antibodies can be readily generated against such hapten groups, this would provide a simple procedure to isolate the receptor protein by affinity chromatography.

Although both [2,4-NAPS-Trp^9][^{3}H]ACTH–BSA and [2,5-NAPS-Trp^9][^{3}H]ACTH–BSA could be cleaved at the thioether link between the Trp residue of the hormone and the NAPS group (Figure 4) by thiolysis, the reaction was incomplete. Reduction of the disulfide link between the Trp residue of ACTH and the photoreactive group [2,4-NAPSS-Trp^9]-ACTH–BSA proceeded more smoothly, resulting in complete reaction in 6 h. This result eliminates the need for radioactive ligands in photoaffinity labeling. Thus, receptor protein(s) may be covalently labeled with nonradioactive NAPSS derivatives of tryptophan- or cysteine-containing ligands, subjected to reduction with thiols, and then localized on Na-DodSO$_4$ gels or columns by reaction with specific antibodies raised against the haptenic group, 2-nitro-4-(acetylamino)-phenyl. Radioactive protein A may then be used to locate the sites of antibody binding as described by Adair et al. (1978).

Acknowledgments

We thank Professor C. H. Li for his interest.

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Binding of Insulin Receptors to Lectins: Evidence for Common Carbohydrate Determinants on Several Membrane Receptors†

José A. Hedo,* Len C. Harrison, and Jesse Roth

ABSTRACT: Insulin receptors from human placenta and from cultured lymphocytes (IM-9 line) were solubilized in Triton X-100 and applied to agarose columns containing 12 different immobilized lectins. Receptors from both tissues were adsorbed by lectins that bind *N*-acetylglucosamine (wheat germ), mannose (concanavalin A, lentil, and pea), and galactose (ricins I and II) but were unretained by lectins that bind *N*-acetylgalactosamine (horse gram, *S. Japonica*, and soybean), fucose (gorse seed I), and galactose (*B. simplicifolia* and peanut). After desorption with the appropriate monosaccharides, the insulin-binding capacity of the receptor was increased between 5- and 50-fold with recoveries ranging from 7% to 98%. However, when the solubilized membranes from both tissues were chromatographed sequentially on three different lectin columns (e.g., wheat germ, lentil, and ricin I), the receptor showed only a minor increase in purity after elution from the second and third columns. Receptors for multiplication-stimulating activity (MSA) and epidermal growth factor (EGF) in the solubilized placental membranes behaved very similarly to insulin receptors on sequential lectin chromatography. On the other hand, elution from the lectin columns was followed by a clear increase in the affinity of the receptors as evidenced by (1) a decrease in the concentration of unlabeled insulin, causing half-maximal reduction of [^{125}I]insulin binding, and (2) an increase in bound/free [^{125}I]insulin (tracer binding) greater than the increase in binding capacity (saturation binding). This affinity shift was progressive on sequential lectin chromatography. Our findings indicate that the carbohydrate moiety of the insulin receptor contains *N*-acetylglucosamine, mannose, and galactose but that these saccharide residues are neither receptor nor tissue specific. The increase in affinity of the insulin receptor after its desorption from lectins may be due to separation from the binding site of an associated affinity regulator (inhibitor).

The insulin receptor is an integral membrane protein insofar as detergents are required for its solubilization (Cuatrecasas, 1972). While the binding properties of the membrane-bound and detergent-solubulized receptor are identical and have been defined in considerable detail (Harrison et al., 1978), little is known about its chemical structure. Affinity chromatography on insulin agarose is the logical approach to purification of the insulin receptor. However, this technique gives a poor recovery of functional receptor (Jacobs et al., 1977), in large part due to the fact that insulin affinity columns require harsh denaturing conditions to elute bound receptor.

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Chromatography on immobilized lectins has been employed extensively for the purification of membrane glycoproteins, and lectin columns can be eluted with simple saccharides without changes in pH or ionic strength or the use of denaturants (Lotan & Nicolson, 1979). The insulin receptor is assumed to be a glycoprotein since the lectins concanavalin A and wheat germ agglutinin alter the binding of insulin and exhibit insulin-like effects (Cuatrecasas & Tell, 1973). In addition, the Triton-solubilized receptor from rat liver can be adsorbed to agarose derivatives of both lectins (Cuatrecasas & Tell, 1973).

We therefore reasoned that sequential chromatography on a series of different lectin columns would provide a simple scheme for the purification of functional insulin receptors. The present report is a systematic study of the interaction of the solubilized insulin receptor from two different human tissues, placenta and cultured IM-9 lymphocytes, with a panel of 12 lectins. Six lectins bound the receptor and resulted in its significant purification, but sequential lectin chromatography did not result in further purification of either placental or lymphocyte insulin receptor. A similar result was obtained with receptors for multiplication-stimulating activity (MSA)[1] and epidermal growth factor (EGF). The potential usefulness of sequential lectin chromatography therefore appears to be limited by the fact that a number of membrane glycoproteins, including the insulin receptor, share several common carbohydrate determinants whose saccharide specificities are neither receptor nor tissue specific.

Experimental Procedures

Preparation of Solubilized Membranes. Human placentae were collected after normal full-term deliveries, washed in 0.3 M sucrose and 1 mM PMSF[1] (phenylmethanesulfonyl fluoride), and homogenized in an Omnimixer (Sorvall) as previously described (Harrison et al., 1978). After differential centrifugation, a 45000*g* microsomal pellet was obtained and suspended in 50 mM Na Hepes[1] [4-(2-hydroxyethyl)-1-piperazineethanesulfonate] and 10 mM Mg_2SO_4, pH 7.6, at a final protein concentration of 11–14 mg/mL. The suspension was then continuously stirred for 1 h in 1% (v/v) Triton X-100 at 22 °C. Undissolved material, pelleted by centrifugation at 200000*g* for 90 min, was discarded; the supernatants, which contained 50–60% of the protein, were stored at −70 °C.

Human lymphocytes of the IM-9 line were grown at 37 °C in continuous culture (Fahey et al., 1971). The cells were suspended in 40% sucrose, 50 mM Hepes, and 1 mM PMSF, pH 7.6, and 4 °C and ruptured by means of a hand-held glass homogenizer. A 20000*g* crude membrane fraction obtained by differential centrifugation (Lang et al., 1980) was suspended in 50 mM Hepes, 10 mM Mg_2SO_4, and 1 mM PMSF, pH 7.6, at a final protein concentration of 4–5 mg/mL and solubilized in 1% Triton X-100 with continuous stirring for 1 h at 22 °C. After centrifugation at 200000*g* for 90 min at 4 °C, the supernatants containing ~70% of the protein were immediately frozen and stored at −70 °C.

The protein concentration in the solubilized preparations was determined in the presence of Triton X-100 by the fluorescamine method (Udenfriend et al., 1972) using bovine serum albumin as standard.

