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Isolation of the Insulin Receptor of Liver and Fat-Cell Membranes

(detergent-solubilized/[^{125}I]insulin/polyethylene glycol precipitation/Sephadex)

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ABSTRACT **Extraction of liver and fat-cell membranes with the nonionic detergent Triton X-100 prevents specific binding of ^{125}I-labeled insulin to these membranes. This loss of binding to particulate material is quantitatively recovered in a high-speed (300,000 × *g*, 2 hr) supernatant of the extract. Specific and reversible insulin binding to soluble proteins is readily demonstrable by gel filtration. A simple and sensitive assay for detection of specific macromolecule–insulin complexes has been developed based on the selective precipitation of the complex by polyethylene glycol. Extraction of membrane lipids with organic solvents or by phospholipase digestion does not impair the subsequent extraction of the insulin-binding protein with detergent. Binding of insulin to the soluble protein is a saturable and dissociable process having a dissociation constant of about 100 nM. Derivatives of insulin compete for binding in direct proportion to their biological activity; other peptide hormones are without effect. The quantitative features of the detergent extractions and the specific insulin-binding properties of the material so obtained indicate that the protein solubilized is the biologically significant insulin receptor, whose insulin-binding function is essentially unaltered.**

Elucidation of the molecular basis of action of polypeptide hormones requires the identification, isolation, and purification of the specific cellular receptor structures with which the hormone initially interacts. Studies with bulky and insoluble derivatives of insulin (1) strongly suggest that interaction of this hormone with superficial structures of the cell can initiate many, if not all, of the metabolic effects of the hormone. It has recently been possible to measure directly the specific interaction of ^{125}I-labeled insulin with intact, metabolically responsive, isolated cells from adipose tissue (2). The close correlation between the properties of the physical interaction and biological activation under normal conditions (2), as well as when the chemical structure of insulin is altered or when the cell surface is modified by enzymic digestions (3–5), indicates that biologically significant receptor interactions are being measured. After disruption of the cell, the specific insulin-binding structures can be recovered quantitatively in the particulate fraction, and the kinetic properties of insulin binding to this membrane fraction are nearly identical to the kinetics of binding observed with intact cells (2, 6). Furthermore, the insulin-binding properties of isolated liver membranes (6–8) are very similar to those of adipose-tissue cells and membranes, suggesting that the receptor structures in these two tissues may be very similar or identical (7).

This paper describes the use of nonionic detergents in the extraction and solubilization of the above-described insulin-binding structures of liver and fat-cell membranes. A sensitive and simple assay procedure to measure the solubilized presumed receptor is presented that greatly facilitates further characterization of these structures. Subsequent papers will describe in detail various molecular and chemical properties of this receptor, the kinetics of its interaction with insulin, and useful purification procedures.

Although all of the experiments described have been performed on both liver and fat-cell membranes, data will not be presented for both tissues for every experiment since the results are, without exception, qualitatively identical with both preparations.

METHODS AND RESULTS

Liver membranes (manuscript in preparation) from Sprague-Dawley rats (80–140 g) are obtained by differential centrifugation of homogenates (in 0.25 M sucrose) prepared with a Polytron PT-10 (Brinkmann) at 21,000 rpm for 90 sec. The 600 × *g* (10 min) supernatant is centrifuged at 12,000 × *g* (30 min), adjusted to 0.1 M NaCl and 0.2 mM $MgSO_4$, and centrifuged at 40,000 × *g* for 40 min. The pellet is suspended in 0.05 M Tris·HCl buffer (pH 7.4), homogenized, and recentrifuged; these steps are repeated three times. These membranes contain about 80–90% of the total specific insulin-binding activity detectable in the crude homogenate. Isolated fat cells (9) were homogenized (Polytron) and centrifuged at 45,000 × *g* to obtain a particulate fraction ("membrane fraction"), which contains virtually all of the specific insulin-binding activity detectable in intact cells (2, 6). Protein was determined by the method of Lowry *et al.* (10) after the solution was heated at 100°C for 30 min in 1 N NaOH; bovine albumin was used as the standard. The measurement of specific binding of [^{125}I]insulin to fat-cell membranes (5, 6) and to liver membranes (6, 7) were described. [^{125}I]Insulin was prepared and purified as described (2).

