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Separation of two thyrotropin binding components from porcine thyroid tissue by affinity chromatography: Characterization of high and low affinity sites*

(hormone receptors/membrane proteins/nonlinear Scatchard plot/negative cooperativity)

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ABSTRACT **Two distinct thyrotropin (TSH) binding species have been separated from solubilized porcine thyroid membranes. Membranes were solubilized with 1% Triton X-100, and the supernatant was recovered by centrifugation at 105,000 × g. Scatchard analysis of thyrotropin binding to solubilized membranes (SM) yielded a nonlinear plot with K_d values for the high and low affinity components similar to those of intact membranes. Chromatography of the SM preparation on concanavalin A-Sepharose 4B resulted in the retention of 10–20% of the binding activity. Upon elution of the column, a peak of binding material (5–7% of total activity) was eluted at 0.3 M α-methyl-D-mannoside. This concanavalin A (Con A) bound fraction exhibited a linear Scatchard plot with a K_d value similar to that of the high affinity component of the SM. The protein fraction that did not bind to Con A (Con A unbound) also exhibited a linear Scatchard plot, but with affinity similar to that of the low affinity component of SM. Discontinuous sucrose density gradient ultracentrifugation revealed the presence of two major binding peaks in the solubilized membrane preparation. The slowly sedimenting peak corresponded to that seen in the Con A bound fraction, whereas the rapidly sedimenting peak corresponded to that of the Con A unbound fraction. Sepharose 6B chromatography indicated that in the case of the Con A unbound fraction, a single peak of specific binding activity was eluted in the void volume, and in the case of the Con A bound fraction, one major peak with an approximate Stokes radius of 67 Å and several other minor peaks were eluted. These results demonstrate the physical separation of two distinct TSH binding species from thyroid membranes and provide further support for the model of multiple classes of binding sites.**

Evidence has accumulated demonstrating that thyrotropin (TSH) binding to thyroid plasma membranes is not a simple bimolecular process (for review, see ref. 1). Previous attempts to describe the interaction of TSH with its receptor by equilibrium saturation analysis have yielded nonlinear (concave upward) Scatchard plots (1–3). Examination of the kinetics of TSH binding has shown that the complex binding behavior is best explained by assuming the existence of two or more classes of binding sites with different affinities and capacities (1, 3). Characterization of these binding components from human (1) and porcine (unpublished data) thyroid revealed that they differ in several aspects, including temperature and pH sensitivity and thermodynamic properties. Furthermore, in recent studies of human thyroid neoplasms (4, 5), it has been demonstrated that the high affinity site is responsible for stimulation of adenylate cyclase.

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The present study demonstrates the separation of two distinct TSH binding species from solubilized thyroid membranes, both of which exhibited linear Scatchard plots with different binding affinities and capacities. The results are discussed in light of the negative cooperativity model.

MATERIALS AND METHODS

Materials. Purified bovine TSH [40 international units (IU)/mg], a gift of John G. Pierce of the University of California at Los Angeles, was iodinated with ^{125}I (Amersham; IM-30) to a specific radioactivity of 60–90 μCi/μg (1 Ci = 3.7 × 10^{10} becquerels) by a modification of the lactoperoxidase method (1). Radiolabeled and radioinert TSH have previously been shown to have equivalent affinities for binding sites on thyroid membranes (4). Partially purified TSH (NIH-TSH-09), follicle-stimulating hormone (FSH) (NIH-FSH-S9), and luteinizing hormone (LH) (NIH-LH-S18) were a gift of the National Pituitary Agency (Baltimore, MD). Thytropar (5 IU/ml) was purchased from Armour Pharmaceutical (Scottsdale, AZ). Bio-Beads SM-2 were purchased from Bio-Rad. All other resins and chemicals were purchased from Sigma.

Preparation of Solubilized Membranes (SM). Porcine thyroids, obtained from a local abattoir, were immediately frozen in liquid nitrogen and stored at −90°C. Partially purified membranes (PPM) were prepared and solubilized as described (3, 6). PPM (12 mg of protein) were solubilized by incubation in 30 ml of 50 mM Tris acetate, pH 7.4/1% Triton X-100 for 1 hr at room temperature. The SM were then centrifuged at 105,000 × g for 30 min at 4°C, and the supernatant was incubated with Bio-Beads SM-2 (0.3 g/ml of supernatant) for 30 min at 4°C. This procedure reduced the concentration of Triton X-100 to 0.2%. The Bio-Beads were removed by filtration through cheesecloth. Protein concentrations were measured by a modification of the Lowry technique that minimized interference in the color development by Triton X-100 (7).

