Purification of the Insulin Receptor from Human Placenta by Chromatography on Immobilized Wheat Germ Lectin and Receptor Antibody*

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Human placental membranes were labeled with Na$^{125}$I using lactoperoxidase, solubilized in 1% Triton X-100, and applied to a column of immobilized wheat germ lectin. After washing, 1 to 2% of the applied radioactivity could be eluted from the column with 0.3 M N-acetyl-d-glucosamine, of which 0.38 to 0.44% was immunoprecipitated by autoantibodies specific for the insulin receptor. Acrylamide-sodium dodecyl sulfate gel electrophoresis of the immunoprecipitates under reducing conditions and autodigestion resulted in three bands with apparent molecular weights of 126,000, 90,000, and 42,000. The elution of unlabeled membrane glycoproteins from wheat germ lectin resulted in an ~20-fold purification of the insulin receptor with near complete recovery, and exclusion of "insulinase" activity. The wheat germ eluate was passed through a column of immobilized normal IgG and then applied to a column of immobilized IgG containing antibodies to the insulin receptor. Insulin receptors desorbed from the antibody column with 2.5 M MgCl$_2$, pH 6.5, showed a further 20-fold increase in binding capacity and a 38-fold increase in immunoreactivity. However, these elution conditions were also shown to decrease irreversibly the binding capacity of crude receptors by up to 80%. Electrophoresis of the antibody-purified receptor revealed two major Coomassie-staining bands with apparent molecular weights of 126,000 and 42,000, and a minor band of 90,000 to 94,000 which may be a dimer of the $M_r = 42,000$ species. Assuming a molecular weight for the insulin-binding subunit of ~129,000, the antibody-purified receptor is ~20% functionally pure, which is substantially more than that achieved by conventional insulin affinity chromatography. Molecular purification may, however, be close to homogeneity given that the deleterious effect of MgCl$_2$, pH 6.5, leads to an underestimate of receptor activity and that only two or three major proteins are recovered. The molecular weights of these purified proteins are identical with those determined for insulin-binding subunits using affinity-labeling methods.

Purification of Triton-solubilized rat liver insulin receptors using a combination of DEAE-cellulose ion exchange chromatography and affinity chromatography on insulin-agarose was first reported by Cuatrecasas (1) and later by Jacobs et al. (2), but the functional purity and the yield of receptor appeared to be only a few per cent. The limited success in purifying a significant amount of functional receptor may be due to inherent problems with ligand-specific affinity chromatography. These include the necessity to use denaturants such as urea to achieve desorption (1-3), the co-purification of "insulinases," and possible irreversible binding or modification of receptor function (4). Heinrich et al. (5) recently obtained substantial purification of rat adipocyte insulin receptors by eluting chemically cross-linked insulin-receptor complexes from insulin antibodies. This approach, however, does not provide quantitative amounts of pure native receptor. The present study concerns the quantitative purification and characterization of the human placental insulin receptor after sequential affinity chromatography on wheat germ lectin and specific receptor antibody.

EXPERIMENTAL PROCEDURES

Solubilized Placental Insulin Receptors—Solubilized receptors were obtained by Triton X-100 extraction of crude microsomal membranes prepared from normal human full term placentae as described previously (6). In brief, villous tissues dissected from fresh placenta were thoroughly rinsed and homogenized with an Omni-mixer (Sorval) in 50 mM Heps' buffer, pH 7.6, containing 250 mM sucrose and 1 mM phenylmethylsulfonyl fluoride, a serine protease inhibitor. The homogenate was centrifuged at 600 × g, and the resulting supernatant was centrifuged at 12,000 × g. The supernatant was adjusted to 0.1 M NaCl and 0.2 mM MgCl$_2$ and centrifuged at 45,000 × g to obtain a microsomal membrane pellet. The membranes were washed three times with 50 mM Heps buffer, pH 7.6, and diluted to a final protein concentration of approximately 20 mg/ml. For solubilization, the membrane suspension was adjusted with 1% Triton (v/v), mixed for 45 min at 24°C, and centrifuged at 105,000 × g for 90 min at 4°C. The supernatant contained solubilized insulin receptors (recovery, 60 to 70%) with binding properties identical with those of membrane-bound receptors (6). The protein concentration of the solubilized fraction was 10 to 15 mg/ml. Its receptor-binding activity was stable for at least 4 months when stored at ~70°C. During subsequent purification steps, the solubilized membranes were supplemented with PMSF (1 mM) and aproamin (500 kallikrein inhibitor units/ml), although the latter protease inhibitor was later omitted from the final step because it was found to read in the fluorescamine assay for protein.

