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Clinical and Equilibria Studies of Thyroid Function Using the Trisorb Resin Sponge

D. L. Tabern, Ph.D.,[1,4] John Kearney, M.D.,[2,4] and Anne Dolbow, B.S., MT (ASCP)[3,4]

Chicago, Illinois

INTRODUCTION

During the past year, well over twenty five hundred procedures have been carried out, employing the Trisorb resin sponge system. Roughly, half of these involved individual patient sera in standard techniques, as a diagnostic procedure. One quarter were concerned with developing a more reliable procedure and the remainder were equilibria studies designed to better understand the mechanisms involved. As a result of the first two, we are proposing a highly reproducible standard serum and a simple low temperature reaction system, which, in our hands, has also improved reliability materially.

During this work, it became obvious to us, that the sponge containing a reactive, but mechanically firmly bound, anion exchange resin, potentially provided a new and unique tool for equilibria studies, formerly restricted to much more laborious dialysis experiments.

A principal advantage stems from the fact that it is a fixed geometry column, of excellent porosity, onto which the substances may be placed, within the time span of a few minutes to a few hours and from which they may be eluted, at any convenient subsequent time, by a variety of agents. Using I-131 labeled compounds and T-3 and T-4 in particular, these changes can be quantitatively followed, simply by rapidly washing the sponge and counting in a conventional well.

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[1]Technical Director, Radioisotope Laboratories.
[2]Internal Medicine and Radioisotopes.
[3]Senior Radioisotope Technologist.
[4]Louis A. Weiss Memorial Hospital, Chicago.

Several sponges, as well as a sponge and another physically different adsorbent, may be placed in the same solution, to study the rates and end points of the equilibria. The only reaction vessel required, is the plastic test tube supplied, facilitating maintenance of fixed temperatures. As many as 20-25 samples can be run in parallel. Washing is complete, in less than a minute, without centrifuging. Assay accuracy is of the order of 1-2%.

Fortunately, the rate of adsorption in the 0-25 degree range, is such that timed experiments are easily quantitated. The rate is slower than that of the usual fine ion exchange resin powders, but more rapid and less subject to dilution errors than is dialysis.

From the theoretical standpoint, such a sponge represents an interesting example of a way of modifying the effective surface of a resin and comparing its equilibrating properties with the same or similar resins in different physical forms. These studies, undertaken originally to better understand the equilibrium changes induced by low temperature adsorption of T-3, suggest so many other applications, that they are being presented at this time.

MATERIALS AND METHODS

The sponge and T-3 solution were secured as the standard Triosorb Thyroid Function Kit[1] and used within 7-14 days. The amount of T-3 per procedure averaged 0.1μc in .003 micrograms. There was less than 2.3% iodide. T-4 of essentially the same composition was also available. In clinical studies, 1 co of patient serum and 0.9 cc of the T-3 solution were used. This solution is sufficient to slightly more than saturate and cover the sponge. In equilibration studies, it was found advantageous to add an additional 1 cc of 7.2 Tris Maleate buffer, insuring a constant pH in that range. This had essentially no effect on sponge uptake and was kept constant.

To maintain exactly 0° C even during 24 hour studies, we used a heavily insulated and covered metal "ice bucket" in which a test tube rack was set. The tubes were thus completely surrounded by ice and water at all times, yet were readily available for use.

Where tube rotation was required, we used a Nuclear Consultants rotator placed in a fixed position in a refrigerator, giving 4° C ± 1 degree. All counting was carried out using a Spectroscaler III with automatic background subtract and using a spectrometer window of 100 KEV. In clinical work, the original samples averaged 20,000-40,000 counts, in the standard time of two minutes.

SECTION I

CLINICAL TRIOSORB RESIN STUDIES

In the clinical evaluation of thyroid disease, the basic and most reliable method is the 24 hour uptake. The values are secured in numerical form and by totaling uptake and excretion, using fixed geometries, one can account for

[1] (Abbott Laboratories).

a high percentage (80-100%) of the administered activity. Thus, the data does not need to be related to any secondary standard. There are only a limited number of situations, each well known, such as iodide or thyroid ingestion, thyroiditis and pregnancy, where the method is not applicable.

Of the other procedures employed, by far the most widely used is some version of the "T-3 Resin Uptake" (References 1-a,b,c). Its apparent simplicity and convenience to the patient, has given rise to its application beyond that justifiable, on a scientific basis. It, and its non-isotopic analog, the PBI, are subject to error, in at least as many situations as the uptake and new sources of error are coming to light with increased use. One of these: antiovulatory steroids, has caused us so many problems, that we have included a detailed study of it in this work.

A. *Physiologic Basis*

In this T-3 test, a known small amount of high specific activity T-3 is added to a carefully measured volume of fresh patient serum. The common impression, abetted by loose terminology in some papers (Ref. 2-a,b), is that while sufficient of the T-3 goes to the strongly binding proteins to saturate them, the remainder remains essentially "free" in the serum, to be taken up by the Trisorb sponge which is added for this purpose. This, of course, is quite contrary to many theoretical papers appearing in recent literature. For instance, Lee et al (Ref. 2-c), stress the involvement of nonspecific bindings like albumin.

We have carefully followed the sponge pickup of T-3 activity with time, over periods of up to 24 hours. The rate of adsorption is roughly proportional to the amount of T-3 remaining on the protein, approaching a plateau only after 20-24 hours. Obviously, we are dealing with the continuous competition of binding sites on the resin, for the T-3, held by a variety of binding sites on several proteins. At the point of equilibration, at 25°, some 80% is on the resin.

As can be seen from Figure 1, the curves for sera from different patients (and even from a variety of animals), are remarkably parallel, and the ratios between them are not much different at 30 minutes and 24 hours, as long as temperatures remain the same. We have found, in these studies, that, rather surprisingly, temperature changes materially shift equilibria and in a very favorable manner.