Crystalline porcine zinc–insulin (24 units/mg) was purchased from Eli Lilly, Triton X-100 from Beckman, polyethylene glycol (Carbowax 6000) from Union Carbide, and crystalline bovine albumin and bovine gammaglobulin (Fraction II) from Miles. Insulin derivatives were kindly provided by Dr. R. E. Chance, Eli Lilly.

Solubilization of Insulin-Binding Structures with Nonionic Detergents. The nonionic detergent, Triton X-100, causes general dissolution of fat-cell membranes and destroys the ability of these membranes to bind [^{125}I]insulin specifically (Fig.

1). The fall in membrane turbidity is disproportionately greater than the loss of insulin binding at concentrations of detergent less than 0.5% (v/v), but with higher concentrations the total loss of binding activity is more profound than is the fall in turbidity. Similar results are obtained with liver membranes, except that less membrane turbidity is lost under comparable conditions. Although higher membrane concentrations require more detergent for solubilization, 1% (v/v) Triton X-100 destroys more than 80% of the binding activity of fat- or liver-membrane suspensions having protein concentrations as high as 15 mg/ml; this concentration of Triton X-100 has therefore been used in most experiments.

The loss of binding of insulin to membranes observed upon extraction with Triton X-100 is accompanied by the appearance of insulin-binding material in the high-speed (300,000 × *g*) supernatant of the extract (Fig. 2). The [^{125}I]insulin peak that appears in the void volume on gel filtration virtually disappears if the Triton supernatant is incubated with native insulin for 3 min before addition of [^{125}I]insulin (Fig. 2 *A*). Rechromatography of the rapidly emerging first peak results in its reappearance in the same position, although a small retarded peak is detected that presumably reflects free insulin that has dissociated from the larger complex (Fig. 2 *B*). However, if the first peak is incubated at 37°C in the presence of native insulin before rechromatography, the radioactivity is nearly completely displaced from the larger complex to the position occupied by free insulin (Fig. 2 *C*). Furthermore, the effluent present in the void volume of this column, upon incubation with [^{125}I]insulin and chromatography, demonstrates significant binding once again. These experiments demonstrate that a specific insulin–macromolecular complex is formed with high-speed supernatant extract, that insulin binds with high affinity and reversibly, and that this binding occurs even in the presence of 0.5% (v/v) Triton.

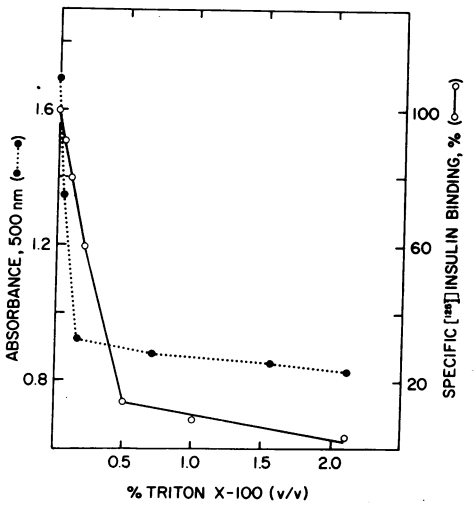

FIG. 1. Effect of Triton X-100 on the turbidity of fat-cell membranes and on the specific binding of [^{125}I]insulin to the membranes. Suspensions of membranes (0.4 mg of protein/ml) in 0.05 M Tris·HCl buffer (pH 7.4) were incubated at 24°C with Triton X-100 for 30 min, and the absorbance at 500 nm was measured. The pellets obtained after centrifugation at 220,000 × *g* for 70 min at 4°C were suspended in Krebs–Ringer bicarbonate buffer, sonicated for 3 sec, and assayed for specific binding of insulin.

