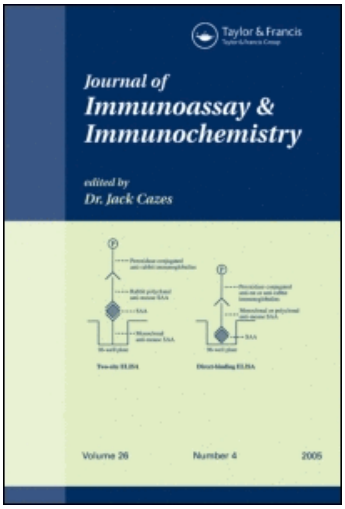

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SENSITIVE HUMAN THYROTROPIN IMMUNORADIOMETRIC ASSAY SET UP BY THE IDENTIFICATION AND MINIMIZATION OF NONSPECIFIC BINDINGS

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ABSTRACT

An immunoradiometric assay (IRMA) of human thyrotropin (hTSH), based on magnetic solid phase separation, was studied especially in terms of its nonspecific bindings (B_0) which were identified as a product of the interaction between an altered form of radioiodinated anti-hTSH monoclonal antibody (^{125}I-mAB) and the uncoupled magnetizable cellulose particle (matrix). Preincubation with the same matrix, solid phase saturation with milk proteins, tracer storage at 4°C and serum addition during incubation were found to be particularly effective in preventing their formation.

These findings were used to reproducibly decrease nonspecific bindings to values < 0.1 % (or < 70 cpm), thus increasing the signal-to-noise ratio (B_{60}/B_0) up to values of 300-500. This way hTSH radioassays were obtained with functional sensitivities of about 0.05 mIU/L and analytical sensitivities of the order of 0.02 mIU/L. Such sensitivities, and, more importantly, a general improvement in assay performance, were obtained in a highly reproducible manner and all over the useful tracer life.

(KEY WORDS: human thyrotropin, immunoradiometric assay, magnetic solid phase, nonspecific binding, sensitivity).

INTRODUCTION

The hTSH assay can provide the single most sensitive and specific index of thyroid status, being an efficient first-line test of thyroid function in ambulatory patients (1-4) and for the screening for neonatal hypothyroidism (5-7). For this reason, numerous hTSH assay set ups, comparisons and applications have been reported, especially with respect to analytical and functional sensitivity that, when sufficiently high, can also discriminate between euthyroid and hyperthyroid individuals (1,8-10).

Spencer et al. (2) have classified hTSH immunoassays into different generations, each representing an approximately 10-fold improvement in functional performance over the previous generation. A third generation hTSH assay, such as the immunochemiluminometric assay (ICMA) described by these authors, may provide a functional sensitivity of 0.018 mIU/L and an analytical sensitivity of 0.003 mIU/L. These two different definitions of sensitivity normally present a 5-10 fold difference in their calculated values. The American Thyroid Association (ATA) gives preference to the functional definition of sensitivity which should be clearly defined on the basis of interassay precision characteristics (9). In the present paper we will continue to use the analytical definition since this is how many authors and manufacturers still refer to the detection limit of their hTSH assays.

While the early hTSH radioimmunoassay (RIA) with an analytical detection limit of the order of 0.5-1 mIU/L clearly belongs to the so-called first generation, second and third generation assays should present sensitivities approximately one or two orders of magnitude higher, i.e. down to 10^{-2} and 10^{-3} mIU/L, respectively. The second generation should include most of the isotopic immunometric assays (IRMAs), but some of the most popular kits vary widely in declared sensitivity values (up to 10-30 fold), even when these are basically determined by the same method (11-21).

Besides the obvious difficulty in classifying systems through such highly variable sensitivity data, nonisotopic immunometric assays such as Delfia, using high specific activity labels and being therefore the best candidates as 3rd generation assays (19,22), very frequently perform like 2nd generation assays, while some isotopic IRMAs, like RIA-gnost (11-15), approach the detection limits of the 3rd generation.

