Properties of the Insulin Receptor Isolated from Liver and Fat Cell Membranes*

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SUMMARY

The insulin-binding proteins isolated from membranes of rat liver and of fat cells with the nonionic detergent, Triton X-100, appear to have similar or identical physicochemical and kinetic properties. The denaturation of the soluble receptor by reagents, such as sodium dodecyl sulfate, urea, and guanidine HCl, is reversible if moderately low concentrations of the reagents are used. The binding protein is relatively stable to storage at 4 or -20°C.

Digestion of the water-soluble receptor with phospholipase C and neuraminidase does not affect its capacity to bind 125I-insulin. High concentrations of NaCl (2 M) are virtually without effect on the insulin-receptor-binding interaction. The specific insulin-binding activity of the receptor is completely destroyed by digesting with concentrations of trypsin which are too low to modify the native receptor in the intact membrane but which are equally effective in destroying the receptor of phospholipase-treated membranes.

Gel filtration experiments on columns of Sepharose 6B calibrated with several standard [14C]-labeled proteins indicate that the soluble insulin receptor has a molecular radius (Stokes radius) of about 70 Å. This large molecular size is not changed by delipidation of the membranes before extracting the receptor, by phospholipase digestion of the soluble receptor, or by performing the chromatography in buffers containing varying amounts of detergent, 2 M NaCl, or 10% sucrose. In the absence of detergent highly aggregated forms of the receptor are observed.

Sedimentation in 5 to 20% sucrose gradients containing 0.1 to 1.0% Triton X-100 reveals that the water-soluble receptor has a sedimentation constant of 11 S, as judged by the behavior of various standard [14C]-labeled proteins under similar conditions. As in the gel filtration experiments, no change in sedimentation occurs after delipidation procedures or by varying the detergent concentration of the sucrose solutions.

On the basis of gel filtration and sedimentation experiments, the receptor has a molecular weight of about 300,000, a frictional ratio of about 1.5, and an axial ratio (prolate ellipsoid) of about 0. The highly asymmetrical character of the protein is probably not explained by unusually high values of solvation or of partial specific volume (ρ). The insulin-receptor sediments in cesium chloride solutions having densities of 1.228 and 1.298, consistent with the view that it is not a lipoprotein.

Scatchard plots indicate the presence of a kinetically homogeneous binding function with a dissociation constant near 10^-10 M. The rate constants of insulin-receptor complex formation and dissociation are 2 to 3 × 10^4 mole^-1 sec^-1 and about 4 × 10^-4 sec^-1, respectively. Complex formation does not result in detectable alteration of the insulin molecule.

No specific insulin-binding activity is detected in the cytosol of fat cell homogenates, and after homogenization of fat cells treated with trypsin or agarose-trypsin, it is possible to show that intracellular membranes or particles have no significant and specific insulin-binding activity. These results argue strongly for the exclusive localization of the receptor to the cell surface.

In recent studies the insulin-binding molecules of liver and fat cell membranes have been quantitatively extracted in water-soluble form by using the nonionic detergents, Triton X-100 and Lubrol-WX (1). The water-soluble insulin-binding material obtained in this way does not sediment significantly upon centrifugation for 6 hours at 300,000 × g, and it passes unhindered through membrane filters having pores with a diameter of 0.2 μm. The specific binding of insulin to this soluble protein is a reversible and saturable process which is unaffected by a variety of unrelated peptide hormones. Chemical derivatives of insulin, however, compete with native insulin for binding in direct proportion to their biological potency. Several features of the extraction procedures, and some of the properties of the binding interaction, indicate (1) that this solubilized material represents those structures which have previously been identified in liver and fat cell membranes as being the biologically significant insulin receptors (2–6).

The present studies present more detailed properties of the insulin-binding protein solubilized from liver and fat cell membranes with Triton X-100. The results further strengthen the

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contention that this insulin-binding material is the insulin receptor of liver and adipose tissue. These studies provide a rational foundation for the purification of this receptor and for the detailed elucidation of the nature of the insulin-receptor interaction.

**MATERIALS AND METHODS**

Crystalline pork zinc-insulin (24 units per mg) was purchased from Eli Lilly; ultrapure urea, guanidine HCl, succrose, cesium chloride, bovine thyroglobulin, and sodium dodecyl sulfate from Schwarz-Mann; polyethylene glycol (Carbowaax 6000) from Union Carboide; crystalline bovine albumin, bovine γ-globulin, and apoferritin (Fraction II) from Miles; trypsin and neuraminidase from Clostridium perfringens from Worthington; phospholipase C from C. perfringens from Nutritional Biochemicals; and phospholipase A from Vipera russelli from Boehringer; microfine silica (QUSO G-32) from the Philadelphia Quartz Co.; tate tablets (25 mg) from Gold Leaf Pharmaceutical Co., Inc.; [1-3C]acetic anhydride from Amersham-Searle; and carrier-free Na125I in 0.1 m NaOH from New England Nuclear or Union Carboide.

Liver membranes from Sprague-Dawley rats (80 to 140 g) were prepared by differential centrifugation (1, 7). Fat cell membranes (4) represent the crude particulate fraction (23,000 g) of homogenates of isolated adipose tissue cells. Protein was determined by the method of Lowry et al. (8) after heating at 100° for 30 min in 1 N NaOH; bovine albumin was used as the standard. Trypsin-agarose was prepared by the cyanogen bromide procedures described earlier (3), the derivative contained 5.9 mg of trypsin per ml of agarose.