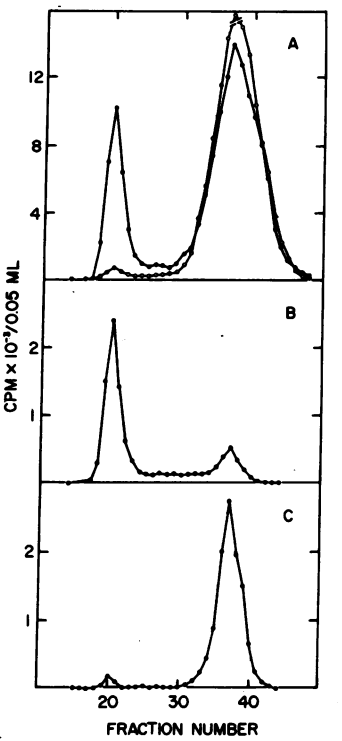

FIG. 2. Gel filtration patterns demonstrating specific and reversible binding between [^{125}I]insulin and material solubilized from liver-cell membranes with Triton X-100. Liver-cell membranes (6 mg of protein/ml) suspended in 0.05 M Tris·HCl buffer (pH 7.4) were incubated for 20 min at 24°C with 0.5% Triton X-100 and centrifuged at 300,000 × *g* for 70 min. The supernatant (0.5 ml) was incubated for 30 min at 24°C with 1.6 nM [^{125}I]insulin, cooled in ice, and applied to a column (45 × 1 cm) of Sephadex G-50 (medium), equilibrated and run at 4°C with Krebs–Ringer bicarbonate–0.1% albumin (w/v)–0.5% Triton X-100 (*A*, ●——●). The flow rate was 4.5 ml/hr, and each fraction contained 0.8 ml. A separate sample (0.5 ml) was processed identically, except that it was incubated with native insulin (50 μg/ml) for 3 min before addition of [^{125}I]insulin (*A*, O——O). The effluent buffer containing the first radioactive peak (tubes 18–21 in *A*, ●——●), which corresponds to the column void volume, was pooled. 1 ml of this material, containing 126,000 cpm, was immediately rechromatographed on the same column (*B*); the first radioactive peak in this column contained a total of 92,000 cpm. Another 1 ml of the pooled material of the first peak was incubated with 10 μg of native insulin for 50 min at 37°C before chromatography (*C*). The material appearing in the void volume of this column, after incubation for 30 min at 37°C with fresh [^{125}I]insulin (1.7 nM), followed by chromatography, was capable of again binding [^{125}I]insulin.

All of these studies were performed with Triton X-100 extracts subjected to centrifugation at 300,000 × *g* for 70 min; centrifugation for 6 hr does not result in significant sedimentation of the insulin-binding material.

Assay of Soluble Insulin–Receptor Complex. A simple but sensitive assay procedure was devised to measure soluble insulin–macromolecular complexes. The procedure is based on the selective precipitation of these complexes, but not of free insulin, by polyethylene glycol. The possible usefulness of this reagent was suggested by its capacity for selective protein fractionation (11), and by its recent application to radioimmunoassay of peptide hormones (12).

TABLE 1. *Effect of Triton X-100 on assay of soluble insulin–receptor complexes*

Triton concentration (%(v/v))	Specific binding of [^{125}I]insulin (cpm)
0.025	22,430 ± 820
0.05	23,100 ± 710
0.15	21,800 ± 960
0.5	12,700 ± 210
1.0	6,410 ± 170

A liver-membrane suspension, extracted and solubilized with 0.5% (w/v) Triton X-100 as described in Fig. 2, was assayed in the presence of different concentrations of the detergent in the assay mixture. The incubation medium contained 220,000 cpm of [^{125}I]insulin in a total volume of 0.25 ml. Values are means ± standard error (n = 3).