Chart I: Lectins and the Corresponding Inhibitor Monosaccharides Used in this Study

LECTIN	MONOSACCHARIDE
Concanavalin A	α-methyl-mannopyranoside
Lens Culinaris Agglutinin (Lentil)	α-methyl-mannopyranoside
Pisum Sativum Agglutinin (Pea)	α-methyl-mannopyranoside
Wheat Germ Agglutinin	N-acetyl-glucosamine
Ricinus Communis Agglutinin I (Ricin I, $Ricin_{120}$)	β-methyl-galactopyranoside
Ricinus Communis Agglutinin II (Ricin II, $Ricin_{60}$)	β-methyl-galactopyranoside
Bandieraea Simplicifolia Lectin	α-methyl-galactopyranoside
Peanut Agglutinin	α-methyl-galactopyranoside
Dolichus Biflorus Agglutinin (Horse Gram)	N-acetyl-galactosamine
Sophora Japonica Agglutinin	N-acetyl-galactosamine
Soybean Agglutinin	N-acetyl-galactosamine
Ulex Europaeus Agglutinin I (Gorse Seed)	L-fucose

Lectin Chromatography. Free lectins were purchased from Sigma (St. Louis, MO) and from Vector Laboratories (Burlingame, CA). Lectins coupled to agarose beads were obtained from Vector Laboratories. Some batches of immobilized wheat germ, lentil, and ricin I were purchased from Miles Laboratories (Elkhart, IN). The amount of lectin coupled varied between 1 and 5 mg of protein per mL of settled gel. The 12 lectins employed and the monosaccharides used to compete for binding to these lectins are listed in Chart I.

In the individual experiments, 1 mL of lectin–agarose that had been stored at 4 °C in the appropriate monosaccharide solution (0.3 M) was packed into columns 0.9 cm in diameter and washed at room temperature with 10 mL of 0.15 M NaCl, 50 mM Hepes, 0.1% Triton X-100, and 0.01% sodium dodecyl sulfate ($NaDodSO_4$), pH 7.6. Subsequently, the columns were washed with 100 mL of 0.15 M NaCl, 50 mM Hepes, and 0.1% Triton, pH 7.6, and finally with a similar amount of this buffer containing 10 mM Mg_2SO_4. Solubilized membranes (~6 mg of protein) were recycled 5 times through each column; each final flow-through was saved, and the columns were washed with 50 mL of 0.15 M NaCl, 50 mM Hepes, 10 mM Mg_2SO_4, and 0.1% Triton, pH 7.6, at 4 °C. Monosaccharides used for elution were applied at a concentration of 0.3 M in 2 mL of this buffer, and the flow of the columns was stopped for 30 min. In control experiments, washing and eluting the columns in this fashion did not lead to any detectable leakage of the coupled lectin as measured by the presence of protein in the eluate. Monosaccharides in the eluates were removed by extensive dialysis against 50 mM Hepes, 10 mM Mg_2SO_4, and 0.1% Triton, pH 7.6, at 4 °C. Control samples and the flow-throughs were also subjected to the same treatment. Binding assays were performed not later than 3 days after chromatography.

In other experiments, wheat germ columns (4 mL of settled gel) were loaded with solubilized placental membranes (~24 mg of protein) and desorbed, after washing, with linear gradients (from 0 to 0.1 M or from 0 to 0.01 M) of *N*-acetylglucosamine.

[1] Abbreviations used: PMSF, phenylmethanesulfonyl fluoride; Hepes, 4-(2-hydroxethyl)-1-piperazineethanesulfonate; MSA, multiplication-stimulating activity; EGF, epidermal growth factor; R_0, receptor binding capacity estimated from the abscissa intercept of a Scatchard plot of competitive equilibrium binding data; $INS_{50\%}$, concentration of unlabeled insulin that produced half-maximal inhibition of [^{125}I]insulin binding in steady-state competition experiments.

In sequential chromatography experiments, three lectin columns (5-mL bed volume each) were used in series, with the application of approximately 30 mg of solubilized membrane protein. PMSF was added routinely to all buffers. Chromatography at either 22 or 4 °C gave the same results. Eluates were usually dialyzed before being applied to the next column, but, as described under Results, removal of monosaccharide was not essential and omission of the dialysis steps shortened the procedure considerably. The solubilized membranes used as control were exposed to the monosaccharides and subjected to the same dialysis treatments.

Binding Assays. Insulin Binding. [^{125}I]Insulin (100–150 μCi/μg) was prepared by a modification of the chloramine-T method (Roth, 1975). The standard assay was performed by incubating aliquots of crude solubilized membranes (90–120 μg of protein) or eluates from the lectin columns (5–10 μg of protein) with a tracer amount of [^{125}I]insulin (40–60 pg) and increasing concentrations of unlabeled insulin (0–10 000 ng/mL) in a total volume of 200 μL in 50 mM Hepes, 10 mM Mg_2SO_4, and 0.1% bovine serum albumin, pH 7.6. The final detergent concentration was always finally adjusted to 0.1%. Samples were incubated for 15 h at 4 °C; receptor-bound insulin then separated from free insulin by precipitation with poly(ethylene glycol) (Desbuquois & Aurbach, 1971) (final concentration 12.5%) in carrier human γ-globulin (final concentration 0.05%). After incubation of the samples (final volume 600 μL) for 10 min at 4 °C, the precipitates were collected by centrifugation for 5 min in a Beckman Microfuge B and counted for radioactivity in a Searle autogamma counter. Nonspecific binding was defined as the radioactivity precipitated in the presence of 10 μg/mL unlabeled insulin (always <20% of total tracer bound) and was subtracted from total bound radioactivity to yield specific binding. Degradation of the insulin tracer during the assay was measured in the supernatant as the percent increase in radioactivity soluble in 5% trichloroacetic acid. At the temperature of the assay (4 °C), insulin degradation did not exceed 10% of the total radioactivity, and the difference between degradation by crude solubilized membranes and the lectin eluates was <4% of the total radioactivity. Insulin-degrading activity was therefore assessed independently by incubating samples with tracer [^{125}I]insulin for 30 min at 37 °C.

The results of the competition–inhibition assays were analyzed by the method of Scatchard (Scatchard, 1949) and corrected for protein concentration. The concentration of receptor sites (R_0/milligram of protein) was calculated by extrapolation of the Scatchard plot to the intercept on the abscissa. The scatchard plots were normalized by dividing both ordinates and abscissas by the concentration of receptor sites (R_0).

EGF and MSA Binding. [^{125}I]MSA and [^{125}I]EGF were kindly provided by Drs. Peter Nissley and Harry Haigler (National Institutes of Health), respectively. Specific activities were 90 μCi/μg for MSA and 125 μCi/μg for EGF, and both were used at a final concentration of 0.2–0.3 ng/mL. Unlabeled MSA (Buffalo rat liver cell conditioned medium) and EGF (mouse submaxillary glands) were obtained from Collaborative Research (Waltham, MA). Binding assays to solubilized membranes were performed as described above for insulin binding. Specific binding of MSA and EGF was determined by subtracting the radioactivity that remained bound in the presence of 1 μg/mL and 2.5 μg/mL of the respective unlabeled peptide.