In the present study we started from the concept that one of the factors that strongly influences sensitivity, together with assay design, antibody affinity, incubation time and label specific activity, is the amount of nonspecific binding (B_0), which directly determines the signal-to-noise ratio (19,22,23). Thus, we studied the origin and influence of B_0 on a system particularly sensitive to it, a magnetic solid phase hTSH IRMA, in an attempt to improve assay performance in general and sensitivity in particular. In our opinion the generally poor performance of magnetic phase hTSH IRMA is mostly due to its high B_0, ranging from about 0.4% (16,24) to about 1% (6).

MATERIALS AND METHODS

Materials

The pituitary hTSH standard (IRP-1) was kindly provided by the National Hormone and Pituitary Program / NIDDK (Rockville, MD, USA) and used together with a secondary standard of recombinant hTSH (25).

Anti-hTSH monoclonal antibody (mAB) for radioiodination (detecting antibody) (batch code TSH. 0584 0001, Ka = 3.8×10^{11} LM^{-1}) was purchased from Serono Diagnostic (Woking, Surrey, UK).

The magnetic anti-hTSH solid phase was prepared in our laboratory from magnetizable M-174 cellulose (Scipac, Sittingbourne, Kent, UK) and from a polyclonal anti-hTSH preparation kindly donated by Dr. A. Bulatov, National Research Center for Endocrinology, Moscow, Russia. 1-1'-Carbonyldiimidazole (CDI) and bovine serum albumin (BSA) were obtained from Sigma (St Louis, MO, USA). Acetone, grade Anala R,

was purchased from BDH Ltd (Poole, UK) and bovine non-fat dry milk (Molico) was from Nestlé (São Paulo, Brazil).

Sephadex G-200 and G-25 were products of Pharmacia-LKB (Uppsala, Sweden).

Radioiodination and Tracer Purification

The ^{125}I-labelling of anti-hTSH mAB was carried out as previously described (25). The reaction mixture was purified on a 1.5 x 80 cm superfine Sephadex G-200 column and the product (^{125}I-mAB) was stored frozen (-20°C), lyophilized (4°C) or in liquid form (4°C).

The tracer was repurified on the same Sephadex G-200 column or on a small Sephadex G-25 column (1.5 x 5.0 cm) used only to remove free ^{125}I.

^{125}I-mAB was also pre-incubated with the same matrix as used for the assay (uncoupled magnetizable cellulose). This procedure, defined as "tracer cleaning", was carried out by 4 hours incubation at room temperature on a rotary mixer using 50 µl of tracer (corresponding to 5-10 x 10^6cpm) and 2.5 mg (50 µl) of magnetic matrix in 400 µl of 0.05 M phosphate buffer pH 7.4. Tracer and solid phase were separated using an Amerlex-M (N 4001) magnetic batch separator from Amersham (Aylesbury, Buckinghamshire, UK).

Magnetic Solid Phase and IRMA

Polyclonal anti-hTSH antibody was covalently linked to magnetizable cellulose particles M-174 (75% cellulose + 25% Fe_3O_4) via a CDI coupling reaction (26) according to Scipac and NETRIA protocols (27), using 0.6 ml polyclonal antibody per gram of CDI-activated cellulose and adding a first washing step with 1% BSA and a second with 1% dry milk, both in bicarbonate buffer, after the ethanolamine blocking reaction. The obtained antibody-coupled magnetizable cellulose particles were stored at 2-8°C in phosphate buffer containing 0.5% BSA (storage dilution: 25 mg coupled particle/ml).

The ideal amount of solid phase to be used in the assay was determined by analyzing the range of 0.25-5.0 mg/tube. Percent radioactivity (^{125}I-mAB) bound at zero (B_0) and at 60 mIU/L (B_{60}) hTSH under normal assay conditions was used to calculate the signal-to-noise ratio (B_{60}/B_0), a quality parameter already usefully applied by other authors (24).

Assays were carried out as previously described (25) by allowing the preparation to sediment for 10 min on the Amerlex-M magnetic separator after overnight incubation and washing twice with 0.05M phosphate, pH 7.4, plus 1% sodium azide and 0.5% Triton-X.