Of all the proteins involved in binding, i.e., albumin, "prealbumin", and at least two potent "thyroid binding globulins", only the first has been carefully studied by physical chemical means. (Ref. 3) The best evidence suggests a rather compact spherical "macro-molecule" having a molecular weight of around 65,000. There is no indication of significant spatial alterations in the 6-7 pH range. The more active binding proteins seem to have a much smaller average molecular weight and their shape is quite unknown.

Our experience with the slow formation of precipitates in mixed sera from a number of individuals, with simultaneous unpredictable changes in the T-3 values and the inability to predict quantitatively the T-3 values of mixtures, strongly suggest that the active binders are much more labile. There seem to be interactions and changes about which we know little. This is, of course, of importance in the selection and processing of standard sera for T-3 tests.

It should be stressed that even in the relatively simple albumin molecule,

there are a number of binding sites, each having its own degree of specificity for each hormone. In the light of this, the above prolonged, continuous "protein elution" (or in reverse terminology, "resin pickup") curves, are understandable. Such a situation suggested that the most reliable and meaningful T-3 clinical values would be secured under the mildest practical conditions.

B. *Experimental*

(1) *The Standard.* For optimal accuracy, in the presence of so many variables, it is essential that each day's determinations include, in duplicate, samples of a known, continuously used, standard serum. Values should be expressed not as a simple percent of the initial counts transferred to the sponge but rather as the ratio of the patient's percent adsorption, to that of the standard.

At the start of this work, using the standard serum pool made available to us by the manufacturer, duplicate determinations agreed poorly. Numerous patient pools gave 2-5 "percentage points" lower values than this serum and the values stated on the package. Other groups informed us that they encountered the same problem. Reference standards, as then available, were equally erratic and were physically unstable once constituted.

Hoping to secure a constant source of supply, we promptly made a study

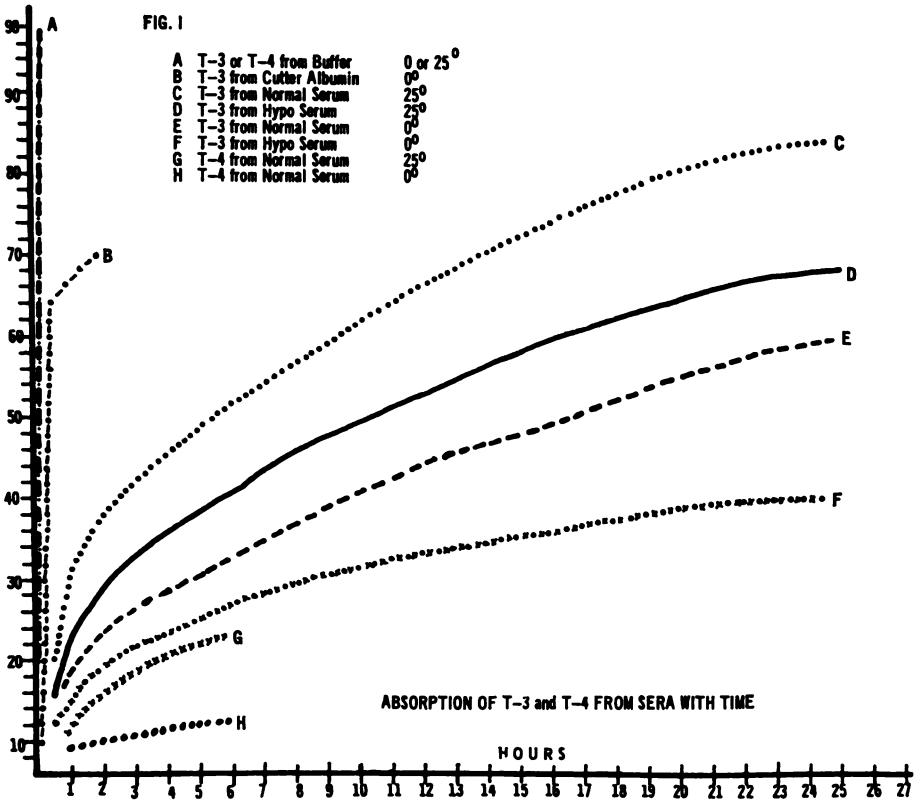


Fig. 1.

of the T-3 values from six animal species. These ranged from 52% for the dog to 19% for the goat. Sera from a number of sheep were measured and all fell within the "normal human" range of 26-30% (Ref. 4). Two males were selected having values of 27-29 "percent" which corresponded with our best laboratory pools. They were set aside and bled at three week intervals. Over four months, they gave quite consistent values. The electrophoretic patterns for our sheep and a human male pool, were strikingly similar.

Such sera did not change in "T-3 value" on repeated freezing and thawing for several weeks, or on heating to 40° for 10 days. A pool (SP-1) made from several such lots of the same animal was used as a standard between February and July. The values secured at 25°, were plotted. It was obvious that there was a progressive shift, presumably due to small changes in the properties of sponge lots. However, these were minor, in view of the fact that duplicate values on any day became more consistent.

Later, a larger pool (sheep mixture) of sheep serum from a local slaughtering establishment was made by mixing male and female specimens (SP-2). The value changed slightly on aging and clarification prior to freezing, but since it provided values highly consistent with all newer data, finer adjustment was not deemed necessary. (As will be shown in Section II, the "T-3 value" of any serum can be adjusted, either upward or downward, by any of several simple methods.)