Results

Effect of Lectins on [^{125}I]Insulin Binding to the Solubilized Placental Receptor. Four out of 12 lectins, concanavalin A, lentil, pea, and wheat germ, altered the specific binding of [^{125}I]insulin to solubilized placental membranes (Figure 1). Wheat germ agglutinin at concentrations ≤25 μg/mL increased insulin binding up to 25% but at higher concentrations markedly inhibited binding (≃75% decrease). Concanavalin A, lentil, and pea agglutinins caused a dose-dependent reduction of insulin binding to a maximum of 50% of total. Addition to the assay medium of the appropriate sugar, *N*-acetylglucosamine (wheat germ) or methyl α-mannoside (concanavalin A, lentil, and pea), blocked completely the effect of the lectins (not shown). Specific binding of insulin was not modified by ricins I and II, *B. simplicifolia*, gorse seed, horse gram, peanut, *S. japonica*, and soybean, even when added to concentrations as high as 500 μg/mL.

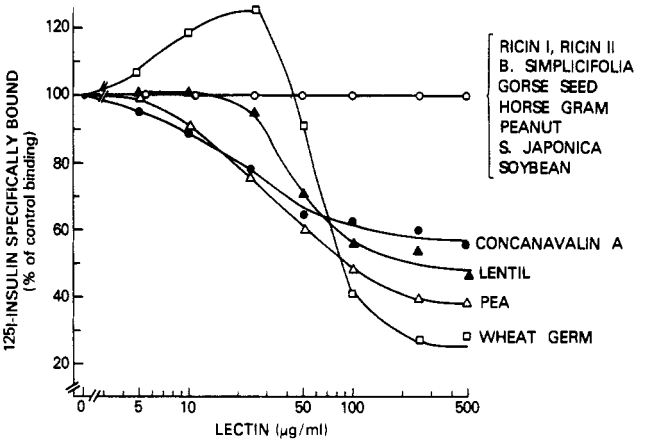
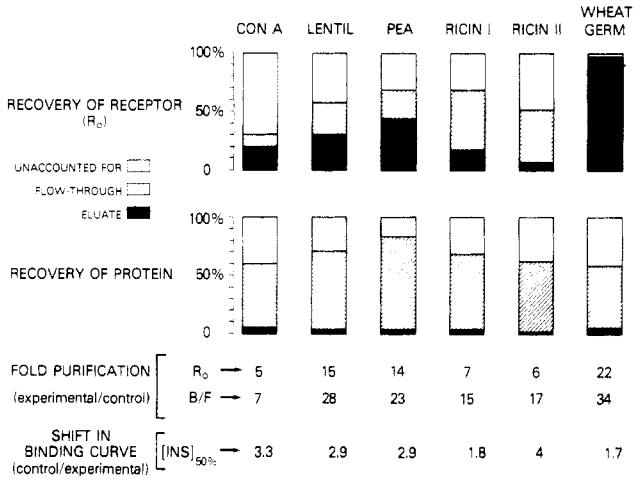


FIGURE 1: Effect of lectins on [^{125}I]insulin binding to the solubilized placental receptor. Soluble membranes (100 μg of protein) and [^{125}I]insulin (50 pg) were incubated at 22 °C for 1 h in 200 μL of 50 mM Hepes buffer, 0.1% bovine serum albumin, and 0.1% Triton X-100, pH 7.6, containing various concentrations of lectins as indicated, with or without an excess of unlabeled insulin (10 μg/mL). Bound radioactivity was separated by precipitation with poly(ethylene glycol) as described under Experimental Procedures. Specifically bound [^{125}I]insulin is expressed as a percentage of control binding in the absence of lectin. Specific control binding amounted to 30% of the total radioactivity; nonspecific binding was 5–6% of the total.

Chromatography of the Insulin Receptor on Individual Lectin Columns. Placental Receptor. When solubilized placental membranes were chromatographed on 12 different immobilized lectins, the insulin receptor was not adsorbed to columns containing *B. simplicifolia*, gorse seed, horse gram, peanut, *S. japonica*, and soybean; 90–95% of the receptor was recovered in the flow-through, and its binding properties remained unaltered. In contrast, insulin binding activity was retained by the other six lectin columns (concanavalin A, lentil, pea, ricins I and II, and wheat germ) and subsequently eluted by using the appropriate monosaccharide (Chart I). The insulin binding capacities (R_0) per milligram of protein of the eluates were increased between 5- and 22-fold, with recoveries ranging from 7% to 98% (Chart II). Optimum purification (22-fold) and recovery (98%) of receptor were obtained with the *N*-acetylglucosamine binding lectin, wheat germ agglutinin. Of the lectins with specificities for mannose and glucose, pea and lentil behaved similarly (15-fold purification, 30–45% recovery) whereas concanavalin A retained 90% of the receptor but only 20% could be desorbed with methyl α-mannoside. The least efficient were the galactose binding lectins, ricin I and ricin II (Chart II). A variable fraction of the total binding activity applied to the lectin columns was not retained, varying from less than 1% for wheat germ up to 50% with ricin I (Chart II). The binding profile of unretained receptors were always exactly superimposable on those of the control prep-

Chart II: Purification of the Insulin Receptor from Human Placenta by Chromatography on Individual Lectin Columns[a]



[a] Recovery of receptor was calculated from binding capacities (R_0) and obtained from Scatchard analysis of equilibrium binding data. Fold purification was estimated both as the increase in binding capacity (R_0) per milligram of protein and as the increase in [^{125}I]insulin bound/free per milligram of protein. The shift in the binding curve is an estimation of the relative change in the affinity of the receptor and was calculated by dividing the concentration of unlabeled insulin that produced half-maximal inhibition of binding ($[INS]_{50\%}$) in the control membranes by the corresponding value for the lectin purified membranes.

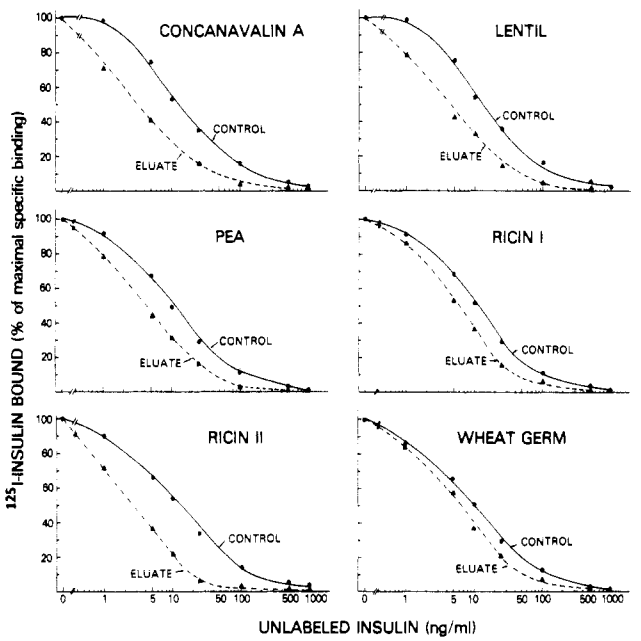


FIGURE 2: Competition inhibition curves of insulin binding to placental receptors purified by chromatography on individual lectin columns. Binding assays were performed as described under Experimental Procedures. Specifically bound [^{125}I]insulin is expressed as a percentage of the initial maximal bound and amounted to 30–40% of the total radioactivity; nonspecific binding was 3–5% of the total radioactivity.

arations (not shown). Furthermore, unretained receptors could subsequently be adsorbed if applied to regenerated columns containing the same lectins.