Sensitivity Evaluation

The analytical sensitivity of the assay was determined by two well accepted methods: using 20 replicates of zero dose + 2SD or according to Rodbard's definition (28) still using 20 replicates of zero and minimal doses. Functional sensitivity was calculated from the intercept on the inter-assay (n = 4 assays, with a duplicate run of each point of the standard curve) precision profile with a CV=20%.

RESULTS

Table 1 summarizes the results of a study carried out to determine the origin of nonspecific binding (B_0) in hTSH magnetic IRMA. Two components are required to interact in order to produce nonspecific binding, i. e. ^{125}I-mAB or something derived from it and the solid phase which, in this specific case, is magnetite (Fe_3O_4) adsorbed to cellulose. The presence of a coupled antibody for capture does not seem to influence B_0. Radioactivity, or a labelled protein per se, does not produce B_0, as confirmed by the practically zero binding of ^{125}I and ^{125}I-BSA. An analogous experiment, carried out on finely dispersed plain magnetite or on silanized magnetite particles, has shown that Fe_3O_4 itself, rather than cellulose, is responsible for high rates of nonspecific binding.

TABLE 1

Origin of Nonspecific Binding in hTSH Magnetic IRMA

Incubation conditions	B_0 (%)	B_{60} (%)	B_{60}/B_0
^{125}I-mAB without solid phase	0.04	0.07	1.7
^{125}I-mAB + uncoupled solid phase	1.42	3.42	2.4
^{125}I + AB bound solid phase	0.06	0.05	0.8
^{125}I-BSA + AB bound solid phase	0.03	0.05	1.7
^{125}I-mAB + AB bound solid phase	1.05	31.30	29.8

Different storage conditions of anti-hTSH ^{125}I-mAB were also studied, analysing the variation in specific (B_{60}; signal at 60 mIU/L) or nonspecific (B_0) binding for a period of up to two months (Figure 1). It was found that B_{60} decreases with practically the same kinetics (from -0.33 to -0.38 percent/day, $P<0.01$) under the three different storage conditions, and that B_0 increases at the same rate in the frozen or lyophilized form (0.04 and 0.05 percent/day, respectively, $P<0.001$). In contrast, B_0 at 4°C was practically stable at values around 0.3 - 0.6% with no correlation with time. At 4°C a slightly lower stability of ^{125}I-mAB seems to occur, as indicated by the lower values of B_{60}.

Possible modifications in the chromatographic profile of ^{125}I-mAB kept at -20°C or at 4°C were investigated by analysing this product after two months storage, on a high resolution superfine Sephadex G-200 column (Figure 2). While a clean, symmetric peak of ^{125}I-mAB and a quite small peak of free ^{125}I were observed immediately after labelling, some different components appeared after long-term storage. The tracer stored at -20°C or at 4°C presented approximately the same amount of radioactivity in the main peak of ^{125}I-mAB (55% and 50% respectively), but while the radioactivity associated with a higher molecular weight form (possibly an aggregate of ^{125}I-mAB), was 17% in the former, it was only 5% of the total in the latter. At the same time a type of BSA-bound radioactivity, already described for other tracers (29) and not appearing in