Insulin Binding Studies—Pork insulin was iodinated with carrier-free Na$^{125}$I to a specific activity of 120 to 140 μCi/μg, using a modified chloramine T method (7). Insulin binding to the solubilized receptor was measured at steady state in a standard competition assay, where

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1 The abbreviations used are: Heps, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; $R_b$, receptor-binding capacity estimated from the abcissca intercept of a Scatchard plot of competitive equilibrium binding data; SDS, sodium dodecyl sulfate.

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the binding of a fixed tracer amount of $^{125}$I-insulin (10,000 cpm; 0.04 ng) was measured in the presence of increasing amounts of unlabeled insulin. The residual radioactivity bound at the highest concentration of unlabeled insulin used (10,000 ng/ml) was taken to represent nonspecific binding and was always <15% of total tracer bound; this was subtracted from all other values to give specific binding. The total assay volume was 200 µl and contained 0.02 to 0.1% Triton and 0.1% bovine serum albumin in 50 mM Hepes buffer. The binding assays were carried out under steady state conditions at 4°C for 18 h, where binding affinity was optimum (minimum dissociation rate) and insulin-degrading activity was negligible (6). Receptor-bound $^{125}$I-insulin was separated from free $^{125}$I-insulin by the addition of cold polystyrene glycol 6,000 (final concentration, 12.5%, v/v) and carrier γ-globulin (final concentration, 0.05%, v/v). The mixture was agitated and, after standing for 10 min at 4°C, was centrifuged at 10,000 rpm in a Beckman Microfuge. The supernatants were aspirated and the pellets were counted in a γ counter. Alternatively, in some cases, the bound and free hormones were separated by adsorption of the free hormone onto charcoal. A suspension of activated charcoal in 0.1% Triton buffer was added at 4°C (final charcoal concentration, 2%) and the mixture was immediately centrifuged. The bound hormone remained in the supernatant and was identical with that determined in parallel by the polystyrene glycol method. Although only used in the later stages of these studies as a check against the polystyrene glycol method, charcoal separation is a simple and rapid procedure. Data from the binding studies were analyzed by the method of Scatchard (8) in order to obtain estimates of receptor binding capacity ($R_o$).

Insulin-degrading activity was assessed by measuring the radioactivity not precipitated by 5% trichloroacetic acid following incubation of $^{125}$I-insulin (325 pg/ml) with solubilized membrane fractions for 20 min at 37°C.

Receptor Immunoprecipitation and Radioimmunoassay—$^{125}$I-insulin receptor complexes were immunoprecipitated using receptor autoantibodies in the sera of two patients (B-2 and B-5) with a form of severe, insulin-resistant diabetes (9, 10). These antibodies have been shown to be specific for the insulin receptor by a number of criteria, including the ability to cause its selective precipitation (11). In the later stages of these studies as a check against the polystyrene glycol method, separation of receptor-bound antibody was determined by subtracting the radioactivity precipitated with antireceptor sera in the presence of 1 mM PMSF. This suspension was incubated with shaking for 2 h at 24°C, and then for 16 h at 4°C. The beads were pelleted by low speed centrifugation and incubated for a further 2 h at 24°C with 1 mM ethanoloamine to block unreactive groups. They were then washed five times, alternately with 0.1 M Na acetate, 0.5 M NaCl, pH 4.5, and 0.1 M Na borate, 0.5 M NaCl, pH 8.5, and finally washed and stored in 50 mM Hepes buffer, 0.1% Triton, 1 mM Na azide, pH 7.6. From the radioactivity present in an aliquot of the washed beads, the coupling efficiency was determined routinely to be >80%. The binding capacity of the coupled IgG was determined by direct "solid phase" immunoprecipitation of $^{125}$I-insulin-receptor complexes using the same conditions described above for indirect immunoprecipitation with second antibody. When soluble IgG was tested in the latter procedure, 200 µg precipitated all the insulin-binding sites in 1 ml of crude solubilized membranes, whereas approximately 570 µg of the bound IgG was required in the solid phase procedure. This represents a 85% loss of antireceptor activity upon coupling to Sepharose.