As is evident from Chart II, there is a discrepancy between the estimates of receptor purification determined by the increase in binding capacity (R_0) vs. the increase in bound/free [^{125}I]insulin, the latter being always greater. This difference is due to an increase in the apparent affinity of the desorbed receptors, reflected by a leftward shift in their competition binding curves and a 2–3-fold decrease in the concentration

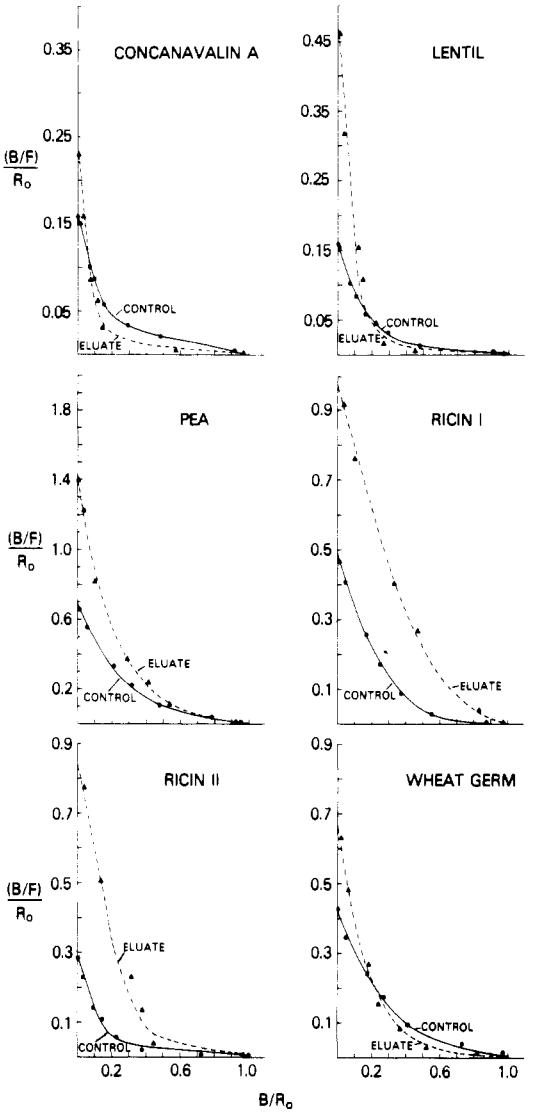


FIGURE 3: Scatchard analyses of the results shown in Figure 2. The binding data were corrected for protein concentration. The binding capacity (R_0) of the receptor in control solubilized membranes ranged between 10 and 24 ng of insulin per mg of protein. Each plot has been normalized by dividing both ordinates and abscissa by the binding capacity (R_0).

of unlabeled insulin, causing half-maximal inhibition of [^{125}I]insulin binding (Figure 2). Correspondingly, the Scatchard plots of insulin binding to the lectin-purified receptors show consistently steeper profiles than those of the controls (Figure 3).

The six lectin columns separated the insulin receptor from the majority of the insulin degrading activity which was unadsorbed by the columns. After incubation of tracer [^{125}I]insulin for 30 min at 37 °C with crude solubilized membranes, between 40% and 50% of the radioactivity was soluble in trichloroacetic acid while equivalent amounts of binding sites in the different lectin eluates yielded always less than 10% of soluble counts.

In the above experiments the lectin columns were eluted with a single batch of buffer containing 0.3 M monosaccharide. In an attempt to improve purification, the wheat germ column was eluted with linear gradients of *N*-acetylglucosamine, from 0 to 0.1 M and from 0 to 0.01 M (Figure 4). However, the gradient elution of both receptor activity and protein was parallel; the binding capacity (R_0) of the receptor peak, estimated from saturation binding analysis, was no greater than that of the receptor eluted batchwise.

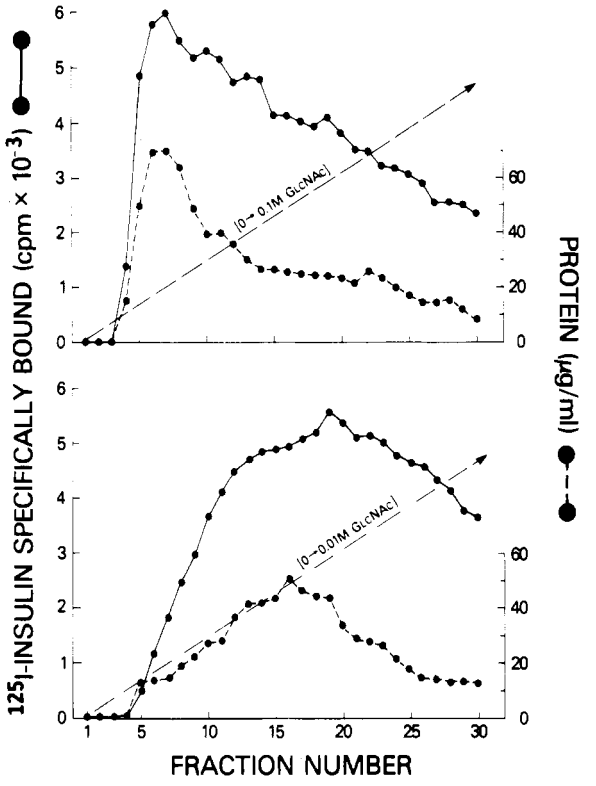
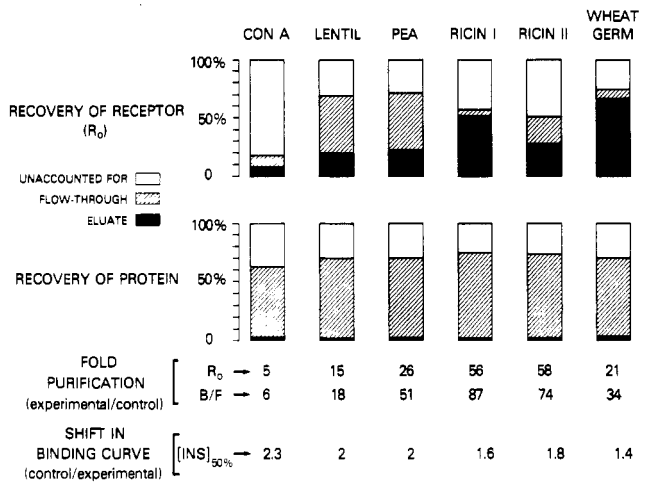


FIGURE 4: Elution of insulin receptor from wheat germ agarose columns using gradients of *N*-acetylglucosamine. The lectin columns (4 mL of settled gel) were loaded with solubilized placental membranes as described under Materials and Methods and eluted with linear gradients (60 mL) of *N*-acetylglucosamine into 2-mL fractions. [^{125}I]Insulin binding in the eluted fractions was expressed as bound radioactivity (cpm) per 100 μL of sample, after subtraction of nonspecific binding.