A reaction mixture containing 0.2 ml of Krebs–Ringer bicarbonate buffer, 0.1% (w/v) albumin, 5–50 μl of detergent-extracted supernatant, [^{125}I]insulin (10^{-9} M–10^{-12} M), and 5–10 μg of native insulin (in control tubes only) is incubated at 24°C until equilibrium is achieved (20–50 min). 0.5 ml of ice-cold 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1% (w/v) bovine gammaglobulin is added, and the tubes are placed in ice. 0.5 ml of cold 25% (w/v) polyethylene glycol is added (final concentration, 10%), and the tubes are thoroughly mixed and placed in ice for 10–15 min. The contents of the tubes are then filtered under reduced pressure on cellulose acetate (EH) Millipore filters; the filters are washed with 3 ml of 8% polyethylene glycol in 0.1 M Tris·HCl buffer (pH 7.4). The filters are counted at 53% efficiency in a Nuclear-Chicago well-type gamma counter. Control tubes are always present in which native insulin (25–50 μg) is added before [^{125}I]insulin; this measures the free [^{125}I]insulin that precipitates under the conditions of the assay or that binds nonspecifically to proteins and filter. Specific binding is determined by subtraction of the radioactivity of the control samples.

Under these conditions less than 0.5% of the total free [^{125}I]insulin is precipitated or nonspecifically adsorbed, whereas nearly quantitative precipitation of the insulin–receptor complex occurs. Concentrations of polyethylene glycol less than 8% (w/v) incompletely precipitate the complex; concentrations higher than 12% significantly precipitate free insulin. The presence of gammaglobulin is essential as a carrier for the precipitation reaction, but concentrations above 0.1% (v/v) cause precipitation of free insulin. Phosphate buffers (0.1 M, pH 7.4) can also be used effectively in the incubation medium. If the pH of the buffer containing the gammaglobulin is above 8 or below 7, the complex is less effectively precipitated. Concentrations of Triton X-100 in the assay mixture in excess of 0.2% (v/v) result in decreased insulin binding (Table 1). Triton extracts of membranes are, therefore, diluted before assay so that the final concentration of detergent is usually less than 0.1%, and always is less than 0.2%.

The specific binding of insulin to the extracts, measured by the polyethylene glycol assay, was compared to the specific binding detected by Sephadex G-25 chromatography under conditions similar to those described in Fig. 2. Although very similar binding activities are obtained by the two assay procedures, about 20–30% less binding is detected by the gel filtration method. This result is consistent with some dissociation of the complex during chromatography.

Relatively large concentrations of [^{125}I]insulin are generally used in the assay, so that specific binding is linear over at least a 10-fold range of protein concentration (Fig. 3). Under the usual conditions used to achieve binding equilibrium, there is no significant inactivation of the free [^{125}I]insulin present in the medium, and the insulin that can be dissociated from the complex is not demonstrably altered (13).

The membrane extracts solubilized with Triton X-100 were passed over Sephadex G-50 (medium grade) columns equilibrated with buffers free of detergents to determine the effect of removal of most of the detergent. Cloudy suspensions are obtained that contain slightly less total binding activity than comparable solutions obtained by passage of extracts over identical columns chromatographed with buffers containing 0.1–0.5% Triton X-100. However, about 20% of the binding activity is present in the visible precipitate, which is readily separated by centrifugation. The remaining binding activity precipitates progressively with time in the absence of added detergent. This aggregation into large molecular species is easily detected by molecular sieving experiments and by sucrose gradient centrifugation (13). The receptor can be maintained in soluble form over prolonged periods if it is present in 0.1% (v/v) Triton X-100–0.1 M sodium phosphate buffer (pH 7.4).

Quantitation of Receptor Solubilization. It is important to demonstrate that the Triton-solubilized material that binds insulin quantitatively reflects removal of the insulin-binding structures from intact membranes (Table 2). With increasing concentrations of Triton in the extraction medium, there is excellent agreement between the loss of specific binding of insulin to the residual membrane or particulate pellet and the appearance in the soluble fraction of specific insulin-binding material.