Binding Assays. (*i*) Binding of ^{125}I-labeled TSH to thyroid membranes was performed as described by Powell-Jones *et al.* (1). (*ii*) Binding to SM was measured as described by Cuatrecasas (8). ^{125}I-Labeled TSH (1–2 × 10^5 cpm per tube) was in-

Abbreviations: TSH, thyrotropin (thyroid-stimulating hormone); LH, luteinizing hormone; FSH, follicle-stimulating hormone; IU, international units; PPM, partially purified membranes; SM, solubilized membranes; Con A, concanavalin A; Con A-Seph, concanavalin A-Sepharose 4B; Con A-B, Con A bound proteins; Con A-U, Con A unbound proteins; PEG, polyethylene glycol.

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cubated with SM (≈30 μg per tube) for 1 hr at 22°C in a final volume of 250 μl of 50 mM Tris acetate (pH 7.4) containing 0.25% bovine serum albumin and 0.02–0.05% Triton X-100. After incubation, bound TSH was precipitated with polyethylene glycol (PEG) and separated by filtration on cellulose acetate filters (Millipore EHWP 025000). Nonspecific binding was determined in the presence of excess unlabeled hormone (0.2 IU/ml). All calculations assumed a M_r of 28,000 for TSH.

Results of apparent equilibrium studies were analyzed by the SCATFIT computer program, kindly provided by David Rodbard of the National Institutes of Health.

Concanavalin A-Sepharose 4B (Con A-Seph) Chromatography. SM (2 mg/ml) and Con A-Seph (0.5 mg/ml) were incubated in a 1:1 ratio (vol/vol) overnight at 4°C. This mixture was then poured into a 28 × 0.6 cm column, drained, and washed with 50 mM Tris acetate, pH 7.4/0.2% Triton X-100 until all of the unbound protein material, as determined by absorbance at 280 nm, was removed. The material collected in the pass-through fraction of the column was designated as the "Con A unbound" or "Con A-U" fraction. The column was then eluted with 0.0–1.0 M linear gradient of α-methyl-D-mannoside in 50 mM Tris acetate, pH 7.4/0.2% Triton X-100, and the fractions corresponding to the protein peak that eluted from the column were pooled, concentrated, and designated the "Con A bound" or "Con A-B" fraction.

Sepharose 6B Chromatography. A 30 × 1.6 cm column of Sepharose 6B was poured at 4°C and equilibrated with 50 mM Tris acetate, pH 7.4/0.25% bovine serum albumin/0.2% Triton X-100. One-milliliter aliquots of Con A-B and Con A-U fractions were prelabeled with ^{125}I-labeled TSH (5 × 10^5 cpm) in the presence and absence of excess unlabeled TSH and chromatographed separately on the same column. Aliquots (0.25 ml) of the resulting fractions were precipitated with PEG, and the radioactivity in the precipitates was determined. Specific binding was calculated by subtraction of nonspecific binding from total binding.

Sucrose Density Gradient Ultracentrifugation. Discontinuous sucrose density gradients were prepared from 5%, 10%, 20%, and 50% (wt/vol) sucrose in 50 mM Tris acetate, pH 8.1/1% Triton X-100. Gradients were prepared by layering 1 ml of each sucrose concentration in a 5.6-ml cellulose nitrate tube at 4°C. Samples (1 ml) were layered on top of the gradient and allowed to stand for 30 min at 4°C before centrifugation in a Beckman SW 50.1 rotor at 48,000 rpm for 2 hr at 4°C. In another set of experiments, samples (1 ml) were fractionated on 4-ml continuous gradients of 5–20% (wt/vol) sucrose in 50 mM Tris acetate, pH 8.1/1% Triton X-100 by centrifugation in an SW 50.1 rotor at 39,500 rpm for 12 hr at 4°C. Protein markers of known sedimentation coefficients were analyzed on parallel gradients. Fractions were collected from the bottom of the tubes. Aliquots (100 μl) were then assayed for specific binding of ^{125}I-labeled TSH at a final pH of 7.4.