Chart III: Purification of the Insulin Receptor from Human Lymphocytes (IM-9 Line) by Chromatography on Individual Lectin Columns[a]



[a] See footnote *a* of Chart II.

Lymphocyte Receptor. Chromatography of solubilized lymphocyte membranes revealed the same pattern of lectin specificities for the insulin receptor as shown with solubilized placental membranes. Concanavalin A, lectin, pea, ricins I and II, and wheat germ retained significant amounts of receptor (Chart III). Again, wheat germ gave the highest recovery (70%) and significant purification (21-fold). However, in contrast to placental receptors, treatment of lymphocyte receptors on the galactose-binding lectins ricins I and II gave better purifications (≃50-fold) and recoveries (50% and 30%, respectively). Binding curves of the purified lymphocyte receptors also exhibited a leftward shift, with an average 2-fold decrease in the concentration of unlabeled insulin causing half-maximal inhibition of [^{125}I]insulin binding (Figure 5). Again, Scatchard plots of insulin binding to the purified receptors were curvilinear with clearly steeper slopes than those of the controls (Figure 6).

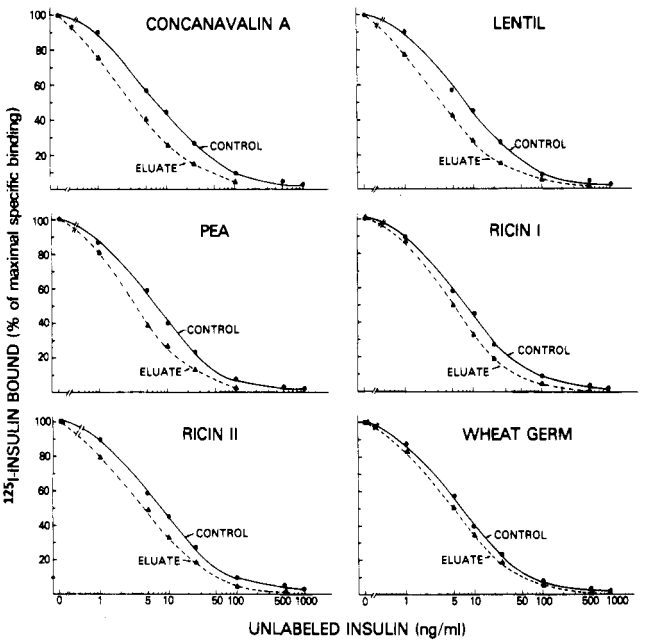
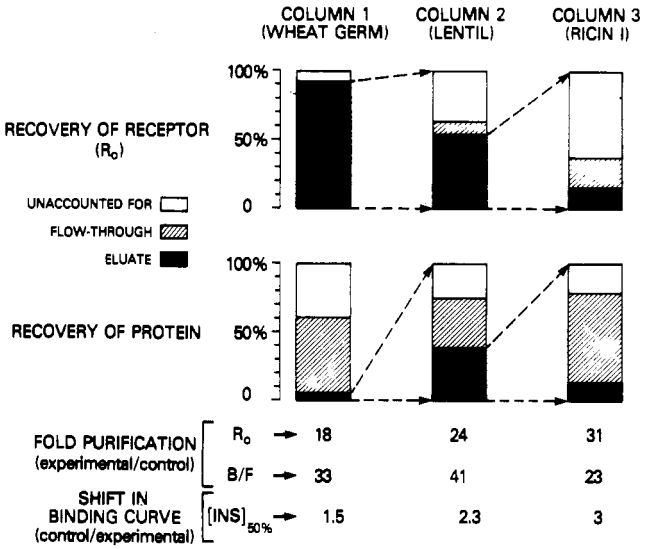


FIGURE 5: Competition–inhibition curves of insulin binding to IM-9 lymphocyte receptors purified by chromatography on individual lectin columns. Experimental details are as described in the legend to Figure 2.

Chart IV: Purification of the Insulin Receptor from Human Placenta by Sequential Chromatography on Lectin Columns[a]



[a] See footnote *a* of Chart II.

Chromatography of the Insulin Receptor on a Series of Lectin Columns. Placental Receptor. Three lectins with different carbohydrate-binding specificities, wheat germ (*N*-acetylglucosamine), lentil (mannose, glucose), and ricin I (galactose), were used in series in an attempt to increase the purification of the insulin receptor over that which can be obtained with a single lectin column. The results are summarized in Chart IV. After elution from wheat germ, the binding capacity of the receptor increased 18-fold, and the receptor activity was almost totally recovered. Chromatography of this preparation on a lentil column was followed by only a very slight increase in the purity of the receptor (24-fold