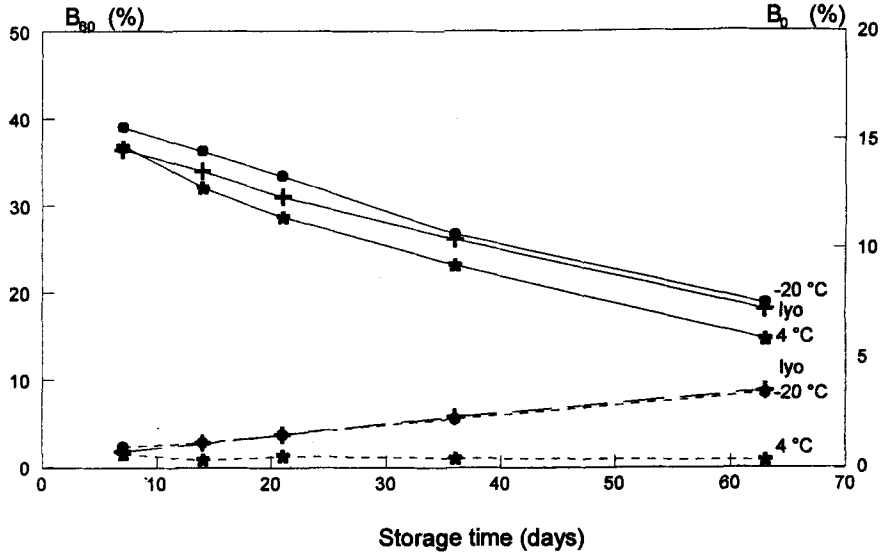
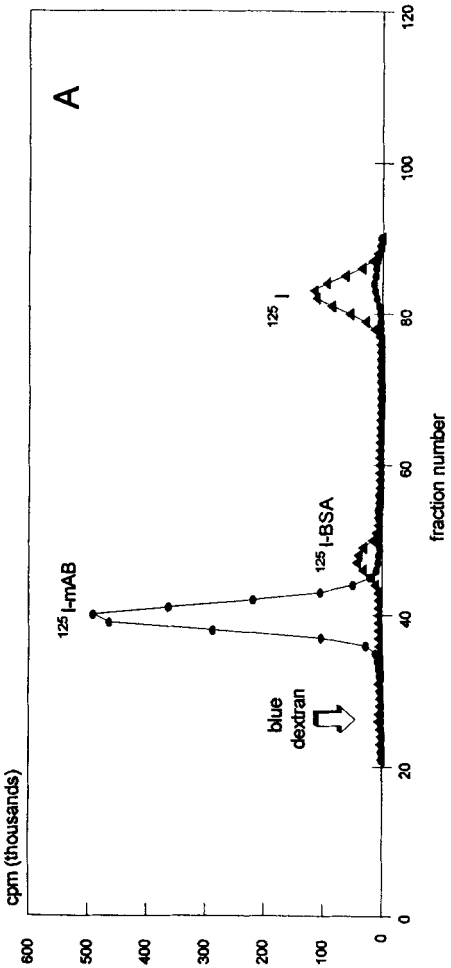
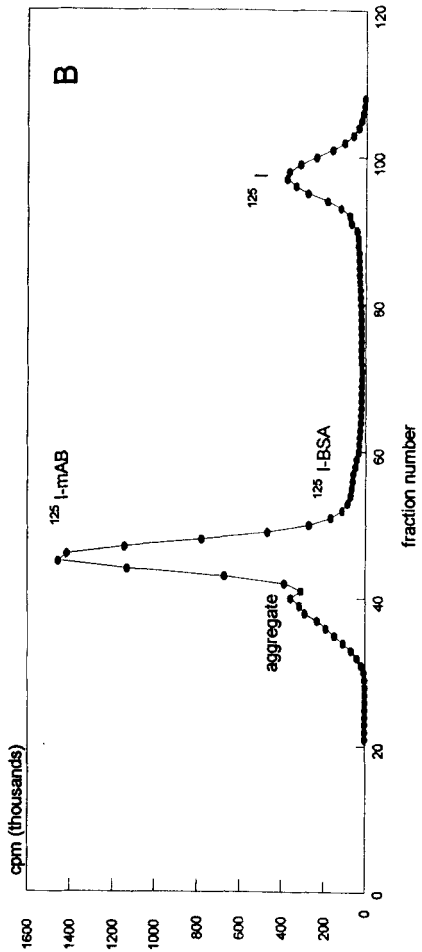


Figure 1. Specific (B_{60}) and nonspecific (B_0) bindings obtained with a ^{125}I-mAB in hTSH magnetic IRMA, under different storage conditions.
—●— -20°C
—+— lyo
—*— 4°C
Solid lines refer to B_{60} and dashed lines refer to B_0.

the frozen product, was remarkably present in that stored at 4°C which also had a higher peak of ^{125}I. When used in IRMA incubation, the fractions corresponding to the hypothetical ^{125}I-mAB aggregate presented high B_0 values (2-5%), suggesting that the nonspecific binding seems to be due to, or at least to be increased by, the presence of this component, whose concentration is higher in the frozen form of the labeled antibody (Figure 1).