The total extractable binding activity is greater than that originally present in the intact membrane (Table 2). This is in close agreement with the known presence of masked binding structures, which can be uncovered by disruption of membrane phospholipids (5). The specific binding of insulin to liver

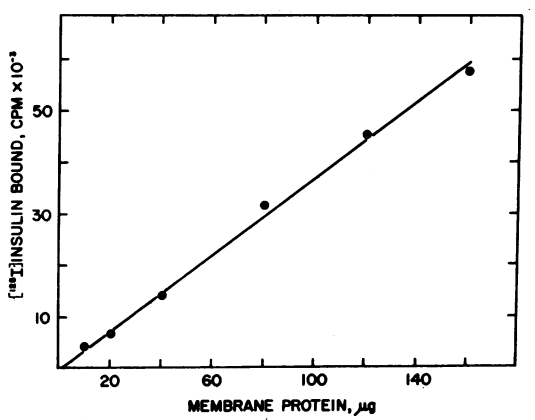

Fig. 3. Specific binding of [^{125}I]insulin to Triton X-100-solubilized receptor from liver membrane (Fig. 2) as a function of the concentration of protein in the medium. The polyethylene glycol assay was used; the concentration of [^{125}I]insulin was 0.7 nM, and 0.25-ml samples were incubated at 24°C for 50 min.

membranes is about doubled by phospholipase digestion or by extraction with organic solvents (5, 7). These effects are even more marked in Triton extracts of fat-cell membranes, in agreement with the greater quantity of normally inaccessible binding structures in these membranes as compared to liver membranes (5, 7).

Another nonionic detergent, Lubrol-PX, is nearly as effective as Triton X-100 in solubilizing the insulin receptor from liver or fat-cell membranes. The presence of this detergent in the incubation medium, however, interferes more seriously with formation of the insulin–receptor complex, as determined by the polyethylene glycol binding assay. For example, concentrations (v/v) of Lubrol of 0.08, 0.16, and 0.5% cause 23, 50, and 85% suppression of insulin binding, respectively (compare with Table 1). Attempts to obtain soluble insulin-binding materials by extraction of membranes with sodium dodecyl sulfate, dimethylsulfoxide, dimethylformamide, hexafluoroisopropanol, and pyridine were not successful. About 10% of the binding activity of liver-cell membranes was extracted by vigorous agitation in distilled water. About 20–30% of insulin-binding activity of liver or fat-cell membranes could be recovered in soluble form after extraction with lithium diiodosalicylate, a reagent used successfully by Marchesi and Andrews to solubilize and purify glycoproteins from erythrocyte membranes (14). This reagent was not used more extensively because of the relatively low yields obtained.

Liver or fat-cell membranes digested with phospholipase C or phospholipase A, or extracted (after lyophilization) with certain organic solvents, display a greater capacity for insulin binding (5). It is significant that membranes subjected to such procedures can be subsequently extracted quite successfully with Triton X-100. In these cases the yield of extraction is quantitative, and there is no increase in activity similar to that described in Table 2.

Solubilization of the insulin receptor appears to be nearly quantitatively reversible, since dialysis of the extracts against detergent-free buffers at neutral pH results in the formation of precipitates that contain virtually all of the insulin-binding activity originally present in the solution. This occurs even in Triton extracts of delipidated or phospholipase-treated membranes.

TABLE 2. *Transfer of insulin-binding activity from liver membranes to supernatant during solubilization with Triton X-100*

% Triton for extraction	Specific binding of [^{125}I]insulin		
	Pellet	Supernatant (cpm/20 μl)	Total
0	7200	200	7,400
0.5	1710	9,200	10,910
1.0	980	11,750	12,730
2.0	460	13,460	13,920

Suspensions of liver membranes (12 mg of protein/ml) were briefly sonicated and extracted with different Triton X-100 concentrations (see Fig. 2). The specific binding of [^{125}I]insulin to the supernatants (300,000 × *g*, 80 min) was measured with polyethylene glycol. The pellets were resuspended in the original volume with 0.05 M Tris·HCl buffer (pH 7.4) and sonicated; aliquots were assayed (5, 6) for specific binding with 400 pM [^{125}I]insulin.