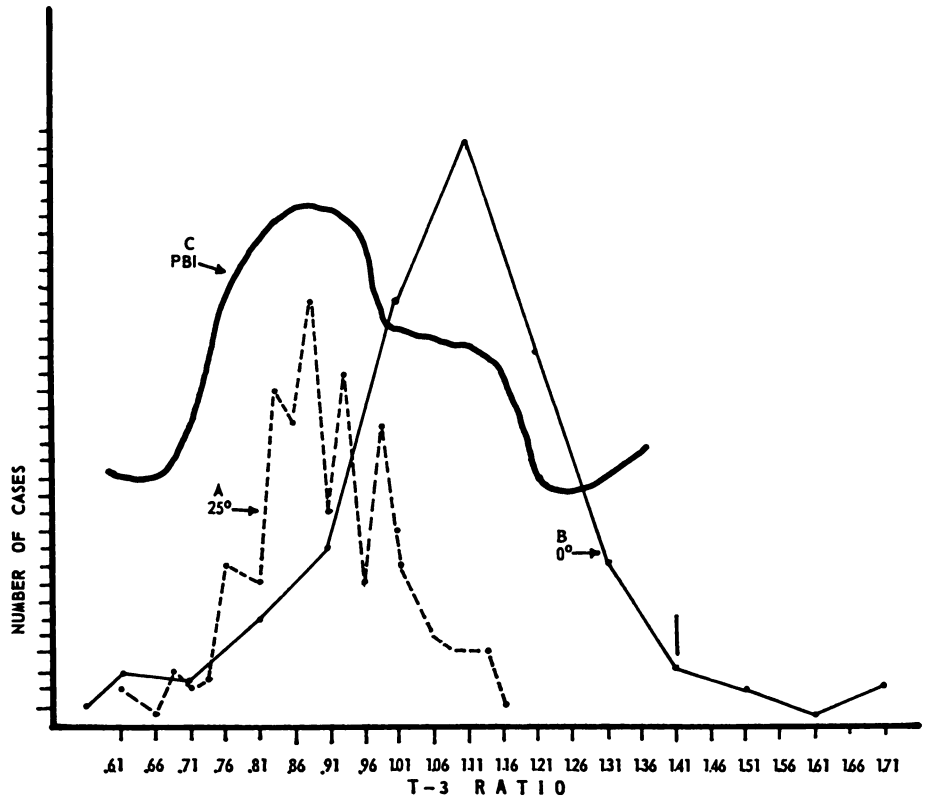


Fig. 2.

(2) *Range of Values.* Since such standards presumably represent individuals and animals with normal thyroid function, we were continuously surprised to note that the "patient average" was significantly lower. Line A of Figure 2 shows the number of cases in various T-3 value ranges, for some 400 consecutive determinations at 25°. The average for females is .88 and that for males .93-.94. Note that the distribution is highly irregular and that a large number of cases are found in the region necessarily selected as the "break point" between normal and hypothyroids. This is obviously a major reason for the difficulty in such discrimination frequently mentioned in the literature.

We plotted in the same way (line C of Figure 2) the distribution of values, in an equal number of PBI's carried out during this same period; and found a similar asymmetric distribution quite pointed to the low side (Av. 4.5 micrograms percent). Though the patients are probably not comparable, we have not seen a similar situation in our 24 hour I-131 uptake plus scan clinical program.

(3) *Zero Degree Equilibration.* Quite a range of temperatures have been used by various workers, usually as a matter of convenience (Ref. 5-a). Since the initiation of this study, Manfredi *et al,* chose to employ an ice-water bath, to minimize the necessity for changes of temperature due to fluctuation of room conditions. They concluded that "the two methods are of equal usefulness," thus obviously having failed to observe the definite advantages brought out in our study. Only Goolden has made a study of the time and temperature relationships (Ref. 5-b).

As stated above, we hoped to improve the reliability through the use of the mildest possible conditions. We found that by placing the tubes vertically in a test tube rack, completely immersed in ice and water, gave exactly 0° over many hours. There is a considerable saving in space and increase in convenience. The results have been highly gratifying.

Adsorption was found to be approximately two thirds that we had observed at 25°. That individual sera values are highly consistent is illustrated by the fact that "SP-1", between April 8 and July 29, had a minimum value of 16.1, a maximum of 17.7 and an average of 16.4. The current standard pool (SP-2) between August 9 and October 26, in 28 separate runs, had a minimum of 18.6 a maximum of 21.1 and an average of 19.7.

To avoid the possibility of species differences becoming a factor, we have prepared by serial bleedings, pools from individuals found to be equivalent to "SP-2" in 0° T-3 values. Several pools have also been secured by combining day to day samples from patients having values between .95 and 1.1. As will be shown later, all of these are stable and they have been introduced, regularly, into many days clinical runs as additional controls. In recent months, commercially available laboratory standard sera have come to essentially this same value. All of this gives us increased confidence in the specific procedure proposed here.

Before changing to the new experimental conditions, we assayed 60 patient sera at both 0° and 25°. These purposely included a wide range of values. In every case, regardless of whether animal or normal human sera was used as a standard, the zero degree value was higher by an average ratio of 1.1 to 1.3. This is shown clearly in Figure 3; in each case, the standard SP-2 is taken as unity. If 0.9 was taken as the lower limit of normal and 1.4 as the upper, every case had

the same diagnostic rating at both temperatures. This spread, at 0°, is obviously wider numerically than the one at 25°.

Our classification ranges are set forth in Table I:

TABLE I

Hyperthyroid	above 1.4
Normal	.90 to 1.4
Borderline low	.86 to .90
Hypothyroid	below .86

Curve B of Figure 2 is made from over 500 clinical cases run by the zero degree procedure. It is free from fluctuations and of equal importance; the curve is falling rapidly at the normal-hypo dividing point, resulting in fewer "borderline" cases. A surprising number of "hypos" are well separated into the mid .70 range and frankly hyperthyroid patients not infrequently have values in excess of 2.0.

It should be noted that the "average" value is now above, rather than below unity, being 1.07 for females and 1.1 to 1.15 for males. The only logical explanation for these facts would be a shift in equilibria with temperature. A study of these and other equilibria is presented in Section II.

Our staff have found no difficulty in changing over to the new values. They find that separation into the correct clinical categories, while not perfect, has been materially improved.

There still remain occasional unexpected differences between the T-3, the uptake and the PBI. A careful check often exposes the cause, usually the use of some drug, or some pathologic state. The existence of these situations, which would, if only one procedure were carried out, result in a misleading diagnosis, is the best argument for multiple tests.

(4) *Possible Variations in Procedure.* While we have seen no compelling reason to increase the duration of the adsorption of T-3, it is obvious from Figure 1 that a two, or even a three hour interval, would give essentially the same ratios. Under these conditions, the samples could be set up before lunch and the readings made in the afternoon. If large numbers are to be done, one lot could be read, while the second is "adsorbing". Even a 24 hours interval, similar to the I-131 uptake is feasible; although, in our experience, the values tend to show greater fluctuation.

