al (*8*). The preparation was carried out at the kilogram scale. After washing with ethyl ether to remove the residual oil traces, the fraction with size ranging from 80 to 100 microns was separated by sieving, sterilized by autoclaving, and stored in dry form at 4°C in the dark.

^{125}I-Triiodothyronine, purified by preparative paper chromatography (specific activity of 25–35 mCi/mg), was supplied by CEA-CEN-SORIN. The tracer was diluted in Michaelis buffer, pH 7.4, containing 0.35% albumin. To prepare reference sera, human blood from healthy donors was collected and pooled. The suitability of this pool as a euthyroid reference standard was checked by usual analytical techniques (PBI measures, T_3 and T_4 tests); hyperthyroid and hypothyroid sera were prepared by the same pool, respectively, by adding thyroxine or treating with anion-exchange resin to lower the endogenous hormone level. Human serum albumin was purchased from ISI, Napoli. The other reagents were of analytical grade.

Serum sample collection. Five milliliters of venous blood were withdrawn and the serum separated by centrifugation. All the samples indicating hemolysis were rejected. The samples were stored at −10°C until assayed (15–30 days).

Separation of MSA-adsorbed from unadsorbed T_3. Five-milliliter plastic syringes were modified by forcing down to the bottom a 3-mm-thick porous plastic disk*. The syringes, each containing a preweighed amount of MSA, were used as incubation vessels. The separation of unadsorbed T_3 was simply performed by discarding the solutions from the syringe.

MSA method for T_3 test. The optimized procedure (see "Optimization of the MSA-T_3 test") used for clinical measurements was as follows:

1. From a bulk suspension of MSA in Michaelis buffer (50 mg/ml), kept under stirring, 2-ml aliquots were delivered into each syringe. The filled syringes were stored at 4°C in the dark until used.
2. Using a precision pipette, 0.5 ml serum were added to 2 ml of solution of labeled T_3 (about

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* Vyon F, Porvair Ltd., 20–50-micron pore diam.

2.5 ng, 0.1 μCi) contained in plastic tubes; after counting, the solution was sucked into the syringe from which the buffer was previously discarded. The empty tubes were counted.

3. The mixture was incubated at room temperature (23 ± 3°C) under agitation; after 1 hr, the solution was transferred into the tubes and counted. Since MSA is retained by the porous disk, the radioactivity of the tube corresponds to the labeled T_3 fraction that is bound to serum proteins.
4. The results are expressed as percentage of T_3 bound to the serum thyroxine-binding globulin (TBG) according to the relationship

$$\text{UTI (unsaturated TBG index)} = \frac{A_x - A_1}{A_0 - A_1}$$

where A_0 is the total activity, A_x the activity of the solution after incubation, and A_1 the residual activity on the empty tube.

RESULTS AND DISCUSSION

Optimization of the MSA-T_3 test. When using a reversible adsorbent, such as MSA, the overall reaction system can be described by the equilibria

$$\begin{aligned} T_3 + TBG &\rightleftharpoons T_3\text{—}TBG, \\ T_3 + MSA &\rightleftharpoons T_3\text{—}MSA. \end{aligned} \qquad (1)$$

A kinetic study of this system for a given amount of MSA was performed by using sera with different T_4 contents. The resulting data, plotted in Fig. 1, indicate that under the experimental conditions used (100 mg MSA, 0.5 ml serum, incubation at room temperature) the equilibrium is reached in all cases within 60 min. These results confirm that the incubation time is not a critical factor when reversible adsorbent such as MSA are used, provided that the incubation is carried out for a time sufficient to reach the equilibrium. The actual time to obtain the true equilibrium obviously depends on the degree of saturation of serum TBG, ranging from 45 to 55 min for sera with high and low T_4 contents, respectively.