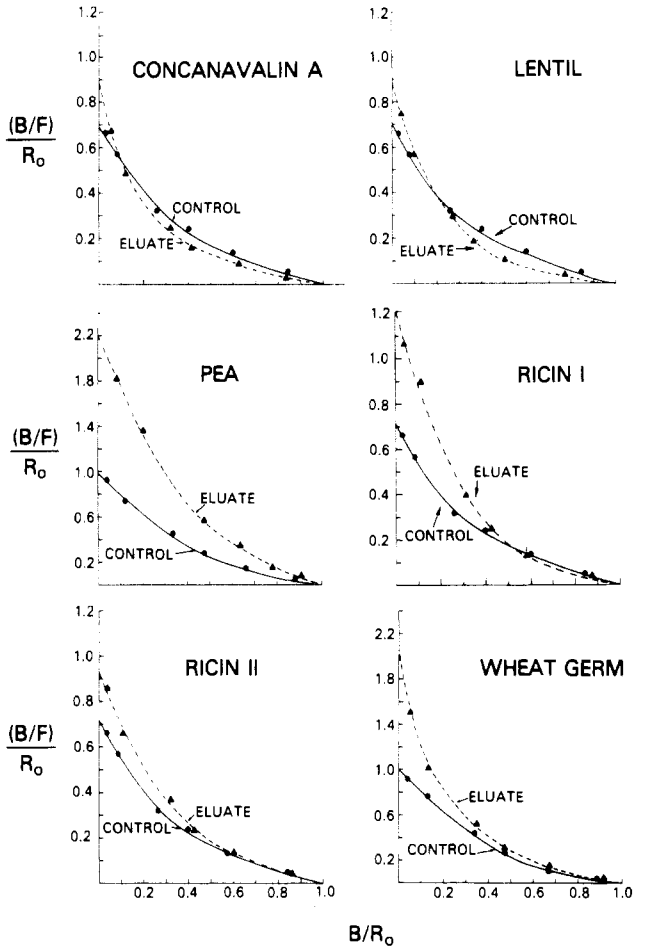
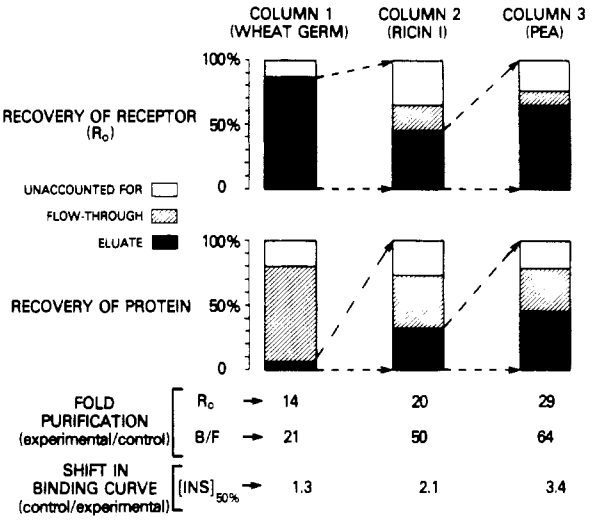


FIGURE 6: Scatchard analysis of the results shown in Figure 5. The binding data were corrected for protein concentration. The binding capacity (R_0) of the receptor in control solubilized membranes ranged between 6 and 9 ng of insulin per mg of protein. Each plot has been normalized by dividing both ordinates and abscissas by the binding capacity (R_0).

over control), with ~50% recovery. When the lentil eluate was then applied to a ricin I column and subsequently eluted, again there was only a minor increase in its purity (31-fold over control), with a 16% recovery. Thus, although the recovery of activity after each lectin column was in good agreement with the individual experiments shown above, the increase in binding capacity was comparable only after chromatography on the first lectin column, and the final purification (around 30-fold) was within the range that can be obtained on a single lectin column. Note, however, that the receptor eluted sequentially from wheat germ, lentil, and ricin I exhibited a progressive increase in apparent affinity (Figure 7). Selection of other series of lectins or alteration of the order of the columns gave comparable results. Overall recoveries could not be improved beyond 28% for the receptor and 1% for protein. In other experiments, sequential chromatography was performed without removing the sugar used for prior elution. Retention of the sugar should have improved specificity, and omission of the intervening dialysis steps reduced glycoprotein loss and degradation. However, the overall results were unchanged when sequential chromatography was performed in this fashion.

Chart V: Purification of the Insulin Receptor from Human Lymphocytes (IM-9 Line) by Sequential Chromatography on Lectin Columns[a]



[a] See footnote *a* of Chart II.

Lymphocyte Receptor. Solubilized lymphocyte membranes were serially chromatographed on wheat germ, ricin I, and pea columns (Chart V). The order of the columns was chosen to maximize the final recoveries. The first column gave a 14-fold purification, but subsequent chromatography on the second and third columns resulted in only an additional 2-fold increase (29-fold over control). The overall recovery of receptor was 27% and that of protein 1%. Again, as with the placental receptor, the apparent affinity of the lymphocyte receptor increased progressively after desorption from each

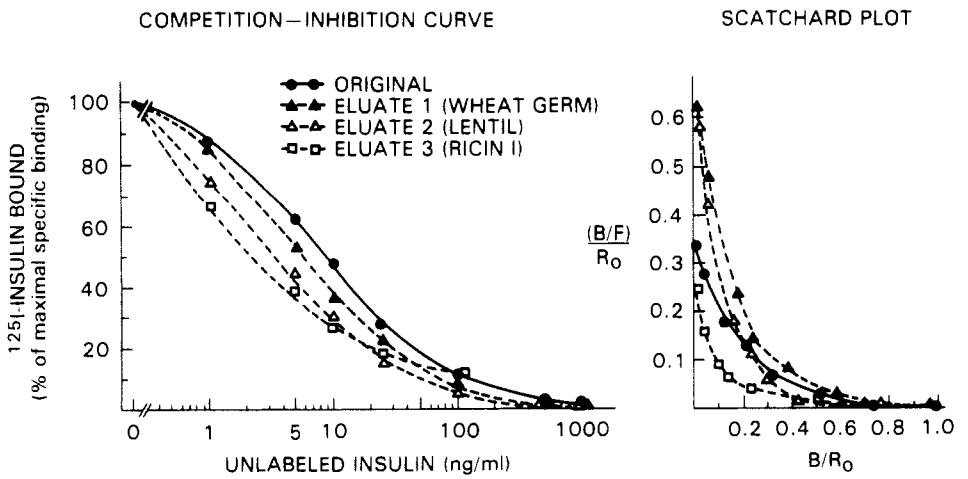


FIGURE 7: Insulin binding to placental receptors purified by sequential chromatography on lectin columns. Maximal specific binding ranged between 30% and 40% of the total radioactivity. Receptor binding capacity of the original solubilized membranes was 11 ng of insulin per mg of protein.

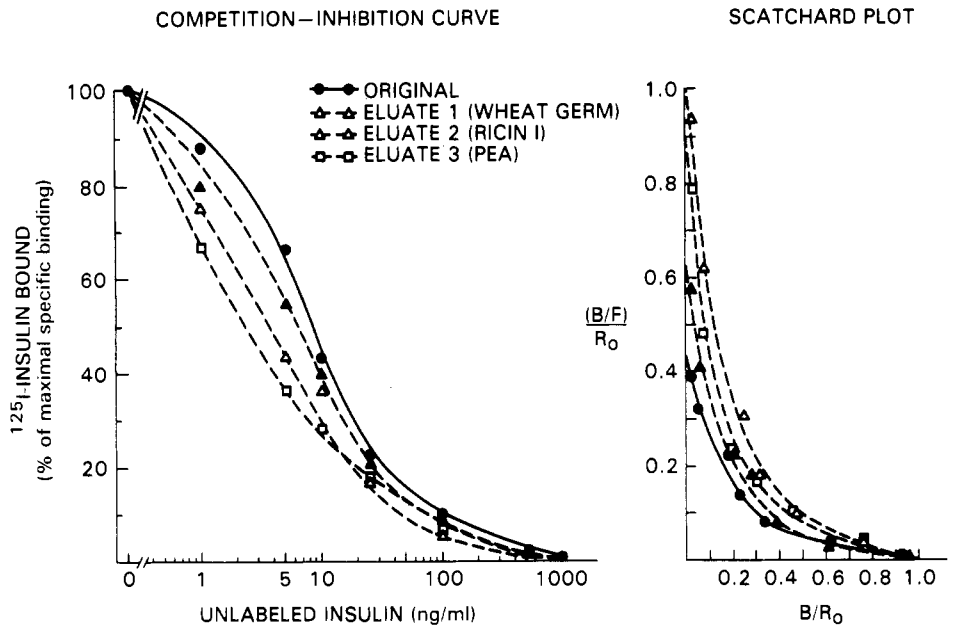
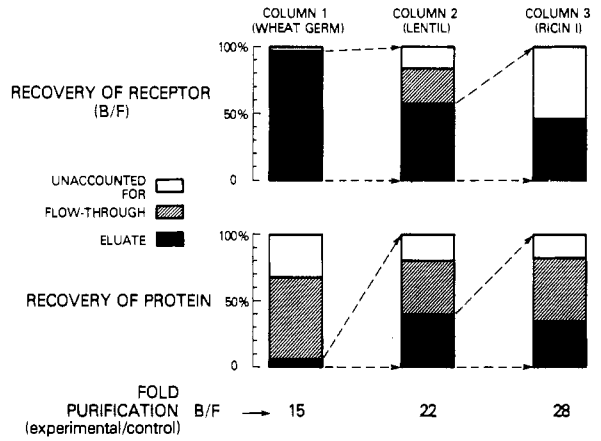