(5) *Steroid Administration as a Source of Error.* The manufacturer quotes from the literature some 10 conditions, or diseases, which have been found to influence the T-3 value. Fortunately, most of these are seldom encountered, or are known to the physician. It is not as well realized that iodides given to the point of thyroid suppression modify both the T-3 and the PBI.

Since pregnancy strongly depresses the T-3 value, it seemed possible that contraceptive steroids might behave similarly. Since the start of our work, Williams *et al* (Ref. 6-a), have demonstrated that this is the case. In addition to the Enovid, of their study, we have extended the study to six additional steroid preparations, Ortho-Novum, Deladrexate, Ovulen, Norinyl, Lyndiol and Sequential. Periods of administration varied from 10 days to several years. Several had received more than one drug.

Cases studied to date exceed 40. Two were in the normal range and two were low borderline. Most of the remainder fell far down in the hypothyroid range (.52 to .80). The effect was full, as early as the tenth day. All steroids used in the series seemed roughly equivalent. We confirm the findings of Williams, *et al*, that the PBI is also abnormal, being in, or near, the "hyper" range (average 7.8). A patient having a value of .52 represented a summation of the steroid effect and hypothyroidism not well controlled by normal doses of thyroid.

Since some five or six million women are reported to be on such steroids and since many of these are in the age group where weight gain is related to thyroid function, we have a potentially serious cause of diagnostic error.

(6) *Preferred Zero Degree Technique.* One cc of fresh patient serum, or serum that has been held in the frozen state and the contents of the T-3 solution syringe (0.9 cc) are placed in the palstic tube supplied. A small amount of hemolysis does not seem to affect the result. One sponge is added and air expelled according to the directions of the manufacturer. The tube is immediately placed in the precooled bath and completely surrounded by ice. Additional tubes are started at three minute intervals. Exactly 57 minutes later, each tube is removed and counted for two minutes, or not less than 15,000 counts, as described in the "materials" section (Fig. 5).

At zero plus sixty minutes the sponge is washed 4-5 times with cold water. When all sponges have been washed, each is recounted and the final value divided to secure the "percent uptake." The value for each patient is then divided

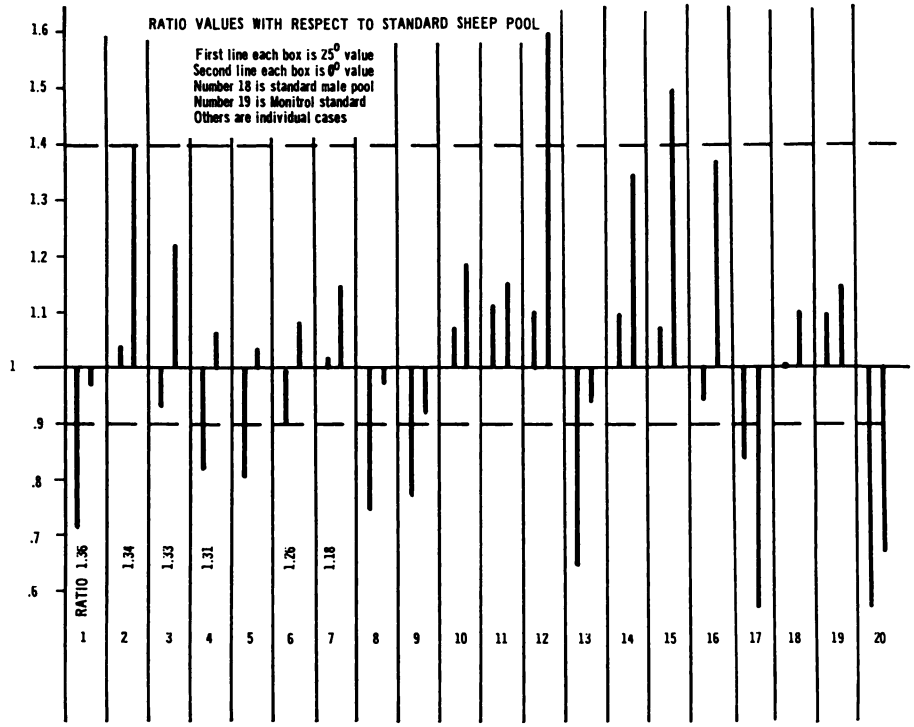


Fig. 3.

by the value secured for the standard in the same group of tubes, to give the "ratio" which is the numerical value reported to the physician. As indicated earlier, our normal "ratio range" is 0.90 to 1.40, with the few in the .86 to .89 group being listed as low borderline.

FINAL INTERCOMPARISON OF PROCEDURES

At the conclusion of these studies we assembled into fifteen pools, sera from well known sources, each of which had been assayed earlier. Each pool was clarified and carefully rerun at both zero and 25 degrees, using as the standard the "SP-2" referred to above. The PBI value of each was also determined in duplicate. The pools were also used for the equilibria studies of Section II. (See Table II.)

Group A contained two composites (2 and 3) made solely from sera assaying near 1.0; No. 5 was an individual pool of similar properties; No. 4 was the PBI laboratory normal standard; Nos. 6, 7 and 8 were current lots of three commercial standard sera. It will be seen that when reassayed at 0° all were remarkably uniform and near 1.0.

In group B, we have two pools (9 and 10) made from sera, indicating two degrees of hypothyroidism and each fits the average values of the samples incorporated. Nos. 11 and 12 are from patients on steroids at two institutions. Note that these are even lower than those having hypofunctioning glands. Pregnancy has been shown to decrease T-3 values due to the production of "abnormal" proteins and so a "prenatal pool" was included as No. 13.