The sequential scheme used for the purification of the insulin receptor is shown in Fig. 1. A 10-ml column of wheat germ-Sepharose was washed and equilibrated with 50 mM Hepes buffer, 10 mM MgCl₂, 0.1% Triton, pH 7.6. Placental membranes solubilized in 1% Triton, usually in a volume of 10 ml, were diluted 2-fold with 50 mM Hepes buffer and recycled four times over the wheat germ column at 24°C. The final flow-through was retained and the column was washed with 200 ml of 50 mM Hepes buffer, 150 mM sodium chloride, 0.1% Triton, pH 7. The glycoproteins bound to wheat germ were eluted with 16 ml of 0.3 M N-acetyl-D-glucosamine in 0.025% Triton. The eluate was dialyzed against 50 mM Hepes buffer, 0.025% Triton, pH 7.6, and concentrated to 4 ml on an Amicon PM-10 membrane. No measurable transfer of Triton occurred during dialysis (estimated by absorption at 274 nm and by the distribution of [1H]Triton) and the final Triton concentration was therefore assumed to be 0.1%. Four milliliters of the wheat germ column eluate were recycled four times over a column of 5 ml of normal IgG-Sepharose at 4°C, and the column was allowed to drain. The flow-through was recycled four times over a 5-ml column of antireceptor-IgG-Sepharose which had been pre-washed with 50 ml of the eluting reagent 2.5 M MgCl₂, 0.2 M Na borate, 0.1% Triton, pH 6.5, and, just prior to use, with 250 ml of 50

![](Fig_1.png)

**Fig. 1. Sequential scheme used for insulin receptor purification.** Details are given under "Experimental Procedures."
mm Hepes buffer, 0.1% Triton, pH 7.6. After recycling the solubilized membranes, the antibody column was washed with 100 ml of 50 mM Hepes buffer, 1 M NaCl, 0.1% Triton, pH 7.6, and then treated with 15 ml of the 2.5 M MgCl₂ eluting reagent. The eluate was immediately dialyzed against 50 mM Hepes buffer, 0.1% Triton, pH 7.6, and finally concentrated to 3 ml.

The choice of MgCl₂ as the eluting reagent was made after comparison with a number of other chaotropic salts, neutral polar compounds, acids, and bases. These included 0.5 M potassium chloride, 0.5 M sodium perchlorate, 1.0 to 2.0 M lithium chloride, 1.0 M sodium citrate, 1.0 to 2.0 M sodium thiocyanate, 1.0 M sodium iodide, 0.5 to 1.0 M acetic acid, 0.1 to 0.5 M ammonium hydroxide, 6.0 M guanidine, 2.0 to 4.0 M urea, 2.0 M choline chloride, 0.05 M diethylamine, pH 11.5, and 0.5 to 2.0 M sodium chloride ± 0.1 M EDTA. All except sodium chloride ± EDTA were capable of dissociating, at least partially, the receptor-antibody complex, but MgCl₂ had the least (irreversible) effect on insulin binding (see under "Results"). Protein was determined against bovine serum albumin standards by the fluorescamine (Fluram) method (15). Aprotinin gave a reading in the assay (1 kallikrein inhibitor unit = 1 μg of bovine serum albumin), but Hepes, Triton X-100, and PMSF did not.

**RESULTS**

125I-labeled placental membranes were solubilized in Triton and chromatographed on a wheat germ lectin column. After extensive washing, 1 to 2% of the radioactivity applied was eluted with 0.3 M N-acetyl-D-glucosamine. The fraction of radioactivity specifically precipitated from the wheat germ column eluate by antireceptor serum (B-2 or B-5) was 0.38 to 0.44%. Acrylamide-SDS gel electrophoresis and autoradiography of immune precipitates revealed three major bands with molecular weights of 126,000, 90,000, and 42,000, and a variable minor specific band of 116,000 (Fig. 2). After immunoprecipitation with control sera, a minor band of 42,000 was also sometimes observed (Fig. 2). However, we believe this to be nonspecific, because it was seen at the front boundary of reduced IgG (heavy chain) only when a large amount of IgG was present in the immune pellet.