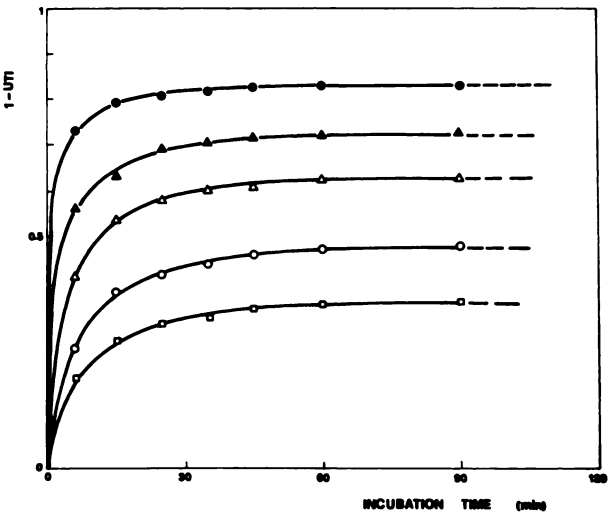

FIG. 1. T_3 uptake of 100 mg MSA as function of incubation time at 23°C, for serum samples with low (open squares), normal (open circles), and high (open triangles) T_4 content. Blank experiments in which 0.5 ml of MSA solution (37.5 mg/ml) (closed triangles) and of Michaelis buffer (closed circles) were used instead of serum are shown as well. All data refer to mean values of ten replicate experiments.

The equilibrium value for the system (*1*) depends on the adsorbent mass; this dependence is shown in Fig. 2, from which it is seen that 100 mg MSA gives for a serum with a normal T_4 content a UTI value of about 0.5. This situation is optimal in terms of assay precision and of discriminating power for abnormal T_4 contents in both senses.

Figure 2 also indicates that the system is nearly insensitive to small variations of the adsorbent amount when 100 mg MSA are used. In this situation, in fact, only a 2% shift of the UTI value results for a 10% mass variation. This enabled a MSA suspension to be used to deliver the chosen amounts into the syringes instead of weighing dry MSA.

The effect of the incubation temperature on the rate at which the system evolves towards the equilibrium is illustrated in Fig. 3 in the case of a serum with a normal T_4 content. As shown in Fig. 4, variation of the equilibrium value with temperature are appreciable, although in the neighborhood of normal room temperature they are much lower than the variations reported for other adsorbents, such as resin or charcoal (*5–7*).

As expected, the plots of Fig. 3 demonstrate that the binding constant of the T_3-TBG complex is lowered by increasing the temperature, while the reaction rate is enhanced. In any case a 60-min incubation is sufficient to reach the equilibrium. From the variations of all the reference sera with the temperature, as shown in Fig. 4, it is possible to establish that the mean shift of the UTI value is about 1% for a 3°C change of the working temperature. Therefore, from a practical point of view, no correction factor is needed when working at temperatures ranging from 20 to 26°C.

From the same Fig. 4 it can be observed that the blank experiments on both MSA and serum albumin are virtually independent of the temperature so that the temperature dependence is to be attributed entirely to serum TBG.

Assessment of clinical validity. In order to estab-

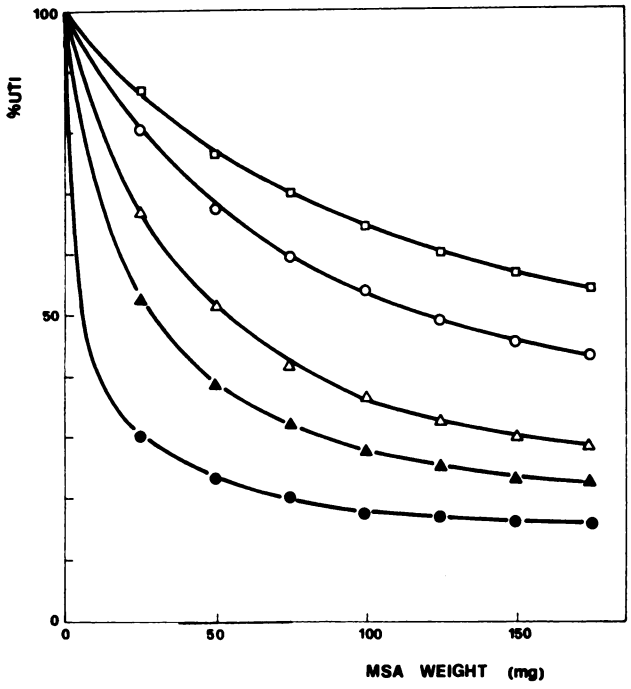

FIG. 2. Effect of increasing MSA weights on dissociation of T_3-TBG complex measured at equilibrium, for serum samples with low (open squares), normal (open circles), and high (open triangles) T_4 content. Blank experiments in which 0.5 ml of MSA solution (37.5 mg/ml) (closed triangles) and of Michaelis buffer (closed circles) were used instead of serum are shown as well. All data refer to mean values of ten replicate experiments.