RESULTS

Binding Properties of SM. PPM from porcine thyroids were solubilized with 1% Triton X-100, and the SM were recovered by centrifugation at 105,000 × *g* for 30 min. This fraction contained ≈50% of the total TSH binding activity and 50–75% of the total membrane proteins. Specific binding of ^{125}I-labeled TSH was not affected by 0.08–0.8% of Triton X-100. Scatchard analysis (9) of specific TSH binding to SM yielded a curvilinear plot with K_d (apparent equilibrium dissociation constant) values for the high and low affinity binding components similar to those observed in intact membranes (Fig. 1).

Con A-Seph Chromatography. Chromatography of the SM preparation on Con A-Seph resulted in the retention of 10–20% of the binding activity on the column. Fifty to seventy percent of the binding activity of SM did not bind to Con A-Seph and was found in the Con A-U fraction. After elution of the Con A-Seph column with an α-methyl-D-mannoside gradient, a peak of TSH binding activity (5–7% of total activity) was eluted at 0.3 M α-methyl-D-mannoside, and it is referred to as the Con A-B fraction.

Scatchard analysis of TSH binding to the Con A-U fraction exhibited a linear plot indicative of a single low affinity binding component with a K_d of 50–60 nM (Fig. 2*A*). This value is con-

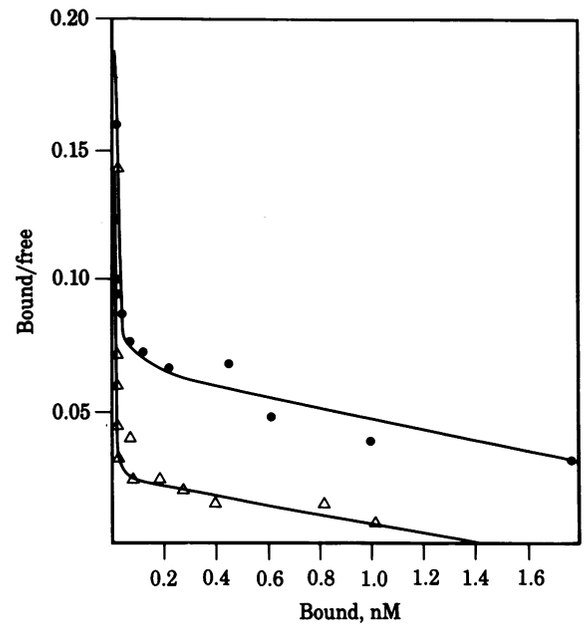

FIG. 1. Scatchard plots of specific TSH binding to PPM (●) and SM (△). PPM (160 μg/ml) and SM (120 μg/ml) were incubated with ^{125}I-labeled TSH (84 pM) and varying concentrations of unlabeled TSH (NIH-TSH-09) for 1 hr at 22°C in a final volume of 250 μl of 50 mM Tris acetate buffer, pH 7.4/0.25% bovine serum albumin. Incubations with SM also contained 0.02–0.05% Triton X-100. After precipitation of SM with 250 μl of 20% PEG, ^{125}I-labeled TSH bound to SM and PPM was separated from free TSH by filtration. Nonspecific binding was determined in the presence of excess unlabeled TSH (0.2 IU/ml). Radioactivity in the filters was counted and specific binding was calculated by subtracting nonspecific and filter binding.

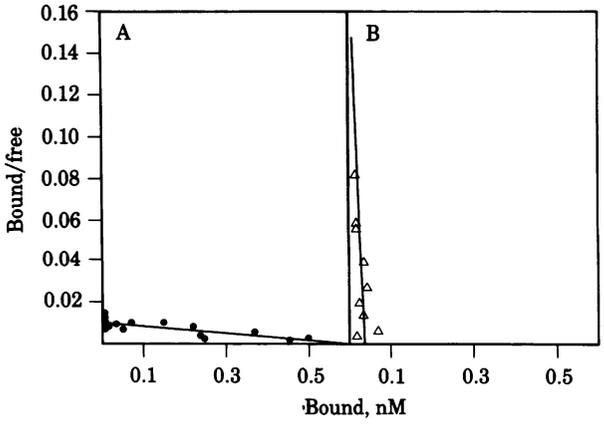

FIG. 2. Scatchard plots of TSH binding to the Con A-U (*A*) and to Con A-B fractions (*B*). Con A-U (0.65 mg/ml) and Con A-B (0.2 mg/ml) were each incubated with ^{125}I-labeled TSH and the same concentrations of unlabeled TSH for 1 hr at 22°C, as described for SM preparation in legend of Fig. 1. Note that in *B*, specific binding was negligible at TSH concentrations >1–2 nM.