Preliminary studies were performed to define the optimum conditions for quantitative elution of receptor from antibody columns. Insulin-binding activity could be recovered from antibody affinity columns using a variety of reagents (see under "Experimental Procedures"). Thus, as shown in Fig. 3, elution with 1.0 M acetic acid, 0.1% Triton, pH 4.0, was associated with a significant increase (~60-fold) in specific activity. However, exposure to all the reagents tested also led to a marked decrease in the insulin-binding capacity of the receptor. Following a 10-min exposure to 0.5 or 1.0 M acetic acid in 0.1% Triton and immediate neutralization with Tris, or dialysis against 50 mM Hepes buffer, pH 7.6, the binding capacity of the crude solubilized receptor decreased by 80 and 90%, respectively. Irreversible denaturation (80% decrease in binding capacity) was also seen after rapid (<3 min) elution of antibody columns with 1 ml aliquots of 1 M acetic acid, 0.1% Triton, pH 4.0, collected directly into 0.35 ml of 2 M Tris for immediate neutralization. Of the various reagents tested, 4.5 M urea, pH 7.3, and 2.5 M MgCl₂, pH 6.5, had the least effect on the binding properties of the receptor (Fig. 4). Even so, binding capacity, estimated from the abscissa intercept (R₀) of the Scatchard plot (Fig. 4, inset) was decreased by up to 80%. In addition, there was an increase in the binding affinity of the receptor as reflected by the leftward shift of the competition curve and by the increase in slope of the Scatchard plot. MgCl₂ (2.5 M), 0.1% Triton, adjusted to pH 6.5 with 0.2 M Na borate, was used for eluting the antibody affinity column in all subsequent experiments.

The results of the sequential purification scheme are summarized in Table I, and the binding curves for the purified receptor are shown in Fig. 5. The wheat germ step gave a high recovery (>75%), removed the majority (95 to 97%) of nonreceptor proteins, and allowed the receptor to be concentrated within a small fraction of glycoproteins. A further increase in receptor specific activity was not achieved by repeated desorption from wheat germ-agarose. As shown in Table II, the wheat germ step also removed insulinase activity that was present in the crude solubilized membrane.

The wheat germ eluate was passed through a column of normal IgG to control for nonspecific and specific (Fc receptor) adsorption of solubilized membrane components. There was a 25 to 30% loss of both protein and binding activity in the filtrate from the normal IgG column, and consequently no change in the specific activity of the receptor (data not shown). The losses in activity were almost entirely accounted for by nonspecific retention and trapping, and were recovered
after washing the column with 50 mM Hepes buffer, 1 mM NaCl, 0.1% Triton, pH 7.6.

The receptor that eluted with 2.5 mM MgCl₂ from the antibody column was enriched a further 20-fold in insulin-binding capacity and a further 38-fold in immunoreactivity (Fig. 4; Table 1). Note also that the apparent affinity of the antibody-purified receptor was increased 3.7-fold over the starting material; its binding curve (Fig. 5) resembled that seen after exposure of the crude solubilized receptor to the MgCl₂ eluant (Fig. 4). Failure of the antibody column to bind all of the applied receptor appeared to be due to "overloading"; the receptor in the flow-through had the same affinity as did that in the starting material and could subsequently be bound to a regenerated column, thereby excluding the possibility that the receptors were immunologically heterogeneous. In contrast to the wheat germ-purified membranes, antibody column eluates contained no receptor-binding activity for either multiplication-stimulating activity (courtesy of Dr. Matthew Rechler, National Institutes of Health, Bethesda, Md.) or somatostatin C (courtesy of Dr. Richard Furlanetto, University of Texas, Galveston, Tex.) (data not shown).

Analysis of both B-2 and B-5 antibody-purified receptors in SDS-polyacrylamide gel electrophoresis under reducing conditions in the presence of PMSF and aprotinin revealed major protein bands with molecular weights of 126,000 and 42,000, and a variable band of 90,000 to 94,000, all clearly purified when compared to the pattern in the wheat germ-agarose eluate. In addition, a band of low molecular weight was observed migrating just behind the dye front, but this has not been analyzed further (Fig. 6).

DISCUSSION

An important step toward understanding the molecular basis of insulin action will be the chemical and structural characterization of the insulin receptor. Unfortunately, the purification of receptors is hampered by several factors, including a relatively low concentration in the starting material.