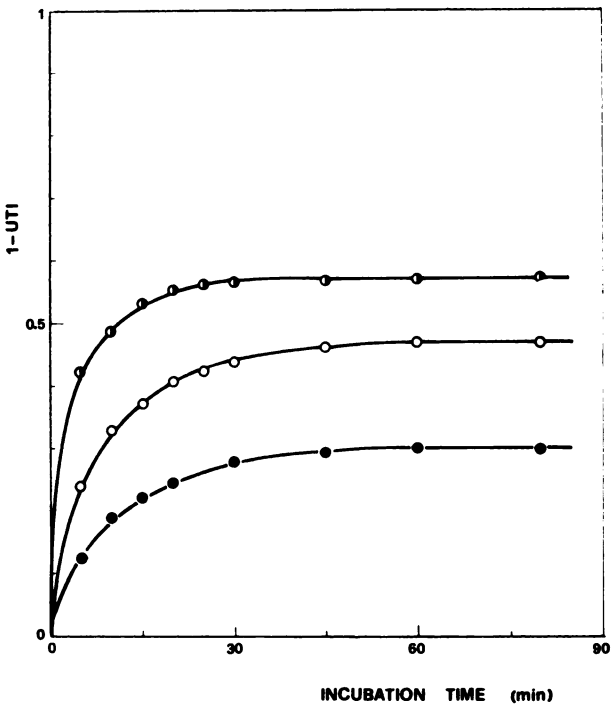

FIG. 3. Effect of 100 mg MSA as function of incubation time at 4°C (top circle), 23°C (open circles), and 37°C (closed circles) for serum sample with normal T_4 content. All data refer to mean values of ten replicate experiments.

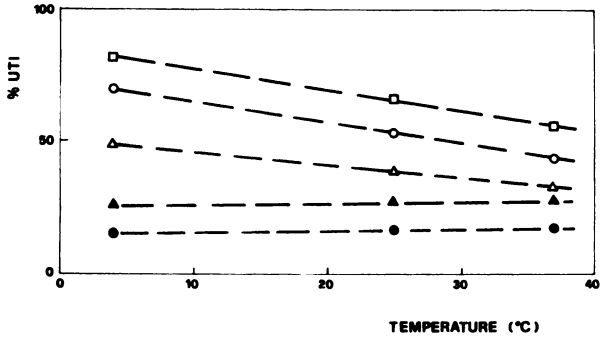

FIG. 4. Effect of temperature on dissociation of T_3-TBG complex in presence of 100 mg MSA, as measured at equilibrium for serum samples with low (open squares), normal (open circles), and high (open triangles) T_4 content. Blank experiments in which 0.5 ml of MSA solution (37.5 mg/ml) (closed triangles) and of Michaelis buffer (closed circles) were used instead of serum are shown as well. All data refer to mean values of ten replicate experiments.

lish the validity of the method and its discriminating ability for different physiopathological situations, 241 subjects were examined (98 males, 143 females, age 10–60). For all the subjects, the clinical status was previously assessed by usual diagnostic tests: protein-bound iodine (PBI) measurement, thyroidal radioiodine uptake (RAI) test at 6 and 24 hr, basal metabolism rate (BMR). Moreover, the thyroidal function tests with Triasorb and Tetrasorb kits* were performed for a number of individuals. Therapy, if any, was taken into account.

All data, together with the subjects' sex and age, were used to subdivide the population examined into groups. A particular group was represented by normal pregnant women. The elaboration of the results obtained was carried out by using a 360/44 IBM computer.

The analysis of variance for duplicate tests both carried out in parallel (within-assay) and at different times (between-assay) on 173 samples using freshly purified labeled T_3, led to the exclusion of any significant between-assay variability ($p < 0.01$) and to establish 1% variation coefficient. By testing daily during 1 month the three reference sera with the same batch of labeled T_3, a systematic shift of the values up to 3% was found. However, this degradation effect was completely avoided when a lyophilized tracer preparation was used ($p < 0.01$). Another source of systematic error was found to be represented by the technique of serum delivery. The values obtained by using a Biopette† showed a significant difference of −2% when compared to those obtained with a precision glass pipette.

* Abbott, Chicago, Ill.