The procedures used to measure the specific binding of 125I-insulin to membranes and particulate materials have been described (3-6). 125I-Insulin was prepared and purified as described elsewhere (2).

The assay used to detect water-soluble insulin-receptor complexes has recently been described in detail (1). The assay is based on the selective precipitation of the insulin-receptor complex, but not of free insulin, by polyethylene glycol under appropriate conditions. Briefly, samples (10 to 200 μg of protein) are incubated for 30 to 60 min at 24° in 0.25 ml of Krebs-Ringer-bicarbonate buffer containing 0.1% (w/v) albumin and 125I-insulin (10-11 to 10-12 M). Determinations are performed in duplicate or triplicate, and for every such determination parallel, duplicate, or triplicate samples are performed in the presence of native insulin (25 to 50 μg per ml) to determine and correct for nonspecific binding of insulin. After incubating the samples sufficiently long to achieve binding equilibrium, an ice-cold solution of γ-globulin is added followed by polyethylene glycol (final concentration, 10%, w/v). The precipitate which forms during 15 min (in ice) is filtered and washed with 8% (w/v) polyethylene glycol over cellulose acetate (EHWP) Millipore membranes, and the radioactivity on the filter is determined in a well-type γ counter. Nearly quantitative precipitation of the insulin-receptor complex occurs, whereas less than 0.5% of the free insulin is precipitated (1).

The detailed procedures for extracting the insulin-binding proteins from membranes with detergents have been described (1). A membrane suspension (1 to 30 mg of protein per ml) in 0.05 M Tris-HCl buffer, pH 7.4, is adjusted with Triton X-100 to a final concentration of 1 or 2% (v/v). The higher concentration (2%) of Triton X-100 is used with suspensions containing more than 15 mg of protein per ml, and the lower Triton X-100 concentration (1%) is used for the less concentrated membrane suspensions; under these conditions more than 80% of the total binding activity is solubilized. The membrane suspension is incubated with Triton X-100 for 20 to 30 min at room temperature. The suspension is then centrifuged for 90 to 90 min at 44,000 rpm in a Spinco 65 rotor at 2°. The supernatant is used directly or, in certain cases, it is dialyzed for 16 hours against 0.1 m sodium phosphate buffer, pH 7.4, containing 0.1% (v/v) Triton X-100. Dialysis under these conditions does not lead to loss of insulin-binding activity although a fine precipitate frequently forms which can be removed by centrifugation at 30,000 rpm for 30 min; this precipitate does not bind insulin.

It has been shown (1) that with these extraction procedures the insulin-binding activity which disappears from the membrane pellet is recovered quantitatively in the soluble fraction. Red blood cell membranes subjected to similar procedures produce solubilized proteins with insignificant capacity for specific binding of 125I-insulin. The concentration of Triton X-100 in the supernatant solution can be decreased by dilution to 0.05% (v/v) without resulting in immediate precipitation of the receptor. With Triton X-100 concentrations of 0.05% or less, however, precipitation of the receptor occurs gradually during storage over several days. Dialysis of the Triton X-100 extracts against sodium phosphate (pH 7.4) or Krebs-Ringer-bicarbonate buffers which contain no detergent results in virtually complete precipitation of the insulin-binding proteins. This facile transition from the soluble to the particulate state, with no accompanying loss in insulin-binding activity, is also observed with Triton X-100 extracts of delipidated or phospholipase-digested membranes.

[14C]Acetyl derivatives of proteins to be used for standardization of gel filtration columns, or for markers in density gradient centrifugation experiments, were prepared by reacting the proteins with [14C]acetic anhydride. γ-Globulin (11 mg), albumin (10 mg), apoferritin (10 mg), or thyroglobulin (15 mg) were dissolved in 0.3 to 1.0 ml of 0.1 m sodium phosphate buffer, pH 8.0, and pooled in ice. Ten micromoles of [14C]acetic anhydride (5 mCi per mmole), in 50 μl of benzene, were added while the protein solution was stirred vigorously, and the reaction was maintained in ice for 90 min. The reaction mixtures were chromatographed on Sephadex G-25 (fine grade) columns (60 × 1 cm) equilibrated with 0.05 m ammonium bicarbonate buffer, pH 8.0. The radioactive material emerging in the void volume of the column was pooled, lyophilized, and redissolved in 2 ml of 0.01 m sodium phosphate buffer, pH 7.4. The specific activity of the standard proteins prepared in this way varied from 0.1 to 0.4 μCi per mg of protein.

Gel filtration chromatography of the insulin receptor for determination of effective molecular radius a (Stokes radius) (9) was performed on agarose (Sepharose 6B, Pharmacia) columns (1.5 × 87 cm) equilibrated at 4° with various detergent-containing buffers as described in the legends to the figures and tables. Samples (0.5 ml) were applied on the column after incubating with 125I-insulin (1 to 5 × 106 cpm) at 24° for 30 min. The columns were calibrated by chromatographing standard [14C]-acetyl proteins in the presence of the same detergent-containing buffers. The gel filtration data are expressed in terms of Kav, the parameter defined by Laurent and Killander (10) as follows.

