Removal of an Endogenous Antigen from an Antibody to Increase Its Effective Affinity Constant, as Illustrated by Triiodothyronine Assay

Lawrence K. Oliver and Carlos Cano

Antisera to triiodothyronine were shown to contain large quantities of the hormone, well in excess of normal circulating concentrations. Extracting the triiodothyronine from the antiserum with alkaline ethanol yielded an antibody of increased affinity. Use of this antibody in a radioimmunoassay resulted in a fourfold increase in sensitivity as compared with the unextracted material.

Additional Keyphrases: immunochemistry • general technique for enhancing sensitivity of radioimmunoassay

The development of radioimmunoassay techniques over the last several years has enabled analysts to detect and measure analytes that are present in very low concentrations in body fluids. Sensitivity is one of the major attractions of radioimmunoassay, and the further lowering of detection limits is the goal of much research in clinical chemistry.

Limits of detection in a radioimmunoassay are governed by many interacting variables, including amount, affinity, and specificity of the antibody; mass and specific activity of the label; and efficiency of separation of antibody-bound label from free. The antibody affinity has generally been considered to be a primary physicochemical constant for each antibody and hence not subject to modification (increase) by normal chemical manipulations. It has been presumed that once the effective affinity constant is maximized by appropriate dilution, it cannot be increased further.

We had been re-investigating ways of improving sensitivity in radioimmunoassays and began to consider means of altering the effective affinity constant.

Recent reports (1-3) have verified a tenet in the folklore of radioimmunoassay: animals immunized to produce antibodies to endogenous antigens may produce extraordinarily high concentrations of those antigens in their own circulation. Herrmann et al. (1) demonstrated that rabbits immunized with a triiodothyronine/bovine serum albumin conjugate had circulating triiodothyronine concentrations that were 600-fold normal. The animals remained euthyroid despite this vigorous synthesis of triiodothyronine. The authors concluded that the excess hormone was protein-bound and not available in active form.

While this work was in progress, Fyhrquist and Wallenius (4) reported a 48-h dialysis procedure which removed bound vasopressin and angiotensin II from their respective antibodies. They observed a marked increase in the affinity constant and a corresponding improvement in assay sensitivity, which they attributed to the unmasking of high-affinity binding sites.

We report here a rapid, simple procedure for removing certain hapitens from an antiserum without harm to the antibody and with an improved effective affinity constant.

Materials and Methods

Reagents

Buffer diluent. Dissolve barbital, 3.12 g (17 mmol), sodium barbital, 17.1 g (83 mmol), sodium azide, 1 g, and polyethylene glycol (grade 6000; Polyscience, Inc., Warrington, Pa. 18976), 10 g, in 1 liter of distilled water. The pH should be 8.6. Adjust if necessary with HCl or NaOH. Add human serum albumin to a final concentration of 100 mg/liter.

Reagent mixture. Add 8-anilino-1-naphthalenesulfonic acid, ammonium salt (G. K. Turner, Palo Alto, Calif. 94303) to buffer diluent to a final concentration of 0.667 g/liter; rabbit gamma-globulin (Schwarz/Mann, Orangeburg, N.Y. 10962) to a final concentration of 333 mg/liter; and [125I]triiodothyronine (Abbott Radiochemicals, N. Chicago, Ill. 60064) to a final concentration of 167 ng/liter.

Antiserum to triiodothyronine was raised in rabbits by the method described by Chopra et al. (5).

Second antibody. Goat anti-rabbit gamma globulin was obtained from Antibodies, Inc., Davis, Calif. 95616.
Procedure

Antiserum stripping procedure. To 0.5 ml of raw antiserum add 4.5 ml of absolute ethanol while continuously mixing with a vortex-type mixer. Add 0.1 ml of 15.9 mol/liter (30%) ammonium hydroxide and mix with a vortex-type mixer. Centrifuge for 5 min at 2000 × g. To determine the amount of extracted triiodothyronine, evaporate the supernatant fluid, dissolve the residue in buffer, and assay this solution. Reconstitute the antibody-containing protein pellet with 0.5 ml of buffer with gentle mixing. This procedure can be scaled up by using proportionally larger amounts of all reagents.