sistent with that obtained for the low affinity component of the Scatchard plots of PPM and SM (Fig. 1).

Saturation analysis of TSH binding to pooled fractions from the Con A-B peak also resulted in a linear Scatchard plot (Fig. 2*B*). Computer analysis by the SCATFIT program showed the presence of a single, high affinity TSH binding site with an approximate K_d of 0.2 nM—virtually identical with that observed in PPM and SM (Fig. 1).

Sucrose Density Gradient Ultracentrifugation. Aliquots of SM, Con A-B, and Con A-U were subjected to discontinuous sucrose density gradient centrifugation, and specific binding of ^{125}I-labeled TSH was measured in each gradient fraction as described. As shown in Fig. 3*A*, the sedimentation profile of specific TSH binding to SM revealed two major binding species. The sedimentation profile of the Con A-U component on discontinuous sucrose gradient in the presence of 1% Triton X-100 exhibited a major binding peak that corresponded to the heavier peak of SM preparation, albeit with some contamination from the low M_r component (Fig. 3*B*).

Similar analysis of ^{125}I-labeled TSH binding components in the Con A-B fraction (Fig. 3*B*) showed the presence of a single specific binding peak with roughly the same sedimentation rate as the low M_r peak of the SM preparation. However, when an aliquot of Con A-B was fractionated on continuous 5–20% sucrose gradient, a heterogeneous population of binding components was detected. Three major specific binding peaks were measured, one of which corresponded to the binding peak observed on discontinuous sucrose gradients. The other two sedimented more slowly. There was no significant difference between gradients made in 0.2% or 1% Triton X-100 (data not shown). This latter experiment was repeated twice and similar peaks were observed.

Sepharose 6B Chromatography. When an aliquot from the Con A-U fraction was chromatographed on Sepharose 6B columns after binding with ^{125}I-labeled TSH, a major peak of bound radioactivity (as determined by PEG precipitation) eluted with the void volume (data not shown), and a free tracer peak eluted with an approximate K_{av} = 0.78. When Con A-U was prelabeled in the presence of excess unlabeled TSH, the void volume peak was reduced to about 20% of the total bound radioactivity, whereas the free tracer peak remained essentially unchanged.

Pooled fractions from the Con A-B peaks were labeled with ^{125}I-labeled TSH in the presence and absence of excess unlabeled TSH and were chromatographed on Sepharose 6B columns. Fractions (0.4 ml) were collected and total binding and nonspecific TSH binding were assayed by PEG precipitation. The elution profile of specific binding activity from one experiment is shown in Fig. 4. The ^{125}I-labeled TSH-binding protein complex was eluted as a major peak with an approximate K_{av} = 0.37. Four or five other binding peaks of varying M_r also appeared to be present. Comparison of the K_{av} value with those of reference proteins (10) indicated an apparent approximate Stokes radius of 67 Å for the major peak. Assuming that the receptor is not significantly asymmetrical or of unusual partial specific volume (8), the M_r of the 67-Å peak is around 300,000. These values are similar to those recently reported for the insulin receptor (8, 11).