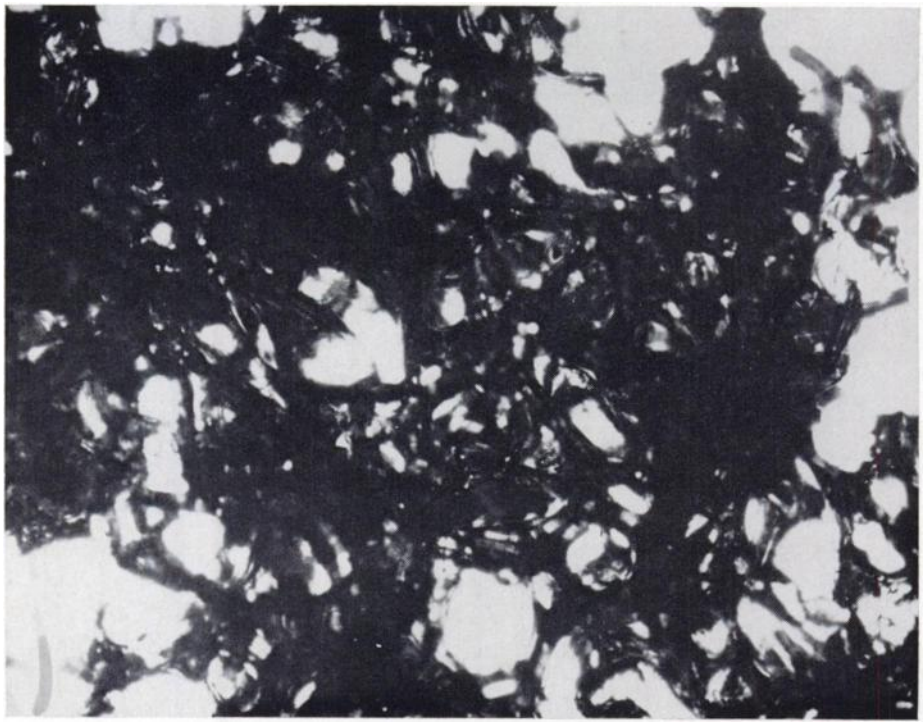


Fig. 4.

In group C, No. 14 represents nearly all patients clearly hyperthyroid. No. 15 is derived from a polycythemia patient, subject to repeated phlebotomies and whose T-3 ratios always were in, or near, the "high" range. It seemed of interest to include as No. 16 an equine pool, since such sera regularly assayed very high. It is in this "C" group that the temperature differential is most clearly seen.

TABLE II

		0°	*25°*	*PBI*	*Free Resin*
1	SP-1	1.0	1.0	10.6	1.0
2	Normal Human Pool# 1	1.01	.94	Decol.	1.07
3	Normal Human Pool# 2	1.03	1.06	Decol.	1.14
4	PBI Std.	1.0	.90	6.0	1.07
5	Pool T	.99	.90	6.4	1.06
6	Monitrol	1.02	1.03	5.2	—
7	Metrix	.99	.95	5.2	1.07
8	Chemtrol	.95	.99	—	1.06
9	T-3 below .8	.74	.72	Decol.	.81
10	T-3 .8 to .9	.81	.78	16.9	.89
11	Weiss steroid	.69	.74	8.2	.86
12	OSU steroid	.68	.73	7.5	.77
13	Pregnancy	.60	.59	7.8	.73
14	T-3 above 1.4	1.69	1.26	Decol.	1.9
15	Pool G	1.38	1.22	8.8	1.67
16	Equine	2.1	1.85	5.2	2.0

COMPARISON WITH THE PBI

The PBI values of these pools deserve consideration. Four decolorized completely, indicating a high iodide content, presumably derived from one or more of the sera incorporated. The PBI values of steroid and pregnancy pools are also higher than one would have reason to expect, and in fact are in, or close to the hyperthyroid range. The normal sheep serum (SP-1) which is, by all criteria, in the normal range, was "hyperthyroid" by the PBI, while the equine serum, very high by all T-3 procedures, was "low normal". All of these clear cut abnormalities serve to point up the fundamental limitations of the PBI in practice, even though, in the days when alternative procedures were not available, it was assumed to be highly accurate.

Finally, each pool was assayed, using T-3 and exactly 100 mg of large particle size IRA 400 resin, plus 7.2 buffer, by rotating for two hours at +4° and counting the washed resin. The values, while somewhat less constant than with the sponge, place each sample in the proper "clinical category" (Table II). Two cc of the supernatant solution was likewise counted, as in the "TBI", but the values failed to show adequate characterization.

SECTION II

EQUILIBRIUM STUDIES

A. *General*

In the original Hamolsky version of the T-3 test, the erythrocytes were regarded as relatively nonspecific locations of alternate binding sites. When resins were substituted, they were assumed to play a similar role. More recently, Goolden *et al* (6), suggest that the mechanism is different, in that while the erythrocyte method measures the final equilibrium, the resin method is a measure