Procedure

To a 10 × 75 mm glass tube, add 0.2 ml of stripped antiserum to triiodothyronine, 0.2 ml of serum sample or standard, and 0.6 ml of reagent mixture, then mix and incubate for 2 h at 37 °C. Add 0.5 ml of goat anti-rabbit gamma-globulin, mix, and incubate for 1 h at 37 °C. Centrifuge, decant the supernatant fluid, and measure the radioactivity of the pellets.

Results

Different aliquots of a single specimen of antiserum were treated with various concentrations of ethanol in buffer (Figure 1). The ammonium hydroxide concentration was kept constant (0.1 ml to 0.5 ml of serum). The amount of triiodothyronine extracted increased with the proportion of ethanol. Cursory checking revealed that antibody activity was present in all cases after such stripping, so we used the highest concentration of ethanol in further investigation.

The antiserum was subjected to successive stripping treatments to determine the total extractable triiodothyronine. The yield of triiodothyronine decreased with each extraction (Table 1); two extractions removed 97% of the extractable hormone. The precipitated protein dissolves readily after one or two extractions, but only with difficulty after three. Therefore, we used two extractions routinely.

When serum from non-immunized rabbits was assayed, triiodothyronine concentrations of 1.2 to 1.9 μg/liter were found. Two ethanol extractions removed more than 95% of this hormone also.

When the extraction was performed at 4 °C, no measureable triiodothyronine was found in the alcohol phase. We conclude that temperature markedly affects the efficiency of extraction, but we did not investigate low temperature further.

The effects of this treatment on the protein constituents of the serum were evaluated by electrophoresis and immunoelectrophoresis. Table 2 summarizes the changes in albumin, total globulins, and total protein concentrations in serum before and after two extractions. No measurable loss of total protein is observed and no significant changes in albumin/globulin ratio. Immunoelectrophoretic scans for the raw and extracted sera were virtually identical, except for a negligible loss of clarity of some of the precipitin lines in the extracted serum.

Specific antibody activity was determined by utilizing the antiserum in a radioimmunoassay for triiodothyronine. Standard curves (Figure 2) were prepared from data obtained with untreated and treated antisera at the same working (9000-fold) dilution. The B/B₀ values at

### Table 1. Triiodothyronine Removed by Successive Extractions

<table>
<thead>
<tr>
<th>Extractions</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trilodothyronine removed, mg/liter</td>
<td>2.40</td>
<td>0.80</td>
<td>0.09</td>
<td>3.09</td>
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### Table 2. Changes in Concentration of Protein Constituents of Serum on Stripping

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>Treated</th>
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<tbody>
<tr>
<td>Albumin (A)</td>
<td>33</td>
<td>36</td>
</tr>
<tr>
<td>Globulin (G)</td>
<td>30</td>
<td>27</td>
</tr>
<tr>
<td>Total</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td>A/G ratio</td>
<td>1.1</td>
<td>1.3</td>
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*Fig. 1. Amount of triiodothyronine removed as a function of final ethanol concentration*

A single specimen of antiserum was used throughout.

*Fig. 2. Standard curves for untreated (A) and treated (B) antisera*

Both antisera came from the same bleeding of a rabbit. B/B₀ values are expressed as per cent.
each concentration of standard for the stripped antisera are lower than for the unstripped antiserum. Conversely, equivalent $B/B_0$ values of 0.90 are found at 75 ng/liter for the stripped antiserum and at 300 ng/liter for the unstripped antiserum, a fourfold increase in sensitivity as a result of stripping.

The same curves shown in Figure 2 were recalculated to construct Scatchard plots (Figure 3). Affinity constants and binding site concentrations derived from these plots are shown in Table 3. The effective affinity constant has doubled, with only 5% loss of binding sites.