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1 B. Desbuquois, F. Krug, and P. Cuatrecasas, manuscript in preparation.
TABLE I

Effect of various protein-denaturing reagents on binding of insulin to water-soluble receptors of liver membranes

Solubilized protein (0.65 mg per ml) from liver membranes was incubated in 0.2 ml of Krebs-Ringer-bicarbonate buffer containing 0.1% (w/v) albumin, 3.2 X 10^{-10} M 125I-insulin, and the indicated denaturant for 45 min at 24°C. Specific binding was measured as indicated in the text with the polyethylene glycol assay. Values are expressed as mean value ± standard error of the mean (three determinations).

| Denaturant                  | Specific binding of 125I-insulin (nmole X 10^3/mg protein) |
|-----------------------------|------------------------------------------------------------|
| None                        | 25.5 ± 0.9                                                 |
| Sodium dodecyl sulfate, 0.05% (w/v) | 10.2 ± 0.7                                               |
| Sodium dodecyl sulfate, 0.1% (w/v) | 5.4 ± 0.4                                               |
| Urea, 0.9 M                 | 26.7 ± 0.7                                                 |
| Urea, 2.0 M                 | 13.8 ± 0.6                                                 |
| Urea, 3.0 M                 | 3.2 ± 0.4                                                  |
| Urea, 5.0 M                 | <0.5                                                       |
| Guanidine HCl, 0.0 M        | 24.9 ± 1.1                                                 |
| Guanidine HCl, 2.0 M        | 9.4 ± 0.5                                                  |
| Guanidine HCl, 3.0 M        | 2.8 ± 0.3                                                  |
| Guanidine HCl, 4.0 M        | <0.5                                                       |
| Glycerol, 10% (v/v)         | 17.3 ± 0.7                                                 |
| Glycerol, 20% (v/v)         | 9.8 ± 0.6                                                  |

TABLE II

Reversibility of prior incubation of water-soluble insulin receptor of liver membranes with protein denaturants

Samples of "Triton"-soluble membrane protein (4.2 mg per ml) were incubated for 20 min at 24°C with the indicated reagent ("Previous incubation"). Of these incubations, 20-μl aliquots were then added to assay mixtures containing 0.18 ml of Krebs-Ringer-bicarbonate buffer containing 0.1% (w/v) albumin and 4.1 X 10^{-10} M 125I-insulin. The samples were incubated for 45 min at 24°C and assayed for specific insulin binding. Values are the mean ± standard error of three determinations.

| Denaturant      | Concentration | Specific binding of 125I-insulin (nmole X 10^3/mg protein) |
|-----------------|---------------|------------------------------------------------------------|
|                 | Previous      | In assay mixture                                          |
|                 | incubation    | mixture                                                   |
| None            | 0.16%         | 0.016%                                                    |
|                 | 0.36%         | 0.035%                                                    |
|                 | 0.48%         | 0.048%                                                    |
| Urea            | 3 μm          | 0.3 μm                                                    |
|                 | 4 μm          | 0.4 μm                                                    |
|                 | 5 μm          | 0.5 μm                                                    |
| Guanidine-HCl   | 2 μm          | 0.2 μm                                                    |
|                 | 3 μm          | 0.3 μm                                                    |
|                 | 4 μm          | 0.4 μm                                                    |
| Glycerol        | 20%           | 2%                                                        |

\[ K_{AV} = \frac{V_a - V_o}{V_i - V_o} \]

where \( V_a \) is the elution volume corresponding to the peak concentration of the solute, \( V_o \) is the void volume of the column as determined by the appearance of blue dextran 2000 (Pharmacia), and \( V_i \) is the total volume of the gel bed.

Density gradient centrifugation on 5 to 20% (v/v) sucrose linear gradients was performed by the procedures described by Martín and Ames (11). The sucrose solutions, in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.1% (w/v) albumin and 0.2% (v/v) Triton X-100, were prepared with a Buchler triple outlet gradient mixer and polysaltic pump. The Triton X-100 membrane extracts were dialyzed for 16 hours at 4°C against the buffer to be used in preparing the sucrose solutions. Samples (0.1 or 0.2 ml) were incubated at 24°C with 125I-insulin (1 to 8 X 10^6 cpm) for 30 min and cooled in ice before layering on top of the gradients. In every centrifugal run control samples were included in which native insulin (20 μg) was added to the membrane-extracted solution before addition of 125I-insulin. A sample was also included which contained 125I-insulin but no tissue extract. Centrifugation was performed at 5°C at 34,000 rpm for 16 hours in a Spinco SW 50.1 rotor with the Spinco model L2-65B ultracentrifuge. Fractions (20 drops each) were collected by puncturing the bottom of the tube with a Buchler piercing unit. In all cases the bottom part of the empty tubes was cut, placed in 0.5 ml of 10% (w/v) sodium dodecyl sulfate, and counted for radioactivity with a liquid scintillation counter in the presence of 10 ml of TLX toluene fluorocount and 2 ml of Bio-Solv Solubilizer BBS-3 (Beckman). Portions (50 to 100 μl) of the fractions were examined for radioactivity with a well-type γ counter (for 125I) or with a liquid scintillation spectrometer (for 14C) using the counting phosphors described above. No significant quenching was detected with sucrose, sodium bromide, cesium chloride, or Triton X-100 under these conditions. Sedimentation coefficients were determined by comparison to the behavior of [14C]-acetyl apoferritin, albumin, γ-globulin, thyroglobulin, and insulin under the same conditions.