† Schwarz/Mann, Orangeburg, S. C.

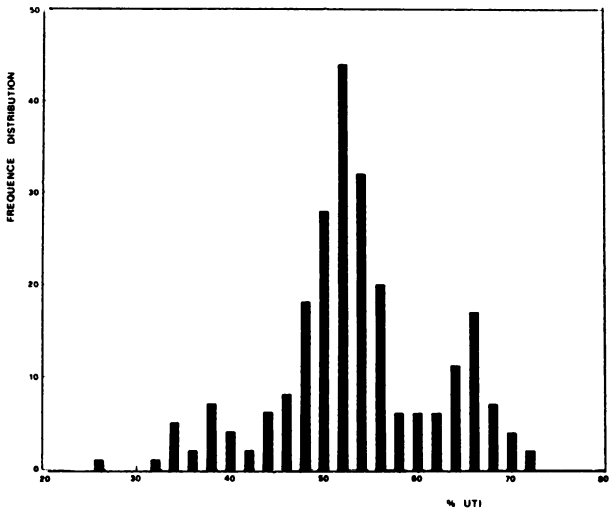

FIG. 5. Overall distribution pattern of % UTI values obtained for 241 subjects.

All the data reported in this paper refer to the former technique.

In Fig. 5 the total distribution pattern for the examined subjects is shown; from this, the histograms of Fig. 6 were obtained according to the group subdivision previously stated. The results of the statistical treatment for the main groups corresponding to euthyroid, hypothyroid, hyperthyroid subjects, and pregnant women are listed in Table 1.

From these data it is possible to observe that only one case out of 68 euthyroid subjects is found beyond the 95% confidence range but lies within the 99%. For the 21 hyperthyroid patients, the single case escaping from the 95% range (but included in the 99%) is, however, found at the lowest end of the distribution. The 95% range includes all of the 12 cases of hypothyroidism. The highest UTI values were found for the group of the 40 pregnant women. Only two cases are not included within the 95% range, but also in these cases the discrimination possibility is not affected since both of them are placed at the highest scale end. The differences between these four groups, as evaluated by the Student's t-test, was shown to be significant at a level $p < 0.01$.

No particular distribution is observed from Fig. 6

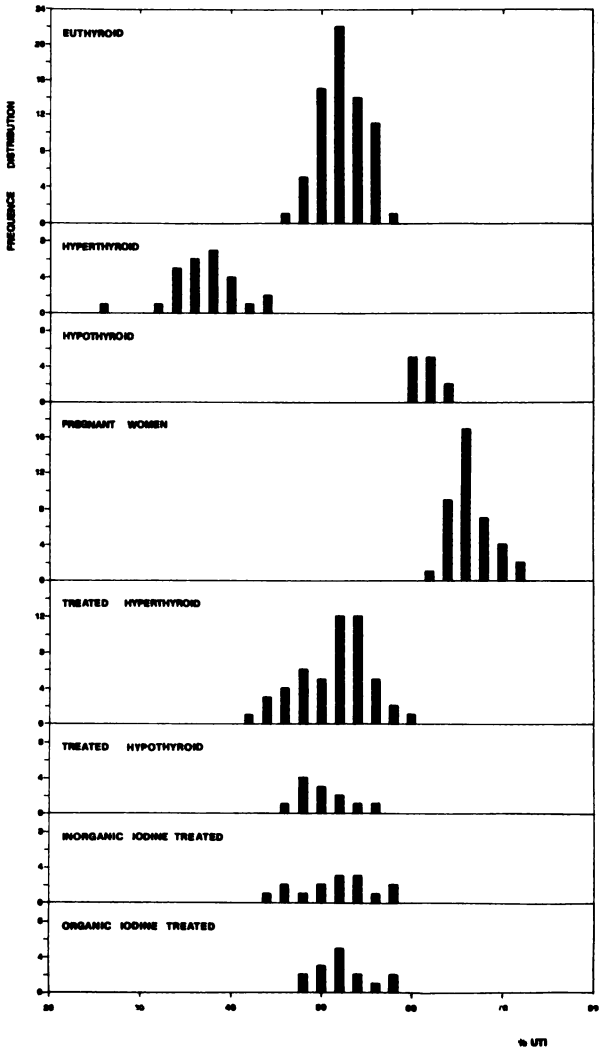

FIG. 6. Distribution patterns of % UTI values subdivided in relation to physiopathologic status. Groups of hyper- or hypothyroid subjects indicated as "treated" received drugs of different kind but excluding iodine-containing drugs. Inorganic and organic iodine refer to same meaning as defined by Sisson (9).