TABLE 3. *Displacement of soluble insulin–receptor complex by insulin derivatives and peptide hormones*

Addition	Specific binding of [^{125}I]insulin (cpm)	
	Fat cell	Liver
No additions	7210 ± 320	5890 ± 430
Insulin, 5 ng/ml	3030 ± 104	2710 ± 350
0.5 μg/ml	110 ± 30	70 ± 25
Desalanine-insulin, 5 ng/ml	3140 ± 78	2940 ± 280
Desoctapeptide-insulin, 50 ng/ml	7010 ± 240	5960 ± 610
200 ng/ml	4840 ± 310	4230 ± 410
Reduced and carboxymethylated insulin, 5 μg/ml	6970 ± 206	5740 ± 350
Proinsulin, 0.1 μg/ml	3560 ± 260	2990 ± 170
Glucagon, 0.1 mg/ml	7440 ± 340	5970 ± 280
ACTH, 0.1 mg/ml	7510 ± 420	5620 ± 350
Oxytocin, 0.1 mg/ml	7320 ± 304	5750 ± 290
Growth hormone, 0.1 mg/ml	7450 ± 220	5730 ± 220

Samples of Triton X-100-solubilized material from fat (30 μg of protein) or liver (55 μg of protein) membranes were incubated for 60 min at 24°C in 0.25 ml of Krebs–Ringer bicarbonate buffer containing 0.1% albumin, 61 pM [^{125}I]insulin, and the indicated hormone. Insulin binding was determined with polyethylene glycol. Values are means ± standard error (n = 3).

Specificity of Binding to Solubilized Receptor. There is good agreement between the biological activity of insulin derivatives and their ability to compete with insulin for binding to the solubilized membrane material (Table 3). Desalanine-insulin is indistinguishable from native insulin, the insulin chains (separately or together) are ineffective, and proinsulin is 20-times less effective than native insulin. Very similar results are observed in binding studies with intact fat cells or membranes, and in biological studies (2, 6). Desoctapeptide-insulin can displace the binding of [^{125}I]insulin, but 50-times higher concentrations are required compared to native insulin; displacement may be due to trace contamination with native insulin. Other peptide hormones are totally ineffective, even at extremely high concentrations.

The specific binding of [^{125}I]insulin to the Triton X-100-solubilized material is a saturable process with respect to insulin (Fig. 4). The dissociation constant calculated from this data is 130 pM, which is similar to the values (50–90 pM) obtained in studies with intact cells (2) or isolated membranes (2, 6, 7). The maximal binding capacity of various Triton X-100 extracts of liver membranes is between 0.25–0.35 pmol of insulin per mg of protein; for extracts of fat-cell membranes, the values range from 0.55 to 0.7.

DISCUSSION

In the context used here, "receptor" refers to those molecules of the cell that are uniquely capable of recognizing, and thus interacting with, insulin with a high degree of selectivity and affinity, and that in addition possess the capability to convey the occurrence of the interaction to biochemical processes that find expression in metabolically significant events. The insulin-binding macromolecular substance "solubilized" from

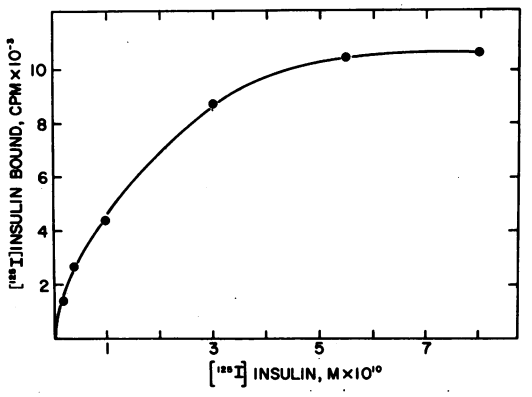

FIG. 4. Effect of the concentration of [^{125}I]insulin on the specific binding to Triton X-100-solubilized material from liver membranes. Assays contained 100 μg of solubilized protein/ml.

membranes by nonionic detergents very likely represents the biologically significant receptor for this hormone if, as appears likely, the specific insulin-binding structures that have been studied in intact cells and in isolated membranes are indeed the receptors for insulin (2, 3–7). The quantitative aspects of the extraction and the close similarity in binding properties strongly indicate that the extracted molecules are the same as those that can be measured and studied in the original particulate systems. The properties of the insulin-binding interaction described in this report, as well as those (kinetic rate constants, effects of salts, enzymic digestions, etc.) presented elsewhere (13), suggest that the binding properties of the receptor are remarkably unchanged upon dislocation from its native environment in the membrane. The identity of the isolated receptor is further substantiated by the failure to detect significant specific binding of insulin to proteins obtained by similar detergent solubilization of erythrocyte ghosts, structures that possess very little or no capacity to specifically bind insulin (2).