Virtually all of the studies to be described were performed on extracts from both liver and fat cell membranes to determine possible differences in the receptor from these two tissues. Although the data for both types of membrane extracts are not presented in every instance, in no case have significant differences in their properties been detected.

RESULTS

Effect of Protein Denaturants—It has been shown previously (1) that the nonionic detergents, Triton X-100 and Lubrol-WX, in concentrations greater than 0.5% (v/v) and 0.2% (w/v), respectively, decrease the binding of insulin to the water-soluble insulin receptor if they are present in the incubation mixture used in the binding assay. However, simple exposure of the membrane proteins to high concentrations (3%) of these detergents does not adversely affect the insulin-binding protein since removal of the detergent by dialysis or dilution fully restores the capacity of this protein to bind insulin specifically. It is of interest that dialysis of the Triton X-100 extracts against detergent-free buffers results in the formation of precipitates which contain all of the insulin-binding activity originally present in the intact membrane or in the detergent-solubilized proteins.

Commonly used protein-denaturing reagents, such as sodium dodecyl sulfate, urea, and guanidine, caused a marked loss in binding activity (Table I). Glycerol in concentrations greater than 20% (v/v) similarly interferes with the insulin-receptor interaction. Some of the adverse effects of these reagents on the insulin-binding protein appear to be reversible by simple dilution.
of the denaturant (Table II). By comparing the data in Tables I and II it is apparent that although 0.16% sodium dodecyl sulfate, 3 M urea, and 20% glycerol cause severe or nearly total suppression of binding, the effects are almost completely reversed by decreasing the concentration of the reagent 10-fold. Exposure of the insulin receptor to higher concentrations of these reagents results in apparent irreversible denaturation.

**Stability of Soluble Receptor to Conditions of Storage**—The insulin receptor extracted from liver and fat cell membranes with Triton X-100 is reasonably stable to storage for at least 4 weeks at 4 or -20°C as long as the concentration of the detergent is greater than 0.5% (Table III). The lower temperature is preferable since greater stability is maintained and fewer problems are encountered with bacterial contamination. During storage, particularly with freezing, a fine precipitate develops which contains no insulin-binding activity and which can be removed readily by centrifugation. It has been shown that repeated (four times) freezing and thawing by itself is not injurious to the receptor. Storage in 20% (v/v) glycerol does not provide protection against the loss of binding activity (Table III). Dithiothreitol at concentrations of 0.1 and 1 mM is also not helpful in maintaining stability during storage at 4 or -20°C. It is also of interest, furthermore, that this reagent does not decrease the insulin-binding activity of the receptor under these conditions.

**Effect of NaCl and EDTA**—Exposure of liver (5) or fat cell (4) membranes to increasing concentrations of NaCl (up to 2 M) results in a 3- to 6-fold enhancement in the specific binding of insulin to these membranes, presumably as a result of displacement of membrane phospholipids and consequent exposure of normally masked receptors (4). In contrast, only small effects on the binding of 125I-insulin to the soluble receptor are observed by varying the concentration of NaCl in the assay mixture from 0.4 to 3 M (Table IV). With 2 M NaCl the specific binding of insulin to the Triton-solubilized membrane proteins is increased by 10 to 20%, and with 3 M NaCl the binding is decreased. In harmony with the evidence that heavy metals are not required for the formation of specific insulin-receptor complexes in intact membranes (4), no significant effects on the binding of insulin to the soluble receptor are detected by adding the metal chelator, EDTA (Table IV).

**Effect of Enzymic Digestions**—The solubilized insulin-binding protein of liver and fat cell membranes is readily destroyed by mild trypsic digestion (Table V). Profound effects are observed with concentrations (0.5 to 5 µg per ml) of trypsin which are too low to modify the insulin receptor in intact liver or fat cell membranes (3). Such low concentrations of the enzyme, however, effectively destroy the insulin receptor of phospholipase-treated or delipidated membranes (3). Furthermore, unlike the effects observed after trypsin digestion of intact fat cells or fat cell membranes (5), exposure of liver (5) or fat cell (4) membranes to increasing concentrations of NaCl (up to 2 M) results in a 3- to 6-fold enhancement in the specific binding of insulin to these membranes, presumably as a result of displacement of membrane phospholipids and consequent exposure of normally masked receptors (4). In contrast, only small effects on the binding of 125I-insulin to the soluble receptor are observed by varying the concentration of NaCl in the assay mixture from 0.4 to 3 M (Table IV). With 2 M NaCl the specific binding of insulin to the Triton-solubilized membrane proteins is increased by 10 to 20%, and with 3 M NaCl the binding is decreased. In harmony with the evidence that heavy metals are not required for the formation of specific insulin-receptor complexes in intact membranes (4), no significant effects on the binding of insulin to the soluble receptor are detected by adding the metal chelator, EDTA (Table IV).
membranes (3), the decreased binding of insulin to soluble proteins treated with low concentrations of trypsin appears to be complete and is not accompanied by detectable changes in the affinity of the binding protein for insulin.