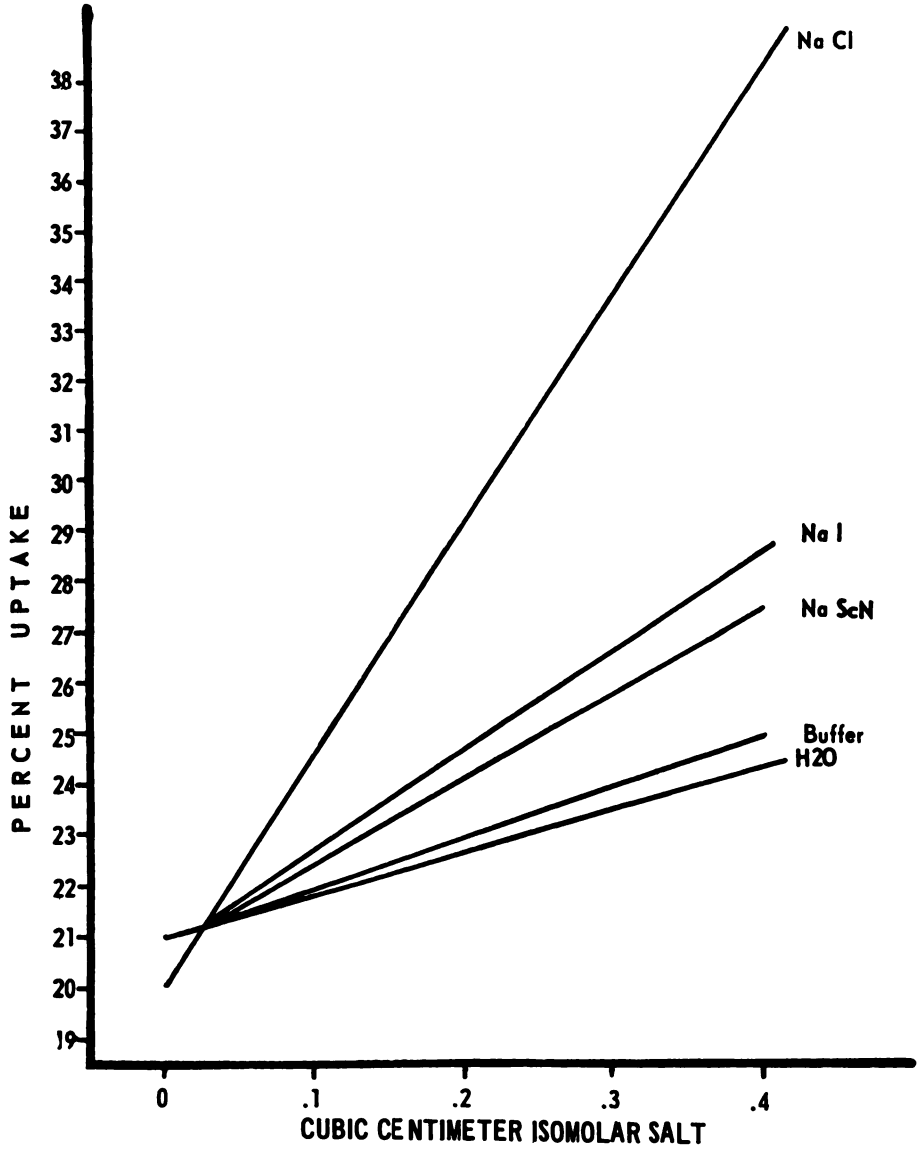


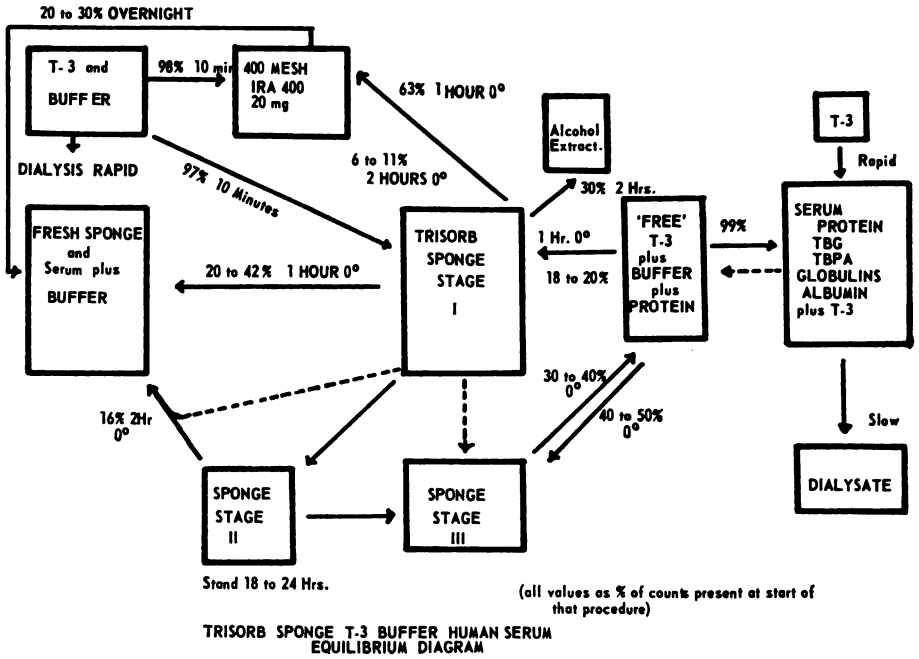
Fig. 5.

of the speed with which the T-3 becomes irreversibly bound to the resin. They further conclude that there are actually two types of resin binding, one reversible, the other irreversible.

The Triosorb sponge is made by suspending a 400 mesh IRA 400 resin in polyurethane. This is then further polymerized and foamed. The result is a continuous fibrous mass, which, as seen under the microscope, consists of a network of slender semi-transparent filaments (Figure 6) which mat together easily. The resin particles are not visible, suggesting that they are an integral part of the structure. The same view is indicated by the low reactivity of the crude sponge. It must be treated mechanically, at a controlled temperature, to "open up" resin availability to a predetermined degree.

Although the resultant sponge gives no indication of "fractures", the rate of T-3 or T-4 uptake is less than half that of an equivalent amount (20 mg.) of the free resin. It would therefore seem probable that there remains over each particle a thin membrane of polyurethane, analogous to those secured by Gottleib and Herbert (7), through the addition of hemoglobin or P.V.P. to Norite carbon. These films have unique "semipermeable" properties and are often "molecular structure" specific. It would not be surprising if they could play an important part in the shifting of equilibria with temperature.

We have carried out resin adsorption and subsequent elution, in the absence and presence of numerous sera and under a wide variety of conditions. Our results indicate that a key factor is time, and that T-3, at first partially removable by fresh serum at pH 7.2, is converted during from 2 to 24 hours into an essentially non-removable state. While this is normally observed to take place in the presence



TRISORB SPONGE T-3 BUFFER HUMAN SERUM EQUILIBRIUM DIAGRAM

Fig. 6.

of serum, it can equally readily take place in the absence of protein, and under conditions which do not result in the transfer of activity to an immediately adjacent sponge. It would, therefore, in modern terminology, seem to result from a rearrangement of binding forces within each resin particle. Actually, small amounts continue to be removable during repeated serum extractions, suggesting that even this "fixation" is an equilibrium. The firmly bound component can be eluted with an organic solvent like ethanol, though physical changes in the sponge leave the mechanism in some doubt.