![Fig. 3. Elution of insulin receptors from a receptor antibody affinity column using 1 M acetic acid. IgG, purified on protein A-Sepharose from antireceptor serum B-5, was coupled to Sepharose 4B as described under "Experimental Procedures." Crude solubilized placental membranes (9.8 mg) diluted to 0.1% Triton in 50 mM Hepes buffer, pH 7.6, were recycled four times over a 2-ml antibody column at 4°C. At A, the column was treated with 10 volumes of 50 mM Hepes buffer, 0.5 mM NaCl, 0.1% Triton, and B, the column was treated with 1 M acetic acid, 0.5 mM NaCl, 0.1% Triton, pH 4.0. Fractions (1 ml) were collected and the acidic fractions were immediately neutralized by the addition of 0.35 ml of 2 M Tris. Insulin receptors were assayed in each fraction by determining specifically-bound ¹²⁵I-insulin, separated using polyethylene glycol or by indirect immunoprecipitation with antireceptor serum B-2, as described under "Experimental Procedures."](image)

![Fig. 4. Insulin binding to unpurified solubilized placental receptors briefly exposed to conditions used for eluting receptor from antibody affinity columns. Solubilized placental membranes in 50 mM Hepes, 0.1% Triton were incubated for 10 min at 4°C with the reagent as indicated, then immediately dialyzed against 50 mM Hepes, 0.1% Triton, pH 7.6. Equilibrium competition binding assays were performed in 0.05% Triton for 18 h at 4°C as described under "Experimental Procedures." Inset: Scatchard plots of competition binding data.](image)

**TABLE 1**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total protein</th>
<th>Bound/free ¹²⁵I-insulin</th>
<th>Insulin binding capacity (Ro)</th>
<th>Purification</th>
<th>Total yield</th>
<th>Receptor recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude solubilized placental membrane</td>
<td>300 (100%)</td>
<td>1.6</td>
<td>12</td>
<td>1-fold</td>
<td>3600</td>
<td>100</td>
</tr>
<tr>
<td>Wheat germ eluate</td>
<td>13.5 (4.5%)</td>
<td>29</td>
<td>200</td>
<td>17</td>
<td>2700</td>
<td>75</td>
</tr>
<tr>
<td>Receptor antibody eluate</td>
<td>0.189 (0.06%)</td>
<td>1550</td>
<td>4000</td>
<td>333</td>
<td>756</td>
<td>21</td>
</tr>
</tbody>
</table>

* From Scatchard analysis of competitive binding assays.

* From competition against crude placental receptor for binding to receptor antibodies (see under "Experimental Procedures" and Ref. 12).

* Unlabeled insulin concentration required for half-maximal displacement of ¹²⁵I-insulin in competitive binding assays.
Insulin Receptor Purification

**Fig. 5.** Insulin binding to solubilized placental receptors after purification on wheat germ lectin and receptor antibody affinity columns. Equilibrium competition binding assays (A) were performed in 0.05% Triton for 18 h at 4°C and the data were expressed in the form of Scatchard plots (B), as described under "Experimental Procedures."

**TABLE II**
Specific binding and degradation of $^{125}$I-insulin by solubilized placental membranes during purification of the insulin receptor

<table>
<thead>
<tr>
<th>Protein concentration</th>
<th>Specifically bound $^{125}$I-insulin</th>
<th>Bound/$^{125}$I-insulin</th>
<th>Free $^{125}$I-insulin soluble in trichloroacetic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/200 µl assay</td>
<td>cpm/µg</td>
<td>µg/µg</td>
<td>%</td>
</tr>
<tr>
<td>Solubilized placental membranes</td>
<td>196</td>
<td>6</td>
<td>0.33</td>
</tr>
<tr>
<td>Wheat germ eluate</td>
<td>9</td>
<td>89</td>
<td>5.5</td>
</tr>
<tr>
<td>Receptor antibody eluate</td>
<td>2</td>
<td>1084</td>
<td>68.5</td>
</tr>
</tbody>
</table>

* The binding assay was performed for 30 min at 37°C by incubating $^{125}$I-insulin (17,000 cpm; 0.07 ng) and the indicated amounts of protein in a final volume of 200 µl of 50 mM Hepes buffer, pH 7.6, containing 0.1% Triton (v/v). Replicates containing excess unlabeled insulin (10 µg) were included to determine nonspecific binding. Bound $^{125}$I-insulin was precipitated with γ-globulin/polyethylene glycol as described under "Experimental Procedures."