A series of experiments was carried out in an attempt to decrease the presence of B_0. An example of the effects obtained with different treatments is presented in Table 2. The saturation of the magnetic particle with BSA and milk proteins after the CDI coupling reaction had a strong





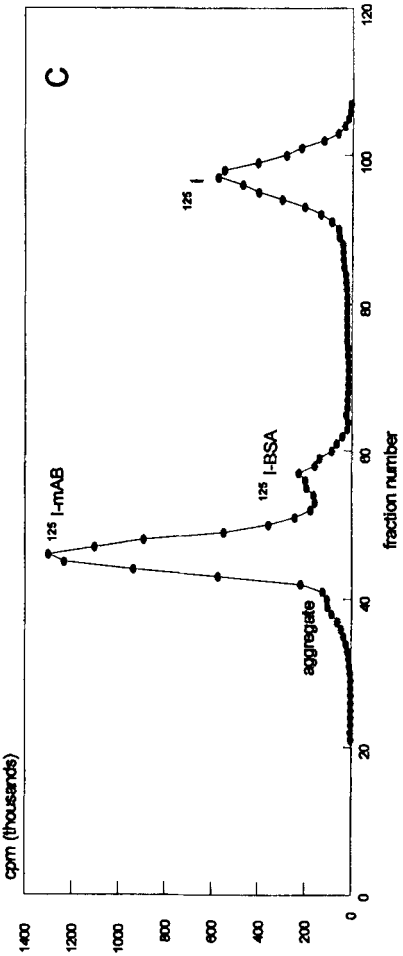


Figure 2. Sephadex G-200 (1.5 x 80 cm) chromatogram of repurified anti-hTSH 125I-mAB:
A. right after radiodination (a previous calibration of the same column with blue dextran, 125I-BSA and 125I is shown: Δ – Δ – Δ – Δ);
B. after two months storage at -20ºC;
C. after two months storage at 4ºC.

TABLE 2

B_0 Lowering Effects due to Different and Independent Treatments

Type of treatment	B_0 (%)	B_{60} (%)	B_{60}/B_0
1. Solid-phase saturation with BSA and milk proteins			
a) without saturation	0.78	29.5	38
b) with saturation	0.17	28.3	166
2. Tracer storage for two months at			
a) -20°C	3.40	18.8	6
b) 4°C	0.33	17.7	45
3. Tracer repurification on Sephadex G-200 after 1 month storage			
a) before treatment	1.54	26.7	17
b) after treatment	0.67	32.4	48
4. Tracer pre-incubation with the magnetic matrix			
a) before treatment	1.20	34.7	29
b) after treatment	0.20	33.6	168
5. TSH-free horse serum addition			
a) incubation without serum	1.20	34.2	28
b) incubation with 20% serum	0.12	24.6	205

positive effect and subsequent experiments demonstrated that the improvement was mainly due to milk proteins, whose efficient blocking action has already been demonstrated in analogous systems (30,31). Tracer storage at 4°C instead of -20°C had a dramatic effect on B_0 even with a certain concomitant decrease in B_{60}. Chromatographic repurification by gel filtration was not so efficient in lowering B_0 even if there was a certain increase in B_{60} due to the elimination of ^{125}I formed during storage. The most efficient treatments were tracer cleaning by pre-incubation with the magnetic matrix and 20% TSH-free horse serum addition to the incubation buffer. The first has no effect on B_{60}, while the