We have encountered still another complicating factor, ie, surface change with repeated adsorption and elution. After several cycles there is usually, but not always, a more rapid, rather than less rapid, adsorption and elution. That this is not solely the result of hydration was established by thorough "soaking" prior to the start of the standard procedure.

Similar but not identical phenomena have been observed using T-4 rather than T-3, and both mono- and diiodotyrosine and large particula IRA 400 rather than the Triosorb sponge.

B. *Experimental*

The materials, equipment and techniques were similar to those described in the preceding section. The primary variation was that, to insure a pH of 7.2 under a variety of conditions, 1 cc of Tris Maleate buffer was usually added. The slightly greater volume decreased adsorption but slightly and to a constant degree.

Triosorb sponge adsorbs T-3 and T-4 from buffer with extreme rapidity, the reaction being 95% complete in 4 minutes at 0 degrees and quantitative in 10. Obviously the sponge is highly porous, and no agitation is required. The rates are the same whether ¼ or twice the standard amount of T-3 in the syringe is used. Hence by regulating the volume of T-3 solution used, any predetermined amount of activity may be adsorbed on any given sponge.

The presence of a nonbinding protein, like gelatin, increases viscosity but does not reduce adsorption materially. Egg albumin permitted a sponge uptake of 85%. Unfortunately, human serum, completely free of binding fractions, was not available. Cutter Laboratories human serum albumin, which, according to its electrophoretic pattern, still contained some globulins, permitted 75% adsorption in 30 minutes. Pure bovine albumin allowed a 1 hour uptake of 80%.

While values for simultaneously run samples usually agreed well, there were day to day variations of a greater magnitude. Hence, a range of values, based upon a number of observations, will be given.

In a typical experiment, T-3 equivalent to either one (or to one-half) of a syringe was adsorbed, in the absence of serum, during one hour, at 0 degrees, to the extent of 97-100%. When promptly extracted for 2 hours with serum and buffer, 42% was removed; two subsequent extractions removed 12 and 10% of the remaining amounts. When the sponge was allowed to stand for 5 hours prior to extraction, only 36% was removed on the first elution and 11 on the second. When the intervening period was extended to 20 hours, the corresponding elutions were only 16 and 7%.

We found that contrary to the observation of Goolden, *et al* (Ref. 5b), ethanol does dissolve some 30% in two hours, but only a small amount (2-3%) on further extractions. Though the values are similar to those found on prompt serum elution, it is not established that the removal mechanisms are related.

Dissociation of T-3 from serum protein and its combination with resin, proceeds slowly; as we have seen, only 19-20% is adsorbed in one hour. If elution is, as above, started promptly, some 30-35% of this is picked up by the fresh serum. Elution at the end of 24 hours or more, removes some 15-18% of that present on the sponge. Doubling the weight of sponge present doubles the percent adsorbed as well as that eluted. Cutting the sponge into small fragments does not enhance adsorption.

Carrying out the procedure at 50 degrees, where uptake is 75%, differentiation between samples no longer takes place. Conversion to the firmly bound form seemingly is also rapid, since less than 5% is desorbed at this same temperature.

If a new sponge is placed in buffer along with the freshly labeled one, the second sponge takes up no activity. However, in the presence of serum and buffer, activity is transferred to the same extent as would be expected from serum elution over a similar period, ie., 30% for freshly started experiments and 13-15% for delayed ones. Obviously, serum binding sites are involved in the transfer.

In runs, using for extraction, sera from patients with high, low, and normal thyroid values, removal was that expected from the work of Scholler (Ref. 8). The relative values were 25%, 14% and 19%, giving essentially the ratios found in the clinical T-3 values for these sera.

When sponges, once serum extracted, are treated with fresh T-3, the amount introduced (adsorbed) is the same as in the original state, as is the amount eluted. After several cycles of adsorption and elution, there often, but not always, ensued a state of "hyperabsorbability" with uptakes of up to 50-60%. This could mean either the exposure of more effective resin surface, or the change of that present. Under no conditions has this been seen on fresh sponges.

Contrary to expectation, extraction of the sponge with cold alcohol or ether, or even heating it with water, followed by drying, did not influence its absorption properties.

When 20 mg of the same 400 mesh resin in the powder state, was used in place of the sponge (mechanical rotation at 4°C), 50-60% was adsorbed, indicating that in being made part of the sponge, activity is reduced by one half to two thirds. When eluted promptly, the usual 30% was removed and 15-20% of the remainder in a second elution. The same change in firmness of binding obviously takes place in the absence of the polyurethane, showing that this is a fundamental property of the IRA 400 resin.

When sponge and serum were added to tagged resin powder, no exchange took place, indicating that the larger surface in effect "holds" all measurable activity tightly.

C. *The Effect of Ions on Equilibria*

It seemed pertinent to study the effects of ions known to influence thyroid hormone synthesis, storage and transport. Cavaleri and Searle (9) found that dilution markedly increased the dialysis of T-3. We find, on the other hand, that dilution with as much as six volumes of water, has no influence on sponge pickup. However, when the diluent is saline, the one hour pickup is tripled.

For a quantitative study, keeping volumes constant, amounts of a standard pool serum (human) from 0.8 to 1.2 ml were diluted with sufficient solutions of isomolar Na Cl, NaI, KSCN buffer and water to make 1.2 ml. Figure 5 shows the one hour 0° pickups. The slope of the water curve obviously represents the effect of continuously changing amounts of binding proteins in the reaction system. Buffer curves are not materially different. The increasing steepness of the curves involving the various ions must be a measure of the equilibrium shift caused by them, resulting in greater sponge uptake.

It is indeed surprising to find that the addition of one half volume of isotonic saline doubles the amount desorbed from serum and made available to the sponge in one hour. The amount going to the sponge is 25% greater at 25° than at 0. The order of effectiveness is the reverse of that which might be predicted on the basis of binding to resin alone.