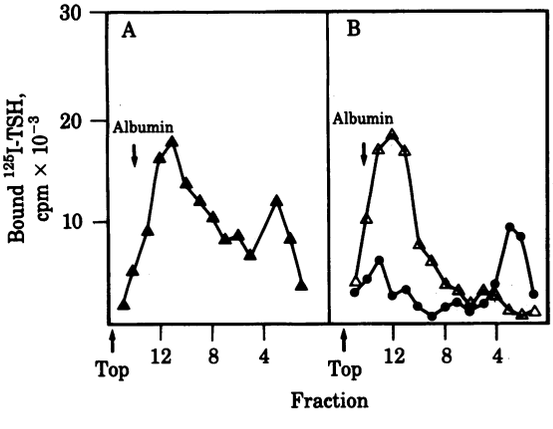

FIG. 3. Sucrose gradient centrifugation of SM (▲), Con A-B (△), and Con A-U (●) fractions. Samples (1 ml) of SM (2 mg of protein per ml), Con A-B (0.2 mg/ml), and Con A-U (0.65 mg/ml) were applied to a 5%, 10%, 20%, and 50% (wt/vol) discontinuous sucrose density gradient (pH 8.1), and centrifugation and ^{125}I-labeled TSH (^{125}I-TSH) binding were performed as described. Albumin, bovine serum albumin.

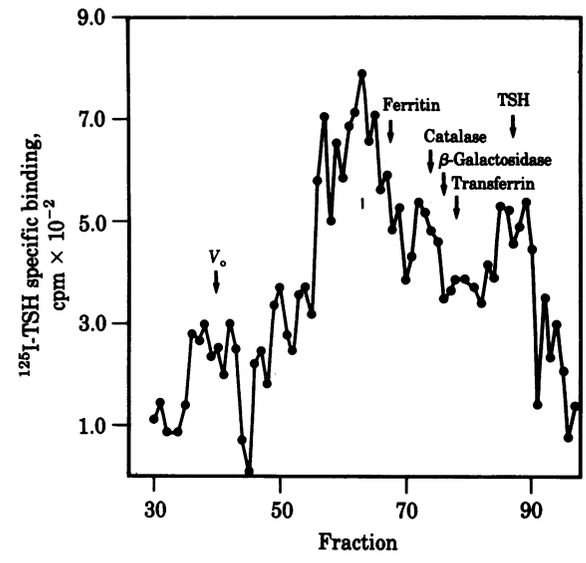

FIG. 4. Gel filtration on Sepharose 6B of Con A-B after binding to ^{125}I-labeled TSH. Pooled fractions of the Con A-B peak were rechromatographed on a 30 × 1.6 cm Sepharose 6B column and specific TSH binding was determined as described. The migration of protein markers was monitored by absorbance at 280 nm.

Properties of TSH Binding Components. *Hormone specificity.* Hormone specificity tests revealed that TSH binding to the high affinity binding species of the Con A-B fraction was highly specific, showing <0.01% crossreactivity with LH or FSH (Fig. 5*A*). In contrast, the low affinity component of the Con A-U fraction demonstrated a decreased hormone specific-

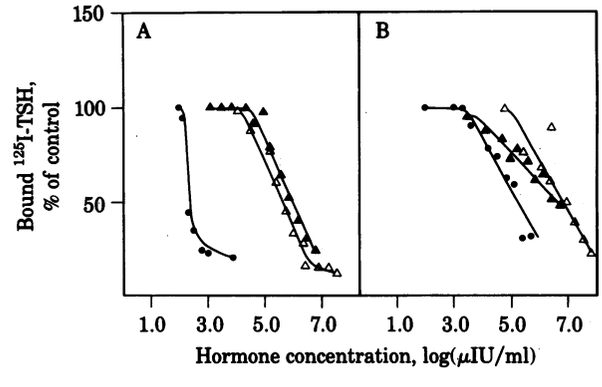

FIG. 5. Binding competition of LH and FSH for the binding of ^{125}I-labeled TSH to Con A-B (*A*) and Con A-U (*B*) fractions. Con A-U (0.65 mg of protein per ml) and Con A-B (0.2 mg/ml) were incubated with ^{125}I-labeled TSH (84 pM) in the presence of increasing concentrations of unlabeled TSH (●), FSH (▲), or LH (△) as described in legend of Fig. 1.