* Degradation of the free $^{125}$I-insulin was estimated as the percentage of radioactivity in the polyethylene glycol supernatant not precipitated by 5% trichloroacetic acid.

and the difficulty of maintaining the integrity and function of these glycoproteins outside their normal phospholipid domain. Despite the report by Cuatrecasas in 1972 (1) describing purification of the rat liver insulin receptor by insulin-agarose affinity chromatography, there has been little progress in this field. Jacobs *et al.* (2) subsequently reported that about 200 µg of receptor could be purified from 50 rat livers by desorption from insulin-agarose with 4.5 M urea, resulting in one major band with a molecular weight of 135,000 in SDS-polyacrylamide gel electrophoresis. They did not present a detailed quantitative analysis of the binding properties of the purified receptor, but stated that it had a specific binding activity of 400 pmol/mg; this represents 5% purity assuming a molecular weight of 130,000 daltons. When samples of this receptor (courtesy of Dr. Steven Jacobs, Wellcome Research Laboratories, Research Triangle Park, N. C.) were studied by us in the radioimmunoassay (12), it was found to be only 1 to 2% pure despite the appearance of the single major band in SDS-polyacrylamide gel electrophoresis. The most likely explanation for this discrepancy is denaturation and loss of activity following desorption with urea (see Fig. 4).

After a number of unsuccessful attempts to improve on the results with the insulin affinity method, and in an attempt to
circumvent problems inherent in ligand-specific affinity chromatography, we adopted the scheme described here. The ability of wheat germ agglutinin to bind the insulin receptor was first described by Cuatrecasas (17) but has not been previously exploited for quantitative purification. Some investigators have employed concanavalin A (2, 3), but with this lectin, only about one-third of the bound receptor can be desorbed by sugar. The receptor can be recovered from wheat germ without loss and purified 15- to 30-fold by desorption with N-acetyl-D-glucosamine. This simple step also separates the binding site from insulinase activity (co-purification of nonspecific and specific immunoglobulins is one potential problem with insulin affinity chromatography). The antibodies used for the subsequent immunofinity purification were purified from the sera of patients with a form of severe insulin resistance (9, 10) and have been shown by several criteria to be highly specific (18). They specifically inhibit the binding of insulin to its receptors in a wide variety of tissues and species, and specifically immunoprecipitate insulin receptors but not related growth factor receptors, as evidenced here by the separation of insulin receptors from migration-stimulating activity receptors and somatomedin C receptors on immunofinity chromatography. Most importantly, we have recently shown that the antibodies recognize the same subunits of the insulin receptor that can be covalently linked with [125I]-insulin (19). Indeed, these are identical with the $M_\text{r}$ = 126,000 and 90,000 components isolated in the present study by immunoprecipitation of [125I]-labeled insulin receptors.

After desorption from an antibody affinity column with MgCl₂, the insulin receptor had a specific insulin-binding activity of at least 4,000 ng/mg (~690 pmol/mg). This value appears to be an underestimate, since radioimmunoassay, which measures the receptor molecule independently of its binding function, indicated a value at least 2-fold higher, i.e. 8,640 ng/mg (1,490 pmol/mg). It might be argued that immunoassay is not a valid assay of receptor purity since the antibodies themselves were used to purify the receptor. The same argument, however, could be applied to the criterion of insulin binding after purification on insulin-agarose. We would argue that immunoassay is not only valid (based on the specificity of the antibodies) but is a more appropriate criterion, since it has been shown to measure the receptor when insulin binding has been compromised, e.g. by tryptophan digestion (12). The difference here between binding activity and immunoreactivity presumably reflects the relatively greater susceptibility of binding to the effects of 2.5 M MgCl₂, pH 6.5. It should be noted that, irrespective of which antibody (B-2 or B-5) was used for purification or immunoassay, the purification results (Table 1) were the same.

The deleterious effects of the eluant on binding were demonstrated in control experiments on crude receptor, where brief exposure resulted in a marked decrease in binding capacity and an increase in affinity (Fig. 4). The Scatchard plot is predominantly linear, in contrast to the curvilinear profile of the crude receptor. De Meyts et al. (20) have shown by kinetic studies that the curvilinear Scatchard plot normally obtained for equilibration insulin binding is consistent with the presence of negative cooperativity. In applying these kinetic criteria, we previously confirmed that increased saturation of the crude solubilized placental receptors accelerated the dissociation rate of prebound labeled insulin (6). The negative cooperativity model predicts the highest affinity of the receptor in its empty state, or $K_e$. The high affinity state seen here after exposure to denaturants exceeds $K_e$, thus indicating the existence of other types of affinity regulation apart from negative cooperativity. Maturow and Hadden (3) also reported that rat liver receptors desorbed from insulin-agarose with 4 M urea exhibited a single order of affinity and claimed to show that the crude receptor contains a glycoprotein which interacts with the binding site to account for its complex binding isotherm. In studies of receptor binding in situ after radiation inactivation, Harmon et al. (21) suggest that affinity is regulated by association of the binding site(s) with a larger molecule ("affinity regulator"). It should not be assumed, however, that we have specifically purified a "high affinity" binding site or have physically separated the recognition subunit from a regulator subunit, since an increase in affinity also accompanied the loss of total binding capacity after simply exposing the crude receptor to a number of denaturants.