The stripping of antiserum does not affect results in this radioimmunoassay (except for increasing sensitivity). Figure 4 presents a scattergram of triiodothyronine values obtained for analysis of patients' samples by use of stripped and unstripped sera. Both were used at a working dilution of 9000-fold. Specimens having less than 300 ng of triiodothyronine per liter are not included because of the lack of sensitivity in the unstripped serum. There is good correlation between the methods ($r = 0.95$) and bias is negligible: the $y$-intercept is at $-70$ ng/liter and the slope of the regression line is 1.03. Specimens having triiodothyronine concentrations between 3.5 and 7.5 $\mu$g/liter (not shown in the figure) also gave equivalent results by the two procedures. At concentrations exceeding 7.5 $\mu$g/liter, the curve developed on using stripped antiserum flattens out and becomes unusable at the working dilution used.

**Discussion**

An animal producing antibodies to an antigen endogenous to the blood stream of that species must have some quantity of that antigen bound to circulating antibody. In principle, the animal can then react in one of two ways: (a) develop symptoms of clinical deficiency of the antigen, because less of the antigen is available for performing its necessary function, or (b) increase its rate of synthesis of the antigen so as to maintain normal circulating concentrations of antigen in its active form in the blood. A large percentage of the thyroid hormones triiodothyronine and thyroxine is bound to specific binding proteins circulating in the blood, the active portion of the hormone being that small fraction which is "free" and in equilibrium with the bound. Generation of an antibody of high enough affinity to compete with those binding proteins merely places a demand on the animal to increase its synthesis of hormone as progressively more binding protein (including antibody) circulates. Thus the animal is capable of maintaining the concentration of free hormones so as to remain euthyroid.

In the opinion of our veterinarian, our rabbits appeared to be euthyroid throughout immunization, so we conclude that they are maintaining normal or near normal concentrations of free triiodothyronine. Herrmann et al. (1) also found that their animals remained clinically euthyroid during immunization with triiodothyronine/bovine serum albumin. Their determinations of free triiodothyronine concentration are of little value because the percentage of free dialyzable triiodothyronine was too low to be accurately measured.

We attempted several other stripping procedures, but failed. Neither activated charcoal nor anion- or cation-exchange resins (both of which remove triiodothyronine from normal serum containing only endogeneous binding proteins such as thyroxine-binding globulin) had enough affinity for the hormone to compete with the antibody. Attempts to displace the triiodothyronine with agents such as 8-anilino-1-naphthalenesulfonic

<table>
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<th>Table 3. Changes In Antiserum from Stripping</th>
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<tr>
<td>$K_{\text{Aff}}$, liter/mol</td>
</tr>
<tr>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Raw antiserum</td>
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<tr>
<td>After ethanol treatment</td>
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Fig. 3. Scatchard plot from data in Figure 2

Untreated antiserum is curve A, treated is curve B. Homogeneity index for both was $\beta > 0.95$

Fig. 4. Use of stripped antiserum in a radioimmunoassay

Results of 300–3500 ng triiodothyronine/liter were used in this plot. The line is drawn at 45°; the regression equation is $y = -70 + 1.03x$
acid or salicylate or to weaken the triiodothyronine protein bonds with chaotropic agents such as NaI were unsuccessful. We tried thyroidectomizing the rabbit to induce release of triiodothyronine from the soluble antigen/antibody complex in the circulation. During the subsequent two weeks (until the rabbit died) we detected no measurable change in total triiodothyronine or any change in antibody concentrations.

 Preferential binding of triiodothyronine to the highest-affinity antibody is a necessary consequence of the mass-action law. As triiodothyronine is removed from these binding sites, an antibody of higher effective affinity constant is produced.

The stripping procedure reported here is rapid and simple: the entire process can be completed within 30 min for 3–5 ml of raw serum. The same technique has been applied in this laboratory to haptens besides triiodothyronine. Four animals being immunized with thyroxine/bovine serum albumin and producing measurable antibodies to thyroxine had an average total thyroxine of 222 mg/liter while the unimmunized rabbits had a thyroxine concentration of 80 µg/liter. Similarly, an aldosterone antiserum produced in rabbits contained 260 µg of aldosterone per liter compared to 90–130 ng/liter in unimmunized rabbits. Thus, the phenomenon of high concentrations of circulating antigen in response to immunization to that antigen appears to be general. Further work is being done to evaluate the use of stripped antisera with other analytes.

References