Digestion of the solubilized membrane proteins with high concentrations of purified neuraminidase from *C. perfringens* (12) does not modify the specific insulin binding to these proteins (Table V). Similarly, digestion with high concentrations of phospholipases A and C are without effect (Table V).

**Gel Filtration and Determination of Stokes Radius**—The agarose filtration chromatography pattern of the solubilized insulin-binding protein (Fig. 1) suggests that it is a protein of large size and that its affinity for insulin must be very great. The nearly total prevention of binding of $^{125}$I-insulin by native insulin is easily demonstrable in these experiments. The reversibility of this insulin-protein complex has been shown earlier by gel filtration experiments on Sephadex G-50 (1). Similar experiments with Triton X-100 extracts of red blood cell ghosts do not result in significant radioactivity which is displaceable by native insulin.

**Effect of Various Chromatographic Buffers and of Delipidation Procedures of Membranes on Gel Filtration Chromatography Patterns of Water-Soluble Insulin Receptor**

Water-soluble Triton X-100 extracts (0.5 ml) of liver and fat cell membranes were chromatographed on the Sepharose 6B column described in Fig. 1 after incubating with $^{125}$I-insulin (5 $\times$ $10^6$ cpm) for 30 min at 24°C. The conditions of chromatography are as described in Fig. 1 except that the column- and equilibrating buffer was altered as indicated in the table. The chromatographic patterns obtained in each case are as described in Fig. 1 except that the distribution coefficient (10) refers to the fast moving peak of Fig. 1 and is the mean value $\pm$ standard error of the mean. The numbers in parentheses refer to the number of experiments.

| Condition of chromatography | Distribution coefficient ($K_{av}$) |
|-----------------------------|-----------------------------------|
|                            | Liver                              | Fat                    |
| Column buffer               |                                    |                       |
| Triton X-100, 0.1% (v/v)    | 0.30 $\pm$ 0.01 (3)                | 0.31 (2)              |
| Triton X-100, 1.0% (v/v)    | 0.29 $\pm$ 0.01 (3)                | 0.30 (2)              |
| No Triton $^a,b$            | 0.21 (2)                           | 0.20 (2)              |
| NaCl, 1.5 M                 |                                    |                       |
| Sucrose, 10% (w/v)          | 0.29 (1)                           |                       |
| Membrane treatment          |                                    |                       |
| Phospholipase C digestion $^c$ | 0.31 (2)                      | 0.30 (1)              |
| Delipidated (EtOH-ether, 3:1)$^d$ | 0.30 (1)                  |

$^a$ The magnitude of the radioactive peak was smaller than that in the presence of 0.1% Triton, indicating less binding.

$^b$ A larger radioactive peak was present in the void volume which could be abolished by previous incubation of the sample with native insulin.

$^c$ The membrane suspension was digested (37°C, 30 min) with 60 μg per ml of phospholipase C from *C. perfringens* before being extracted with Triton X-100.

$^d$ The membranes were lyophilized and extracted with ethanol-ether (3:1) in ice for 30 min (4) before resuspending in buffer and extracting with Triton X-100.

The elution position ($K_{av}$) of the insulin-receptor complex in these agarose chromatography experiments is highly reproducible, and no difference is detected in the behavior of the proteins solubilized from liver or fat cell membranes (Table VI). Furthermore, the chromatographic pattern of the insulin-receptor complex does not vary with the concentration of Triton X-100 in the buffer unless the detergent is omitted entirely or its concentration is decreased below 0.05% (v/v). Under these circumstances highly aggregated forms of the receptor occur (Table VI). No detectable changes occur in the distribution coefficient of the insulin-binding protein by performing the chromatography in buffers containing 1.5 M NaCl or 10% (w/v) sucrose (Table VI). The binding protein obtained by Triton X-100 extraction of phospholipase C-treated or delipidated membranes is indistinguishable by gel filtration from the protein which is similarly extracted from native membranes (Table VI).

It is not possible to determine the molecular weight of the insulin-binding protein by gel filtration chromatography alone since it is apparent that the elution behavior of macromolecules correlates with the Stokes radius of the protein rather than with its molecular weight (9). Accordingly, the Stokes radius of the soluble insulin receptor was estimated by comparing its gel filtration behavior on agarose with that of other standard pro-
Fig. 2. Stokes radius of water-soluble insulin-binding protein of liver and fat cell membranes calculated from gel filtration experiments according to the correlation of Laurent and Killander (10). The standard [¹²⁵I]acetylated (see "Materials and Methods") proteins were chromatographed on the same Sepharose 6B column described in Fig. 1 and Table VI. Two separate column experiments were performed for each standard protein. The distribution coefficient (KdV) for the insulin-binding protein is that described in Table VI. In all cases the column was equilibrated and run with Krebs-Ringer-bicarbonate buffer containing 0.1% (w/v) albumin and 0.05% (v/v) Triton X-100. The linear correlation indicates the validity of the correlation of KdV with Stokes radius persists in the presence of detergent in the buffer. Very similar results were obtained by chromatographing the standard proteins in buffers containing 0.05% (v/v) Triton X-100.