TABLE 1. RESULTS OBTAINED WITH THE MSA METHOD

Group	No. of cases	Percent UTI			Confidence range		Rejected cases 95%	
		Mean	s.d.	Experimental range	95%	99%	No. of cases	% UTI
Euthyroid	68	52.28	2.24	46.60–56.60	47.22–57.34	45.61–59.95	1	46.6
Hyperthyroid	21	37.08	3.97	26.10–44.25	28.43–45.14	25.35–48.81	1	26.1
Hypothyroid	12	61.53	1.15	60.40–63.80	58.88–64.18	57.81–65.24	0	—
Pregnant women	40	66.62	2.08	63.00–71.25	62.23–71.01	60.78–72.45	2	71.2 71.2

concerning the patients to whom drugs containing either organic or inorganic iodine were administered. As for the patients affected by thyroid diseases and under drug administration, most of the cases are included within the euthyroid range but skewed distribution patterns resulted for both hyper- and hypothyroid subjects. This non-normal distribution can be attributed to the different drug dosages and times of treatment. The data as obtained with our method were correlated to results of the other diagnostic tests. A significant correlation coefficient ($p < 0.01$) was found with the Triosorb and Tetrasorb tests. The same correlation level resulted with the PBI measurement, when the samples from iodine-drug treated subjects were excluded. No significant correlation ($p = 0.25$) was obtained with RAI and BMR evaluations. The regression lines for the tests significantly correlated are shown in Figs. 7–9 and the related parameters are reported in Table 2. Assuming the 95% confidence level for our normal range, according to the suggestion of Pain, et al (*10*), the ranges found for the comparative measurements suggest the following: (A) The PBI range is retained, obviously taking into account the limitations of this method, as far as the discrimination of subjects treated with iodine-containing drug is concerned; (B) the upper limit for Tetrasorb was found somewhat shifted downwards, but the discriminating ability on the basis of the clinical status

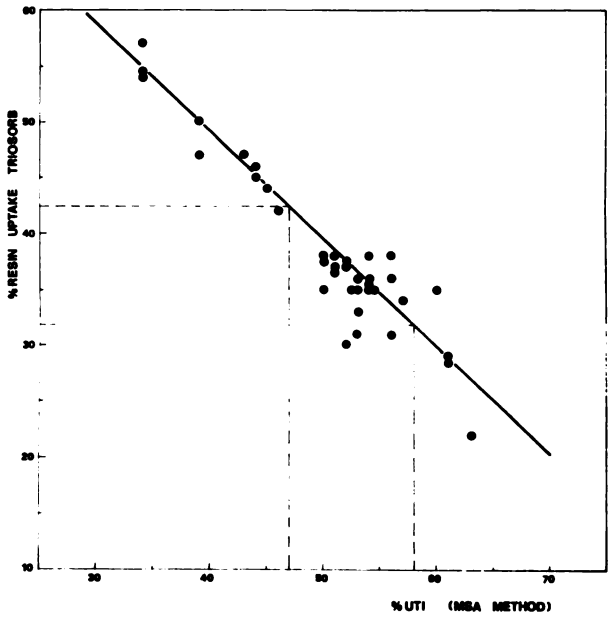

FIG. 7. Regression line and experimental points for correlation with Triasorb T_3 test.

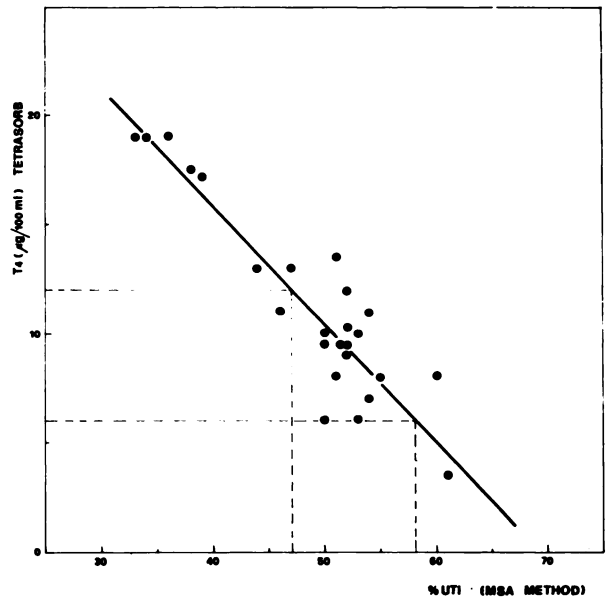

FIG. 8. Regression line and experimental points for correlation with Tetrasorb T_4 test.