TABLE 3

Combination of Different B_0 Lowering Effects

Tracer condition	B_0 (%)	B_{60} (%)	B_{60}/B_0
1. Sephadex G-200 repurification	0.80	34.4	43
2. repurification + "tracer cleaning"	0.30	35.9	120
3. repurification + 20% serum	0.03	25.6	853
4. repurification + "tracer cleaning" + 20% serum	0.01	27.1	2710

TABLE 4

Sensitivities Obtained with Tracers Presenting Different Levels of B_0

Assay number	B_0 (%)	B_0 (cpm)	initial slope of the curve (cpm x L/mIU)	functional*1 sensitivity (mIU/L)	analytical sensitivity (mIU/L)	
					A*2	B*3
1	0.09	55	667		0.024	0.008
2	0.10	62	550	0.05	0.018	0.008
3	0.06	39	508		0.018	0.009
4	0.09	69	516		0.030	0.012
5	0.36	218	356		0.096	0.053
6	0.45	294	557	0.27	0.048	0.016
7	0.65	420	576		0.072	0.021
8	0.66	422	486		0.072	0.021
9	0.86	714	530		0.096	0.033
10	1.13	832	397	0.48	0.240	0.079
11	1.11	724	526		0.144	0.048
12	1.0	582	417		0.120	0.034

*1- Determined on the inter-assay precision profile with a CV=20%;
*2- Determined using 20 replicates of zero dose + 2SD;
*3- Rodbard's definition (28)

second can decrease B_{60} by about 30%. The best serum concentration that could lower B_0 but still maintain approximately the same B_{60} values was found to be 5%, a condition that provided ~ 0.15% B_0 and only a ~ 5% B_{60} decrease.

An example of a combination of different treatments, leading to essentially complete B_0 elimination, is presented in Table 3. All or some

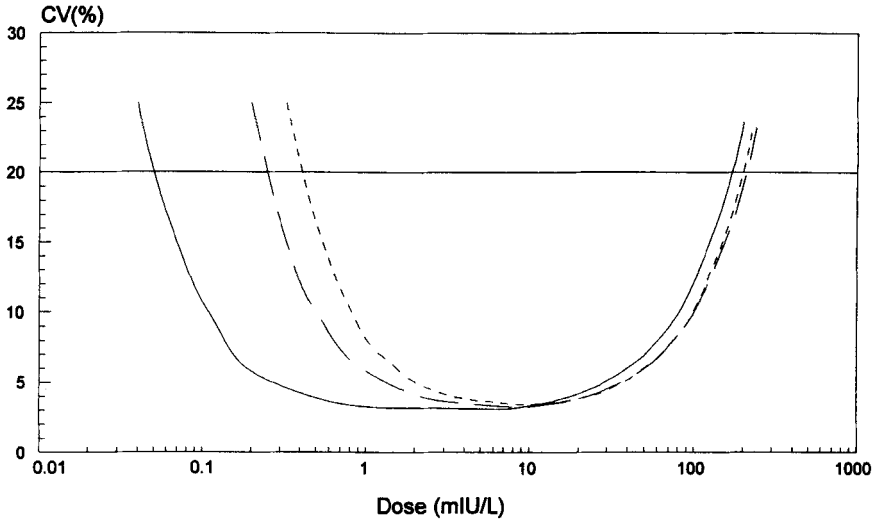


Figure 3. Inter-assay precision profiles obtained with the 3 sets of curves presented in Table 4, using the intra-assay duplicate of each dose:
———————— assays 1-4;
— — — — — — — assays 5-8;
- - - - - - - - - - - assays 9-12

of the described treatments, in combination with storage at 4°C and solid phase saturation with milk proteins, were then routinely applied for the purpose of setting up optimized IRMAS (B_0 always maintained at $\leq 0.1\%$ or ≤ 70 cpm).