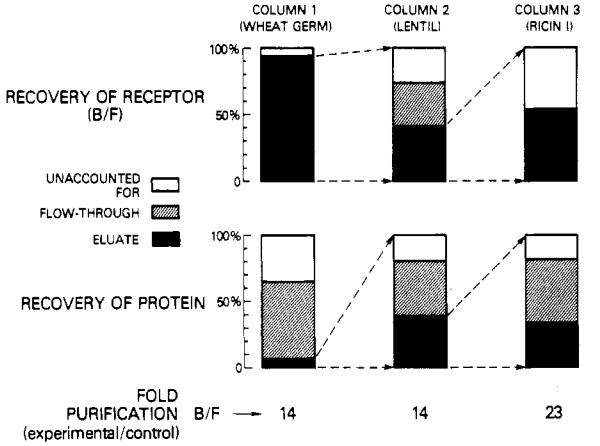
FIGURE 8: Insulin binding to IM-9 lymphocyte receptors purified by sequential chromatography on lectin columns. Maximal specific binding represented 30–40% of the total radioactivity. Receptor binding capacity of the original solubilized membranes was 8 ng of insulin per mg of protein.

Chart VI: Purification of the Placental MSA Receptor by Sequential Chromatography on Lectin Columns[a]



[a] Both recovery and fold purification of the receptor were calculated from [125I]MSA bound/free per milligram of protein for control and purified membranes.

Chart VII: Purification of the Placental EGF Receptor by Sequential Chromatography on Lectin Columns[a]



[a] Both recovery and fold purification of the receptor were calculated from the [125I]EGF bound/free per milligram of protein for control and purified membranes.

of the lectin columns (Figure 8).

Chromatography of the Placental MSA and EGF Receptors on a Series of Lectin Columns. The crude solubilized placental membranes exhibited low but reproducible specific binding for [125I]MSA and [125I]EGF with bound/free ratios per mg of protein of 0.24 and 0.09, respectively, compared to 3–6 for [125I]insulin. The binding activity for [125I]MSA and [125I]-EGF was monitored in the solubilized membranes that had been serially chromatographed on wheat germ, lentil, and ricin I columns (Charts VI and VII). Both receptors were adsorbed and eluted from this series of lectins in a pattern very similar to that observed for the insulin receptor. The first column purified both MSA and EGF receptors ~15-fold, but their specific activities were only slightly improved by further treatments.

Discussion

Our results demonstrate that insulin receptors from two different human tissues, placenta and cultured IM-9 lymphocytes, display similar specificities for a panel of 12 immobilized lectins. Receptors from both tissues were adsorbed by lectins that bind *N*-acetylglucosamine (wheat germ), mannose (concanavalin A, lentil, and pea), and galactose (ricins I and II), but were unretained by lectins that bind *N*-acetylgalactosamine (horse gram, *S. japonica*, and soybean), fucose (gorse seed I), and galactose (*B. simplicifolia* and peanut). These results suggest that the carbohydrate moiety of the insulin receptor contains surface *N*-acetylglucosamine, mannose, and galactose sugars. Futhermore, binding of insulin receptors by ricins I and II, but not by the other galactose-binding lectins *B. simplificolia* and peanut, suggests that it may contain predominantly the β configuration of D-galactoside (Goldstein & Hayes, 1978). Lymphocyte receptors were more efficiently adsorbed by the ricins than were placenta receptors, which may be the consequence of the exposure of more galactose residues due to loss of terminal sialic acid, as described for glycoproteins in other transformed cell lines (Wu et al., 1969; Grimes, 1970). Our results do not indicate that insulin receptors are a heterogeneous population with respect to carbohydrate content. A variable fraction of the receptors applied to the lectin columns was not retained, but could

subsequently be adsorbed if reapplied to regenerated columns, reflecting the limited capacity of the lectin columns rather than the existence of receptor heterogeneity.

On the basis of differential lectin binding, the insulin receptor would possess a carbohydrate composition similar to that of the plasma type (N-linked) glycoproteins (Lotan & Nicolson, 1979). However, it is obvious that conclusions based on lectin binding should be interpreted with caution since the specificities of lectins are complex and not strictly defined. For example, although it is generally accepted that wheat germ agglutinin binds specifically to *N*-acetylglucosamine and its β-1,4-linked oligomers (Goldstein & Hayes, 1978), there is also evidence for the interaction of this lectin with sialic acid (Bhavanandan & Katlic, 1979). In fact, we have observed that removal of sialic acid by treatment of the solubilized placental receptor with neuraminidase markedly reduces its subsequent binding to wheat germ.[2] Four of the six immobilized lectins that retained the receptor (concanavalin A, lentil, pea, and wheat germ) also altered insulin binding whereas ricins I and II failed to exert any effect on binding even when added at very high concentrations. The existence of ricin binding sites on the insulin receptor (or on an associated protein) that are functionally distinct from those for insulin suggests that the ricins could be useful probes of receptor function. It is noteworthy that the same six lectins able to retain the insulin receptor on affinity chromatography appear to exert insulin-like activities in rat fat cells while all of the six lectins unable to adsorb the receptor seem to lack significant effects.[3]