Fig. 3. Sedimentation behavior of insulin-binding protein of fat cell membranes on 5 to 20% sucrose gradients. Fat cell membranes were extracted with 1% (v/v) Triton X-100 and centrifuged for 70 min at 14,000 rpm as described in the text. The supernatant was dialyzed for 16 hours at 4° against 0.1 m sodium phosphate buffer containing 0.2% (v/v) Triton X-100. The supernatant (0.2 ml, containing 0.5 mg of protein) was incubated at 24° for 20 min with [¹²⁵I]insulin (3.5 × 10⁶ cpm) before being subjected to gradient centrifugation for 16 hours (11) under the conditions described in the text (C). Another sample of supernatant was processed identically except that native insulin (90 µg) was added 5 min before incubating with [¹²⁵I]-insulin (B). A sample (0.2 ml) of the dialysis buffer described above was also centrifuged (A) under identical conditions after addition of [¹²⁵I]acetyl apoferritin (6.1 X 10⁶ cpm) and [¹²⁵I]acetyl γ-globulin (7.2 X 10⁶ cpm). The broader appearance of the insulin peak in B compared to that in C probably results from aggregated forms of insulin which form in the former because of its high concentration.

Specific binding of [¹²⁵I]-insulin (displaceable by native insulin) which is detectable by these sedimentation procedures.

To calculate the sedimentation coefficient, s²₀,w, of the insulin receptor it is necessary first to determine whether the sedimentation relationships originally described by Martin and Ames (11) are still valid if detergents are present in the gradient buffers. Comparison of the sedimentation patterns of various standard proteins on 5 to 20% sucrose gradients containing 0.2% (v/v) Triton X-100 reveals that linear relationships indeed exist (Fig. 4) and that relatively reliable sedimentation constants can be determined by these procedures.

The sedimentation constant of the insulin receptor of liver and fat cell membranes was thus determined under a variety of experimental conditions (Table VII). In the absence of detergent in the sucrose solutions highly aggregated forms of the insulin-binding protein appear and virtually no 11 S material is detectable. As in the gel filtration experiments, the binding of [¹²⁵I]-insulin to the aggregated forms of the protein is quantitatively similar to the binding observed with the soluble protein, and the binding is similarly displaced by addition of native insulin. The sedimentation constant of the protein extracted from both liver and fat cell membranes is 11 S, and no change in this value is
Effect of buffer composition and of delipidation procedures of membranes on sucrose gradient sedimentation behavior of insulin receptor

Liver and fat cell membranes were extracted with 1% (v/v) Triton X-100 and centrifuged at 44,000 rpm for 70 min. The supernatants were dialyzed for 16 hours at 4°C against 0.1 M sodium phosphate buffer, pH 7.4, containing 0.27% (v/v) Triton X-100, as described in Table VI before extracting with Triton X-100 as described above; the sucrose solution in these experiments contained 0.2% (v/v) Triton X-100, as described in the text for the standard procedure. The sedimentation coefficient, $s_{20,w}$, was calculated by comparison with the sedimentation behavior of $[^{14}C]$acetyl $\gamma$-globulin and $[^{14}C]$acetyl apoferritin under similar conditions. Every experiment contained control samples in which native insulin was added to the membrane supernatant before addition of $^{125}$I-insulin. The values are expressed as the average ± standard error of the mean when more than two determinations were performed; the latter are indicated in parentheses.

**TABLE VII**

| Condition | Sedimentation coefficient ($s_{20,w}$) |
|-----------|--------------------------------------|
| Addition to gradient buffer | Liver | Fat |
| No detergent | 16.8 (2) | 17.4 (2) |
| Triton X-100, 0.2% (v/v) | 11.0 ± 0.3 (4) | 11.1 ± 0.2 (3) |
| Triton X-100, 0.5% (v/v) | 11.2 (2) | 10.8 (2) |
| Triton X-100, 1.0% (v/v) | 10.9 (2) | 11.2 (2) |
| Sodium dodecyl sulfate, 0.15% (w/v) | | |
| Membrane treatment | | |
| Phospholipase C digestion | 11.1 (2) | 10.8 (2) |
| Delipidation (ethanol-ether) | 10.8 (2) | 11.5 (1) |

* Considerable radioactivity was detected on the bottom of the tube, probably indicating aggregation.

b No significant pattern different from that of the control samples was detected.

The large values of the frictional ratio, $f/f_o^p$, and the unusual combination of large molecular size (Stokes radius) and low sedimentation coefficient ($s$) could be explained if the assumed value for solvation is grossly low, if the $\bar{v}$ of the insulin-binding protein is unusually high, as observed with lipoproteins, if the sucrose present in the sedimentation experiments decreases the molecular size of the binding protein, if the binding protein is significantly adsorbed and thus retarded during gel filtration, or if the gel filtration and sedimentation experiments cause different states of aggregation of the proteins. The last three possibilities are unlikely since the presence of high concentrations of sucrose (10%) and of NaCl (2 M), and varying the concentration of Triton in the buffer 10-fold, do not alter the gel filtration or sedimentation behavior of the protein, since the sedimentation constant of the $^{125}$I-insulin-receptor complex isolated by gel filtration is unchanged from that which is determined directly, and since the quantitative binding relationships determined by the magnitude of the radioactive peaks are similar with both types of procedures.
tion coefficient of the binding protein, suggests that the receptor delipidation procedures do not change Stokes radius or distribution of the membranes with phospholipases and strong digestion of the membranes with phospholipases and strong observed for undenatured proteins (15).