Finally, Table 4 illustrates the effect of three different B_0 levels (represented respectively by assay 1-4, 5-8 and 9-12) on functional and analytical sensitivity, the latter being determined by two well accepted methods. It is also important to emphasize that assays 1-4 were carried out with the same tracer after 11, 24, 31 and 60 days of storage and different B_0 lowering procedures, and essentially the same degree of sensitivity was obtained with the four assays (cf column A, Table 4). Figure 3 shows the interassay (n=4) precision profiles obtained at the

three B_0 levels and illustrates the order of magnitude decrease in functional sensitivity as a result of increasing nonspecific binding.

DISCUSSION

The extensive study carried out on the origin, behaviour and possible chemical nature of the nonspecific bindings which occur in a hTSH magnetic IRMA has shown that these seem to be due to high molecular weight forms of a radioiodinated monoclonal antibody interacting with the solid phase. The possible chemical nature of the binding has not been investigated previously. Hunter et al. (32) found that storage at 4°C was much better than at -20°C, -80°C or as a freeze-dried powder. This prevented a decrease in antibody activity (specific binding), while we showed that storage in liquid form greatly decreased nonspecific binding (or aggregate formation).

The mechanism of formation of this high molecular weight material was not clear. Its kinetics, following tracer deiodination in the same time-related manner in frozen solution or lyophilized form (at a different temperature) is typical of chemical events related to radioactive decay. In previous work (29,33) we tested this hypothesis with another radioiodinated tracer and observed the same deiodination kinetics in the liquid (4°C), frozen (-20°C and -196°C) or lyophilized form (4°C). We assumed that the phenomenon was due to direct radiation effects such as recoil energy or decay catastrophe (34). The clear evidence in the present case that B_0 does not increase during storage at 4°C could not be explained by the protective action of BSA in solution, or by the absence of freezing and thawing effects (data not shown). We can only speculate that the molecular mobility which always occurs in solution, even at 4°C, is responsible for a disaggregation or aggregate deiodination that does not occur in the solid state.

The mechanism related to the efficient procedure of "cleaning" the tracer via pre-incubation with a sample of the same solid phase has been

applied with success also to avidin-coated microtiter plates (30). In view of the high efficiency and simplicity of this treatment it may be universally applicable. Less clear is the action of serum during incubation. This extremely heterogenous component has the positive effect of lowering B_0, but at the same time, the negative effect of lowering also specific binding. Some serum proteins act as competitors in both cases, either occupying nonspecific sites or hindering specific sites. This last possibility had been suggested also by Ho et al. (35) in a study of serum effects on antibody immobilized on different polymeric substrates.

As suggested by Ekins (22) in his elegant analysis of the influence of nonspecific binding on the detection limit of optimized competitive and noncompetitive assays, in the latter systems the potential sensitivity can be improved by a factor "f" which represents the fraction of labelled antibody that is nonspecifically bound. This theory was proved by the practice of our experiment where an approximate 10-fold increase in functional sensitivity corresponded to a 10-fold decrease in B_0. Thus, using the radioisotopic label and without any special assay design modification, but simply increasing the signal-to-noise ratio through the minimization of nonspecific effects, it was possible to set up the most sensitive hTSH magnetic IRMA reported thus far in the literature, both in terms of its functional and analytical detection limit.

Besides the theoretical approach of Ekins (22) demonstrating the influence of nonspecific binding in determining potential assay sensitivity, other authors have emphasized the importance of minimizing this undesirable effect (23,30,31,36,37), but very little has been reported thus far from a practical point of view, especially for radioisotopic systems. The importance given by Wide in his solid-phase assay systems to repeated washing ensuring less than 0.1% nonspecific binding in order to achieve maximal sensitivity has been nonetheless repeatedly stressed (38). The emphasis placed recently by Ciba-Corning on the introduction of repeated wash cycles directed at maintaining levels of $B_0 < 0.1\%$ for its hTSH

magnetic immunoluminometric automated system ACS:180 (36) confirms the importance of this approach. To our knowledge, the present study is the first in which at least five different B_0 lowering treatments have been systematically studied and compared, resulting in an up to 60-fold increase in signal-to-noise ratio with an aproximately 10-fold improvement in functional sensitivity and, even more important, maintaining such assay performance troughout the useful life of the tracer.

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