Dismantling of the cell to extract and isolate the receptor by necessity dissociates the receptor from other structures that are normally necessary to evoke a biologically significant event. It is, therefore, impossible to measure or test with this preparation the second function required of the hypothetical "receptor," that of transmitting to other biologically important molecules information concerning the formation of the hormone–receptor complex. It is, therefore, apparent that identification of the receptor under these circumstances can only be made provisionally, through the considerations discussed above. It may ultimately be possible to demonstrate biological function by reconstitution if the various components of the membrane system can be sufficiently well characterized and isolated.

"Solubilization" as used here refers primarily to the absence of significant sedimentation under a force of 300,000 × *g* for 6 hr, and to unimpaired passage of the material through filters having 0.2-μm pores. Moreover, other physical properties indicate that the soluble binding protein has asymmetric molecular dimensions and a molecular weight of about 300,000 (13). The binding activity is completely destroyed by mild tryptic digestion, suggesting that this receptor is a protein, although there is good evidence from physicochemical studies that it is not a lipoprotein (13).

It is noteworthy that vigorous extraction of membrane lipids with certain organic solvents or by phospholipase digestion does not decrease the yield or significantly alter the properties of the insulin-binding molecules that can be subsequently solubilized with detergents. The insulin receptor is thus an example of a structure which, despite being an integral part of a biological membrane, is not grossly dependent on membrane lipids for at least one of its functions, the recognition of insulin. These favorable properties, and the availability of a simple and reliable assay procedure, suggest that purification of this receptor may be feasible provided the problems posed by its extraordinary scarcity in biological materials can be overcome.

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1. Cuatrecasas, P. (1969) *Proc. Nat. Acad. Sci. USA* **63**, 450–457; Turkington, R. W. (1970) *Biochem. Biophys. Res. Commun.* **41**, 1362–1367; Oka, T. & Topper, Y. J. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2066–2068; Blatt, L. M. & Kim, K. H. (1971) *J. Biol. Chem.* **246**, 4895–4898.
2. Cuatrecasas, P. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1264–1268.
3. Cuatrecasas, P. & Illiano, G. (1971) *J. Biol. Chem.* **246**, 4938–4946.
4. Cuatrecasas, P. (1971) *J. Biol. Chem.*, **246**, 6522–6531.
5. Cuatrecasas, P. (1971) *J. Biol. Chem.*, **246**, 6532–6542.
6. Cuatrecasas, P. (1971) *J. Biol. Chem.*, **246**, 7265–7274.
7. Cuatrecasas, P., Desbuquois, B. & Krug, F. (1971) *Biochem. Biophys. Res. Commun.* **44**, 333–339.
8. Freychet, P., Roth, J. & Neville, D. M. (1971) *Biochem. Biophys. Res. Commun.* **43**, 400–408; (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1833–1837.
9. Rodbell, M. (1964) *J. Biol. Chem.* **239**, 375–380.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
11. Polson, A., Potgieter, G. M., Largrier, J. R., Mears, G. E. F. & Joubert, F. J. (1964) *Biochem. Biophys. Acta* **82**, 463–475; Leberman, R. (1966) *Virology* **30**, 341–347; Chesebro, B. & Svehag, S. E. (1968) *Clin. Chim. Acta* **20**, 527–529; Juckes, I. R. M. (1971) *Biochim. Biophys. Acta* **229**, 535–546.
12. Desbuquois, B. & Aurbach, G. D. (1971) *J. Clin. Endocrinol.* **33**, 732–738.
13. Cuatrecasas, P. (1972) *J. Biol. Chem.*, in press.
14. Marchesi, V. T. & Andrews, E. D. (1971) *Science*, **174**, 1247–1248.