High density solutions of cesium chloride reveal good migration density greater than 1.1 (Table IX). Sodium bromide solutions binding of insulin was severely affected by solutions having a specific to perform these experiments in sucrose solutions since the specific provided the salts required to achieve these densities do not interfere with the integrity of the complex. It was not possible highly unusual solvation factors almost certainly are not involved since it can be calculated that a value of δ greater than 2 g of solvent per g of protein would be necessary to account for the Stokes radius if a frictional ratio in the normal range (1.2) is assumed. This value is an order of magnitude larger than is observed for undenatured proteins (15). A very high value for the partial specific volume (δ) of the insulin-binding protein, indicative of significant lipid content, must be considered seriously. The fact that rather vigorous digestion of the membranes with phospholipases and strong delipidation procedures do not change Stokes radius or distribution coefficient of the binding protein, suggests that the receptor is not a lipoprotein. However, it is difficult to exclude by these procedures the presence of lipid material which may be bound to the protein with extraordinary avidity or in a manner not accessible to enzymes or solvent molecules. For this reason studies were performed to determine directly the density of the insulin-binding protein. Such determinations can be performed (9, 17) on crude protein mixtures by measuring the sedimentation of the 125I-insulin-protein complex in solutions of varying density, providing the salts required to achieve these densities do not interfere seriously with the integrity of the complex. It was not possible to perform these experiments in sucrose solutions since the specific binding of insulin was severely affected by solutions having a density greater than 1.1 (Table IX). Sodium bromide solutions at concentrations greater than 20% (w/v) also interfere with binding. Very high concentrations of cesium chloride, however, do not significantly impair the specific binding of insulin (Table IX).

The sedimentation behavior of the insulin-binding protein in high density solutions of cesium chloride reveal good migration into a solution with a density of 1.228 and very small but definite sedimentation with a density of 1.208 (Table X). These experiments argue against a lipoprotein nature of the insulin-binding protein since even the most dense of lipoproteins (ρ = 1.21 g per cm³ (18)) would not have sedimented significantly in these solutions. The density of the hydrated form of the binding protein is therefore at least 1.25 g per cm³, which corresponds to a maximum hydrated specific volume of 0.80 cm³ per g (9, 19). It is of interest that in these experiments free 125I-insulin (δ = 0.735) sediments with a density of 1.0852 (Table IX), and the indicated concentration of cesium chloride. The samples were centrifuged for the indicated times at 15,000 rpm in an SW 50.1 Spinco rotor as described in the text.

| Compound | Concentration | Densitya | Specific binding of 125I-insulin |
|----------|---------------|----------|-------------------------------|
| None     |               |          |                               |
| Cesium chloride | 20% (w/v) | 1.228   | 12.3 ± 0.4  |
|           | 50% (w/v)     | 1.298   | 29.0 ± 1.4  |
| Sucrose  |               |          |                               |
| 20% (w/v) | 1.2582 | 13.5 ± 0.5 | 28.7 ± 1.8  |
| 50% (w/v) | 1.2836 | 6.2 ± 0.2 | 13.7 ± 0.5  |
| 100% (w/v)| 1.2300 | 4.8 ± 0.3 | 11.8 ± 0.3  |

a At 0°.

### Table X

Sedimentation behavior of insulin-binding protein of liver membranes on high density cesium chloride solutions

Liver membranes were extracted with 1% (v/v) Triton X-100, centrifuged at 40,000 rpm for 70 min (4°), and the supernatant was dialyzed for 16 hours (4°) against 0.1 M sodium phosphate buffer, pH 7.4, containing 0.1% (v/v) Triton X-100. The dialyzed supernatant (8 mg of protein per ml) was incubated in the presence of native insulin (50 µg per ml) with 2.1×10⁻¹⁸ M 125I-insulin for 40 min at 24°. Samples (0.2 ml) were applied on the tops of tubes containing 4.7 ml of 0.1 M sodium phosphate buffer, pH 7.4, 0.1% (w/v) albumin, 0.1% (v/v) Triton X-100, and the indicated concentration of cesium chloride. The samples were centrifuged for the indicated times at 15,000 rpm in an SW 50.1 Spinco rotor as described in the text.

| Cesium chloride | No liver extract | Radioactive peaks | No native insulin | Fast native insulin |
|----------------|------------------|-------------------|-------------------|---------------------|
| 24% (w/v), 40 hrs | 2.4 | 0.8, 2.3 | 2.4 |
| 24% (w/v), 60 hrs | 4.1 | 1.1, 4.1 | 1.0 |
| 30% (w/v), 40 hrs | 1.8 | 0.17, 1.9 | 1.9 |
| 30% (w/v), 80 hrs | 3.4 | 0.32, 3.4 | 3.4 |

a This peak represents the specific insulin-binding protein; all other peaks represent free 125I-insulin.
FIG. 5. Scatchard plot (23) of the specific binding of 125I-insulin to the water-soluble Triton X-100 extract of liver cell membranes. Specific binding was determined with the polyethylene glycol assay described in the text, with 0.15 mg of protein per ml and varying concentrations of 125I-insulin.