A number of clinical sera were evaluated by the standard procedure and by this after adding .4 cc isotonic Na Cl. In the latter case the percent absorbed by the resin was higher (in the 38-45% range at 0°), but the ratios with respect to the standard serum were similar. The change of apparent "T-3 value" with protein concentration has been used to adjust lots so that they corresponded closely with our official standard.

D. *T-4 Equilibria*

A similar, though somewhat simplified study was made of the T-4 sponge equilibrium. Like T-3, it is taken up quantitatively from buffer (that is in the absence of competing protein sites) in well under 10 minutes. On prompt extraction with normal serum (and buffer) 60% is returned in two hours and 25-30% of the remainder, overnight. However, when kept overnight at 0 degrees, in the presence of the original serum, only 30% is subsequently transferred. Obviously, T-4, though less firmly bound at the start, also changes its tenacity of binding with time.

When adsorption takes place in the presence of serum, only 8-10% is taken up in one hour and 14-16% in two hours, indicating a far stronger protein binding, presumably due to the known "preference" of T-4 for "prealbumin" (TBPA). Of this small amount, 20-25% was removed by a two hour extraction. Obviously, the poorer counting statistics, with the usual added activity, make 0 degree adsorption of T-4 less practical as a clinical procedure.

Alcohol extraction of labeled sponges resulted in the removal of 45%, as compared with 25% for T-3, indicating that the T-4 sponge bond is more labile to this organic solvent, as well as to binding proteins.

E. *Studies Involving Simultaneous Equilibria with Labeled and Carrier T-4 and T-3*

If the sponge is first labeled with I-131 T-4 of high specific activity (at zero degrees), adsorption is complete in less than 1 hour and quite surprisingly, none is removed by extraction with as much as 100 micrograms of cold T-4 in buffer. This represents a ratio of carrier to adsorbed material of several thousand to one. If the carrier T-4 is adsorbed first, further adsorption of labeled T-4 proceeds to 93-94% of the extent it would if it alone were present.

The same value is arrived at, if both are mixed and adsorbed simultaneously. Sponges labeled in these three manners, behave in an identical fashion on a 24 hour zero degree serum elution with serum. The loss is 15-17% versus 40-50%, when no carrier is present; again the same "firmness of binding" phenomenon is seen, since after 24 hours only 9-10% is eluted. In the presence of serum, original adsorption dropped from 100 to 59% and elution to 9%.

T-3 (I-131) is likewise readily taken up by a sponge containing 100 micrograms of carrier T-4 and elution is 13% instead of the usual 30-35%.

Working with T-3 alone, a sponge containing 100 micrograms (of T-3) adsorbed 90% of labeled T-3 and lost 19% on elution. Even when the T-3 was increased to 500 micrograms, 85% was adsorbed and 18% eluted.

Very obviously, the Triosorb sponge has an active adsorptive capacity many times that utilized in diagnostic tests and adsorption values are not significantly modified over a wide range of concentrations.

A summation of the principal equilibria studied, is to be found in Figure 6. In the "flow diagram," Stage I represents the readily reversible phase of adsorption, Stage II the more firmly bound state and Stage III the highly reactive form.

F. *Mono- and Di-Iodo Tyrosine Equilibria*

Normally, the amount of the iodo tyrosines present in patient serum is extremely small, but it seems quite probable that they are precursors and possibly degradation products in the thyroid cycle.

In the presence of buffer only, mono-iodo tyrosine is adsorbed to the extent of 60-80% in one hour at 0° and di-iodo to the extent of 87-90%. On prompt elution of both, with serum, 12-18% is removed. Increased firmness of binding with time was also observed. It would thus seem that both adsorption and desorption rates are lower than for T-3 or T-4. In the presence of human serum, adsorption of di-iodo tyrosine fell to the T-3 range, ie, 16-20%. Mono-iodo was adsorbed much more strongly (30-40%).

Most of the pools of Table II were studied, using the iodo tyrosines instead of T-3 in the standard T-3 procedure. We found complete lack of correlation, either with each other, or with T-3 and T-4 values. Even more surprising, was the fact that all animal sera gave very low sponge adsorption values of di-iodod tyrosine, but not the mono-iodo analog. It would seem that the protein binding specificity of these must be entirely different.

CONCLUSION

(1) The mechanisms involved in the T-3 or T-4 Trisorb resin procedure are not simple, as once envisioned, but represent the summation of a number of equilibria, some readily reversible, other essentially unidirectional. Thus, the need for the use of careful temperature and concentration conditions and the inclusion of a single long term, exactly normal standard in every run, is easily understandable. The value of sheep serum for such a standard, has been demonstrated.

(2) It must always be kept in mind that the degree of blood protein saturation by endogenous T-4, is the factor being measured and not the functional state of the thyroid gland. Like the PBI and the I-131 conversion ratio, the T-3 sponge uptake is materially influenced by the types and amounts of abnormal binding proteins produced by physiologic stimuli.

(3) Steroids, and in particular, those now so widely used as contraceptives, pose a newly recognized source of serious error. The PBI is influenced by them, also, but in the opposite direction.

(4) We have established, experimentally, that through carrying out the sponge adsorption at zero degrees C, equilibria are shifted so that the numerical values for each of the usual clinical ranges are increased. The end result is that the distribution curve is falling more rapidly at its lower side, leaving fewer cases in the usual questionable normal-hypothyroid border range. In over 500 clinical cases, sample to sample and day to day reproducibility are improved. The value we report to the clinician, ie, the ratio of the patient serum to that of our standard, is 0.9 to 1.40 for normals, above 1.4 for hyperthyroids and below .89 (or better .86) for hypothyroids.

(5) It is now almost universally agreed, that no single procedure is free enough from errors caused by known, or unknown, clinical situations and drug effects, to be relied on quantitatively for thyroid evaluation. We believe that this T-3 procedure used along with the conventional 24 hour uptake, and where desirable, the scan comes very close to an optimal accuracy.

(6) The various equilibria involved in thyroid hormone adsorption on the Trisorb sponge have been studied individually and as serial phenomena. The effect thereon of chemical and physical factors has been pinpointed.

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