Affinity chromatography on lectins is an excellent one-step purification procedure for the insulin receptor. It gives a 5–50-fold increase in specific activity and separates the receptor from most of the insulin-degrading activity present in the membrane extract. Wheat germ agglutinin is the most efficient lectin in terms of receptor purification (22-fold) and recovery (70–98%). In contrast, concanavalin A which has been previously used together with insulin affinity chromatography for the purification of rat liver insulin receptors (Jacobs et al., 1977) was relatively inefficient. Although most of the receptor activity was adsorbed to concanavalin A, only 10–20% could be recovered by elution with methyl α-mannoside, and purification was only 5-fold. Poor recovery with saccharide elution of concanavalin A columns has also been reported for other membrane glycoproteins (Allan et al., 1972; Nachbar et al., 1976). Elution of the lectin columns using a linear gradient of the inhibitor monosaccharide did not subfractionate the insulin receptor from the other adsorbed glycoproteins, at least in the case of wheat germ agglutinin, suggesting that all these glycoproteins have a similar range of affinity for the lectin. Despite the advantages of lectin chromatography as one step in the purification of the insulin receptor, this procedure alone yields a preparation which is far from homogeneity. With the assumption of a molecular weight of 135 000 for the insulin binding subunit (Jacobs et al., 1979), the receptor eluted from the lectin columns is at best 1% pure as calculated from the Scatchard plots. Another example of the limited usefulness of lectin affinity chromatography has been described in the purification of the glycoprotein hormone erythropoietin (Spivak et al., 1978).

Given that the lectins able to bind the insulin receptor had at least three different carbohydrate specificities, we attempted to amplify the purification by using lectin columns in sequence. Gurd and Mahler have previously pointed out the potential advantage of using different lectin columns in tandem for the fractionation of glycoproteins (Gurd & Mahler, 1974). Unfortunately, this procedure results in only a very small increase in specific activity of the insulin receptor from both placental and IM-9 membranes. Failure of further purification is probably not due to degradation of the receptor during the purification procedure since no significant degradation was observed in control solubilized membranes subjected to the same conditions. In addition, similar results were obtained when the sequential procedure was performed in a single day without dialyses to remove sugars, where recoveries of the receptor were close to 100%. It is more likely that the glycoproteins isolated after the first lectin column cannot be further subfractionated because a large numer of them including the insulin receptor share the same carbohydrate determinants and lectin specificities. It is possible that most surface glycoproteins that serve as receptors have a similar carbohydrate composition, at least on a qualitative basis, as determined by lectin binding. This hypothesis is supported by our observation that placental receptors for both MSA and EGF behaved similarly to the insulin receptor on sequential lectin chromatography and by the fact that the carbohydrate composition proposed for other hormone receptors coincides with that which we suggest for the insulin receptor. Thus, the acetylcholine receptor from rat brain is retained by immobilized concanavalin A, wheat germ, and ricin I, but not by a fucose-binding lectin (Salvaterra et al., 1977). These findings are in keeping with the direct carbohydrate analysis of the purified acetylcholine receptor from the torpedo electric organ which demonstrates *N*-acetylglucosamine, mannose, and galactose (Vandlen et al., 1976). It has also been shown that concanavalin A, ricin, and wheat germ agglutinin block EGF binding to cultured human fibroblasts, while soybean and gorse seed lectins are without effect (Carpenter & Cohen, 1977). Also in agreement with the concept that a significant number of membrane glycoproteins share common saccharide residues is the observation that receptors for several different lectins on the surface of Ehrlich ascites carcinoma cells are not unique glycoproteins as determined by lectin affinity chromatography and gel electrophoresis, but rather have considerable overlap in specificity (Nachbar et al., 1976).

Finally, we have also observed that the affinity of the insulin receptor eluted from the lectin colunns exhibits a clear increase while the binding characteristics of the unadsorbed receptor remain unaltered. Furthermore, this affinity increase was progressive with sequential chromatography on lectin columns. The increase in affinity cannot be due to a decrease in insulin degradation, even though lectin chromatography separated the receptors from most of the degrading activity, because degradation at the temperature of the binding assay (4 °C) was negligible in both crude and purified membranes. Differences in detergent concentration (Harrison et al., 1978) also cannot account for this affinity shift because the binding assays were always performed in 0.1% (v/v) Triton. In addition, we have ruled out the possibility that this phenomenon is due to a direct effect of saccharides or to the dialysis treatments. Leakage of free lectins from the columns cannot explain the affinity shift since it was also observed with the ricins which do not alter insulin binding. On the other hand, we have not been able to reverse the affinity change by mixing the column eluates with the flow-through or wash fractions (data not shown). We postulate that a component of the receptor, devoid of binding activity itself but able to inhibit receptor affinity, is being removed during the affinity chromatographic proce-

[2] J. A. Hedo, A. Itin, and L. C. Harrison, unpublished results.

[3] H. M. Katzen, P. P. Vicario, R. A. Mumford, and B. G. Green, unpublished results.

dures. Along these lines, several investigators (Harmon et al., 1980; Maturo & Hollenberg, 1978; Katzen, 1979) using different experimental approaches have proposed the existence of associated components capable of modifying the affinity of the insulin binding site. Further studies are being performed in an attempt to directly identify such an affinity regulator.

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Inactivation of Chicken Liver Fatty Acid Synthetase by Malonyl Coenzyme A. Effects of Acetyl Coenzyme A and Nicotinamide Adenine Dinucleotide Phosphate†

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ABSTRACT: Chicken liver fatty acid synthetase complex is irreversibly inactivated by one of the substrates, malonyl-CoA. Acetyl-CoA has a dual role. At concentrations less than or comparable to those of malonyl-CoA, the rate of inactivation is enhanced, whereas at acetyl-CoA/malonyl-CoA ratios greater than 2, the rate of inactivation is slowed down. $NADP^+$ at low concentrations (25 μM) affords considerable protection against malonyl-CoA mediated inactivation whereas NAD^+ even at 1.0 mM concentration has no effect. The inactivation process does not lead to the dissociation of the enzyme complex and is accompanied by subtle conformational changes as measured by circular dichroism measurements. Of all the model partial reactions, decarboxylation of malonyl-CoA and the condensation–CO_2 exchange are the only reactions which are not catalyzed by the modified species. The process of inactivation is accompanied by enhanced covalent binding of malonyl groups such that approximately 6 mol of the acyl group is bound per mol of the enzyme at complete inactivation. The available evidence suggests that the inactivation of the enzyme results from the binding of malonyl group(s) at or near the condensing site of the enzyme.

Hepatic fatty acid synthesizing enzymes from avian and mammalian sources have been isolated as homogeneous multifunctional units and in a sequential series of reactions catalyze the addition of "2C" units to the priming substrate acetyl-CoA, resulting in the formation of mainly C_{16} saturated fatty acids: acetyl-CoA + 7malonyl-CoA + 14NADPH + $14H^+$ → palmitic acid + 8CoA + $7CO_2$ + $6H_2O$ + $14NADP^+$.

A number of different approaches are being followed to study the mechanism of action of this complex enzyme. In earlier studies, it was of interest to determine if the enzyme complex of approximately 500 000 molecular weight is constituted from monofunctional enzymes like the *Escherichia*

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