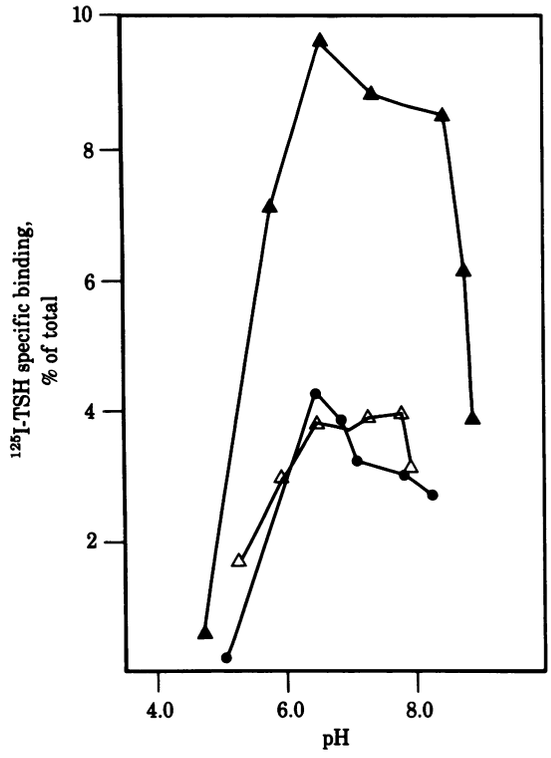

FIG. 6. Effects of pH on the binding of TSH to SM (▲), Con A-B (△), and Con A-U (●) fractions. Aliquots of each tissue preparation were incubated with ^{125}I-labeled TSH (84 pM) for 1 hr at 22°C over a pH range of 4.5 to 9.0. After precipitation with PEG, the samples were filtered and the radioactivity was counted. Each point is the mean of duplicate determinations.

ity, with 100-fold greater crossreactivity for LH or FSH (Fig. 5*B*). This is in agreement with previous studies of intact membranes in which the low affinity site exhibited lower hormone specificity for TSH and higher crossreactivity with other peptide hormones (1).

pH dependency. The effects of pH on the binding of ^{125}I-labeled TSH were determined for the SM preparation and for the high and low affinity binding components isolated from Con A-Seph chromatography (Fig. 6). The SM exhibited near maximal binding over a broad range of pH values, with a slight peak at pH 6.5. This is in contrast to the intact membranes, which have previously been shown to exhibit a very distinct maximum at pH 6.0 (unpublished data). The low affinity species showed a similar profile to the SM, although the peak is slightly more pronounced. The high affinity component also showed a broad peak; however, maximal binding was closer to pH 7.0.

Sensitivity to salts, temperature, or carbohydrate cleavage enzymes. Binding of ^{125}I-labeled TSH to PPM, SM, and Con A-Seph bound and unbound fractions was equally inhibited by the addition of increasing concentrations of $MgCl_2$ (10–150 mM) to the incubation buffer. However, binding to SM and Con A-Seph fractions was less sensitive to NaCl inhibition than was binding to PPM.

All three solubilized preparations exhibited similar patterns of inhibition of TSH binding with increasing temperatures (data not shown). Neither neuraminidase nor β-galactosidase had any significant effect on ^{125}I-labeled TSH binding to the Con A-Seph fractions.

DISCUSSION

These results demonstrate that TSH–receptor interaction in SM is a complex process characterized by nonlinear Scatchard plots (concave upward). Although initially attributed to the presence of heterogeneous binding sites (2), Kohn and Winand (12)—following the lead of DeMeyts *et al.* (13)—proposed that nonlinear Scatchard plots of TSH binding result from negatively cooperative site–site interactions among a homogeneous class of binding sites. Experimentally, a system is described as exhibiting negative cooperativity when the dissociation of bound radioactive hormone is accelerated in the presence of excess unlabeled hormone (13).

While confirming the original observation of accelerated dissociation by excess unlabeled hormone, we and others have presented evidence demonstrating that this kinetic criterion—upon which the negative cooperativity model is largely dependent—inadequately accommodates numerous features of the nonclassical binding of insulin (14), human growth hormone (15), TSH (3, 4), nerve growth factor (16), and β-adrenergic agonist (17). We have shown previously that the enhancement of dissociation of bound ^{125}I-labeled TSH by native hormone is not a valid demonstration of negative cooperativity because (*i*) it was observed in thyroid and nonthyroid plasma membranes that exhibited both linear and nonlinear Scatchard plots (1, 4); (*ii*) the kinetically determined K_d corresponded to a value obtained for the high affinity component of a nonlinear Scatchard plot rather than for the intrinsic affinity constant predicted by the negative cooperativity model (3); and (*iii*) the enhancement effect was observed not only when site occupancy was increased by excess native hormone but also under conditions in which site occupancy was decreased by the addition of native hormone (3). This prompted the return to the original interpretation of heterogeneous binding sites.