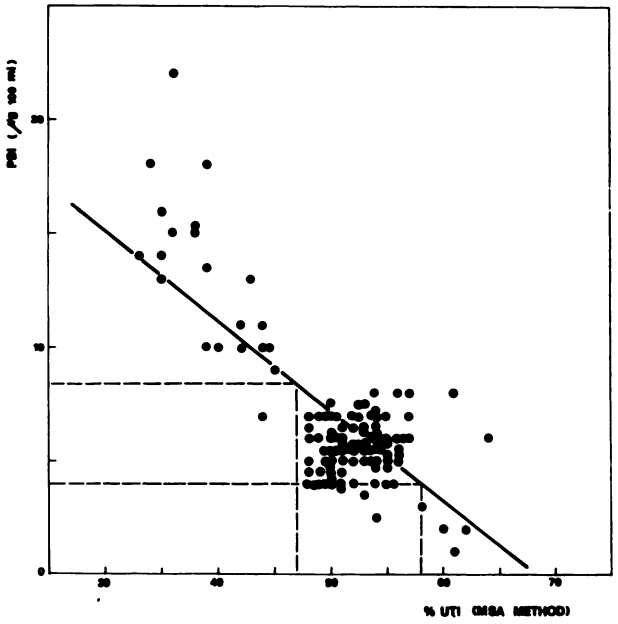

FIG. 9. Regression line and experimental points for correlation with PBI measurements.

TABLE 2. RESULTS OBTAINED WITH THE MSA METHOD

Correlated test	Regression parameters		Normal range*
	Intercept	Slope	
Triosorb T_3	85.96 ± 3.04	−0.945 ± 0.060	31.0–41.0
Tetrasorb T_4	37.32 ± 2.30	−0.540 + 0.082	6.0–12.0
PBI	27.20 ± 2.88	−0.400 ± 0.032	4.0– 8.5

* The data are derived by imposing stated values for normal range of MSA method.

TABLE 3. EVALUATION OF THE INDEX RELATED TO THE FREE T_4 LEVEL ACCORDING TO CLARK, ET AL (REF. 11)

Correlated test	Index of free T_4 level	
	Normal range*	Pregnant women
T_4 test (1-UTI) × T_4	2.32–6.36	2.80–5.70
PBI (1-UTI) × PBI	1.68–4.50	1.96–4.18

* The normal ranges of Table 2 were used for calculations.

is practically the same as for our method; and (c) for Triosorb, a shift of the range towards hyperthyroidism resulted. This can be easily explained by the fact that a large number of the clinically normal cases had resin uptake values close to the upper limit of the normal range or even beyond this limit. When accepting for Triosorb the normal range stated by the supplier, 20% misclassified cases resulted in our hand. As far as sex and age of the subjects examined, no significant difference was found.

The variation coefficient for MSA-T_3 test (1%) is much lower than that reported for charcoal method (3%) (7) and the one found in our hands for the resin sponge (3%). At the 95% confidence limit no overlap was observed among the euthyroid, hyperthyroid, and hypothyroid groups. The values for pregnant women are likely to discriminate as well from hypothyroid subjects by using the MSA-T_3 test and are coherently shifted and reduced to the normal range by the application of the procedure of Clark, et al (*11*) with a T_4 test or PBI measurement (see Table 3). This permits the evaluation of an index related to the free T_4 level, thus eliminating the effect of TBG variation.

SUMMARY

A T_3 test based on the use of microspheres of BSA as reversible adsorbent was set up. The standardization of the method is discussed and the optimized procedure described. The results of clinical tests on 241 sera related to different physiopathologic states are presented, together with the correlation obtained with other diagnostic tests of thyroid function. The method appears perfectly suitable as far as precision, discriminating ability, operative simplicity, and speed are concerned.

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