The increased affinity of the receptor desorbed from antibody is reflected by the relatively greater increase in the bound/free ratio of [125I]-insulin compared to the increase in binding capacity or receptor concentration ($R_0$). Nevertheless, it is apparent that, because of the sensitivity of the receptor to denaturation during elution, the specific activity parameters obtained may underestimate the degree of purification. A number of other factors are also relevant in this regard. For example, it was observed that the half-life of the binding activity of the purified receptor at 4°C was only several days, and that freezing (or thawing) accelerated inactivation. We have also observed a substantial loss of the purified protein after its exposure to plastic or filters, that was not reduced by the addition of glycerol, sucrose, or ethylene glycol in concentrations up to 20%. Finally, since an estimate of purification is critically dependent on protein concentration, it should be pointed out that fluoroscin determinations, while sensitive and precise, are nonspecific and simply reflect the concentration of free amino groups relative to a standard protein, i.e. bovine serum albumin.

The antibody-purified human placental receptor appears to have a specific activity nearly 4-fold greater than reported for the rat liver receptor desorbed from insulin-agarose by 4.5 M urea (2). Based on a molecular weight of 126,000 for the insulin-binding subunit, the antibody-purified receptor is approximately 20% pure. However, in view of the 5-fold decrease in binding capacity after exposure of receptor to the MgCl₂ eluant (Fig. 4), its purity may be closer to homogeneity. This is supported by the finding of only two or three bands after immunoprecipitation of [125I]-labeled proteins or desorption of protein from antibody columns. The component that always purified from the wheat germ eluate in the presence of proteolytic inhibitors has a molecular weight of 126,000. While we have no direct proof that this "heavy chain" is the main subunit of the insulin receptor, its molecular weight is similar to that of the covalently linked [125I]-insulin-receptor complex (5, 19, 22-26) and the rat liver protein purified by insulin affinity chromatography (2, 23). A minor band of $M_\text{r}$ = 116,000 was sometimes present (Fig. 2); this corresponds to the asialo derivative of the $M_\text{r}$ = 126,000 species which has been observed after neuraminidase treatment of [125I]-labeled lymphocytes. In addition, we observed two lower molecular weight bands. That of $M_\text{r}$ = 90,000 was always seen after immunoprecipitation of [125I]-labeled membranes, but on Coomassie staining of antibody column eluates, it was much weaker and was associated with a more intense band of molecular weight 42,000. Bands of $M_\text{r}$ = 90,000 and ~45,000, as well as the $M_\text{r}$ = 130,000 band, have been reported by others under reducing conditions.

cyte membranes (both the $M_1 = 130,000$ and $90,000$ components were precipitated by receptor autoantibodies). In more recent studies of insulin-agarose purified rat liver receptors, Jacobs et al. (23) found $^{125}$I-labeled and Coomassie-stained proteins with molecular weights of 130,000, 90,000, and 42,000 to 45,000, remarkably similar to those seen here; their results from peptide mapping suggest that the $M_1 = 45,000$ species is not a degradation product of the $M_1 = 135,000$ species (27).

The reason for the variability of the $M_1 = 90,000$ and 42,000 bands is not yet clear. Both could represent degradation products of the $M_1 = 130,000$ protein although the results of Jacobs et al. (27) are against this. The consistent appearance of a specific $M_1 = 90,000$ band after covalent labeling with $^{125}$I-insulin (19, 24) suggests that it also may be an authentic subunit of the receptor. Also, we have recently shown a coordinate loss of both the 90,000 and 42,000 bands is not yet clear. Both could represent degradation products of the $M_1 = 130,000$ species after boiling the immune pellets in SDS, and treated and analyzed as in the preliminary experiments in our laboratory indicate that binding function can be preserved by employing monoclonal antibody fragments, whose lower affinity allows receptors to be eluted using non-denaturing conditions.

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