In this study, we present evidence for the physical separation of two distinct TSH binding components by chromatography on Con A-Seph columns, thus confirming the heterogeneity of TSH binding to thyroid plasma membranes. These two components (Con A-B and Con A-U) yielded linear Scatchard plots, with intrinsic K_d values similar to those of the high and low affinity TSH binding components observed in intact and solubilized membranes. In addition, both components were clearly separated by ultracentrifugation on discontinuous sucrose density gradients and by chromatography on Sepharose 6B. Similar findings have been reported recently by Herzberg *et al.* (18), who showed that concanavalin A (Con A) inhibited only the high affinity component of insulin binding to erythrocytes, presumably by selective interaction of the lectin with the high affinity receptor.

The data presented in Fig. 4 demonstrate that the Con A-Seph bound material consists of one major (Stokes radius, ≈67 Å) and several minor binding species of varying molecular sizes. Because detergents are required for solubilization of TSH binding components, it is expected that the apparent Stokes radii measured in this study represent those of the binding proteins plus an unknown quantity of bound detergent (8). When similarly solubilized and analyzed, many of the other peptide hormone receptors thus far examined have similar Stokes radii—ranging between 60 and 72 Å (8, 10, 19)—and many are composed of more than one peptide subunit. For example, Jacobs and Cuatrecasas (11) have reported recently that the insulin receptor—whose Stokes radius is ≈71 Å and M_r is 300,000—is composed of two M_r 135,000 and two M_r 90,000 peptides linked together by disulfide bonds and strong noncovalent interactions. On the other hand, Waters and Friesen (19) have reported that the nonprimate growth hormone receptor—whose Stokes radius is 62 Å and M_r is 300,000—is a tetrameric protein consisting of four M_r 75,000–80,000 peptides linked together by disulfide bonds. Although elucidation of the exact structure of the TSH receptor must await further analysis

(8, 10, 11), it is possible that the major peak observed on continuous 5–20% sucrose density gradients and on Sepharose 6B columns represents the native TSH receptor (M_r, 220,000–300,000) and the other peaks represent proteolytic fragments of the receptor (11, 20). Similar results have been reported previously by Tate *et al.* (20), who showed that the solubilized membrane preparation of bovine thyroid has a heterogeneous thyrotropin binding population with M_r of 286,000, 160,000, 75,000, and 15,000–30,000.

The Stokes radius, hormone specificity, and binding affinity data suggest that the high affinity component is the TSH receptor. Further support for this conclusion has come recently from studies of TSH binding to human thyroid adenoma and carcinoma (4, 5), in which it was shown that adenylate cyclase responds normally to TSH, even though only the high affinity component is present. A similar conclusion has also been reached in studies of insulin binding to monocytes of patients with congenital lipodystrophy (21), in which it was shown that insulin resistance was correlated with the absence of the high affinity sites, whereas the low affinity sites remained unchanged.

In conclusion, these results demonstrate that nonlinear Scatchard plots of TSH binding result from at least two distinct binding species rather than from negatively cooperative site–site interactions as previously hypothesized (13). Though it appears that the high affinity component is the receptor responsible for adenylate cyclase activation, neither the true identity nor the physiological role of the low affinity binding component is yet known. It is possible that the low affinity binding component represents (*i*) aggregates of the receptor formed *in vivo* as a result of clustering or self-aggregation of the receptor—a phenomenon similar to that recently demonstrated for insulin (22, 23); (*ii*) TSH-degrading enzymes; (*iii*) aggregates of the receptor formed as an artifact during solubilization (20); or (*iv*) a receptor–effector complex (24). The effector could be a component of the adenylate cyclase system or of some other system that mediates TSH action by messengers other than cAMP. Previous studies have suggested that there may be at least two distinct effects of TSH on thyroid membranes, including stimulation of ion flux (25) and increase of the turnover of phospholipids (26), which occur independently of cAMP. However, elucidation of these functions must await further purification and characterization of TSH binding species and studies of their possible roles in the action of TSH in a reconstituted system (27).

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