**TABLE XI**

Kinetic constants of interaction of insulin at 24° with receptor solubilized from liver and fat cell membranes with Triton X-100

| Kinetic constant | Units | Liver membranes | Fat cell membranes |
|------------------|-------|-----------------|-------------------|
| Association rate constant (kₐ) | mole⁻¹ sec⁻¹ | 2.3 × 10⁶ (2) | 2.9 × 10⁶ (1) |
| Dissociation rate constant (k₋₋) | sec⁻¹ | 3.8 × 10⁻⁴ (2) | 4.4 × 10⁻⁴ (1) |
| Dissociation constant (Kₐ) | M | 1.3 ± 0.8 × 10⁻¹⁰ (3) | 1.8 ± 0.9 × 10⁻¹⁰ (3) |
| From Scatchard plots | M | 1.6 × 10⁻¹⁰ | 1.5 × 10⁻¹⁰ |

* Number in parentheses refers to the number of experiments performed; when more than three experiments were performed, the data are presented as the mean value ± standard error.

proteins, since the carbohydrate content of the insulin-binding proteins is not known, and since little is known about the preferential interaction terms in CsCl solvents, it is possible that the high density observed reflects compensating contributions of lipid and carbohydrate moieties.

The combined data indicate that the molecular parameters (Table VIII) are probably reasonably valid, and that the insulin receptor is indeed a rather large, asymmetrical protein. The unique combination of a large Stokes radius and a low sedimentation constant similar to that presented in this report has recently been described for the progesterone-binding components of the chick oviduct (20), for the estrogen-binding proteins of calf uterus (21), and for the glucocorticoid-binding component of mouse fibroblasts (22).

**Kinetic Properties of Insulin Receptor Interaction**—It has recently been shown (1) that the binding of 125I-insulin to solubilized liver membrane proteins is a saturable process with a dissociation constant near 10⁻¹⁰ M. A Scatchard plot (23) of the specific binding of 125I-insulin to the Triton-solubilized proteins of liver membranes indicates that a single, high affinity receptor site is being measured and that there is no detectable interaction among the bound molecules (Fig. 5). In this experiment the maximal binding capacity of the soluble protein is about 0.17 pmole of insulin per mg of protein. The average dissociation constants calculated from three separate experiments of this type with liver and fat cell membrane extracts are about 1 to 2 × 10⁻¹⁰ M (Table XI). These values are similar to the values calculated for the insulin-receptor interaction in intact fat cells (2) and in liver and fat cell membranes (5, 6).

The formation and dissociation of the insulin-receptor complex were studied separately as described in Fig. 6. The association process is a bimolecular process, as indicated by the fit of the data to a second order equation (1, 5). Dissociation of the complex is a first order process which is highly temperature-dependent. The rate constants calculated from such data for extracts of liver and fat cell membranes are summarized in Table XI. The similarity of the dissociation constants calculated from rate constants and from Scatchard plots indicate that the rate
TABLE XII

Characterization of $^{125}$I-insulin eluted from soluble insulin-receptor complex of liver membranes

A high speed liver membrane Triton extract containing 8 mg of protein in 11.5 ml of 0.1 M sodium phosphate buffer, pH 7.4, was centrifuged for 20,000 rpm and the supernatant was neutralized with 1.5 ml of guanidine-1 N HCl, conditions which are known to irreversibly denature the insulin-binding protein. The suspension was centrifuged for 20 min at 20,000 rpm and the supernatant was neutralized with 1 N NaOH. The recovery of radioactivity from the pellet was 72%. The eluted and starting $^{125}$I-insulins were compared by physical properties (2, 4) and by the capacity to bind to liver membranes.

| Properties | $^{125}$I-Insulin |
|------------|------------------|
| Percentage precipitable by 8% trichloroacetic acid | 97% | 98% |
| Percentage adsorbed to microsilia (QUSO G-32) | 98% | 98% |
| Specific binding to liver membranes* | $1.8 + 0.3 \times 10^{-7}$ nmoles | $1.0 + 0.3 \times 10^{-7}$ nmoles |

* Amount of $^{125}$I-insulin specifically bound to native membranes with a concentration of $^{125}$I-insulin of $0 \times 10^{-12}$ M.

Table measures the same processes which are ordinarily being studied in the standard binding assays.

The nature of $^{125}$I-insulin which tightly forms a complex with the soluble membrane receptor was examined by precipitation of the complex with polyethylene glycol followed by dissociation of the separated complex with guanidine-HCl (Table XII). The insulin which is eluted in this way is indistinguishable from fresh insulin, indicating that binding to the soluble proteins is not associated with detectable degradation. Furthermore, complex formation is not accompanied by the formation of stable covalent bonds or disulfide interchange reactions.

**DISCUSSION**

Nonionic detergents such as Triton X-100 quantitatively extract in soluble form the insulin-binding structures of liver and fat cell membranes (1). The present studies provide further evidence that the detergent-solubilized molecules are very similar or identical with those high affinity binding structures which can be measured and studied in biologically responsive intact fat cells (2) and in isolated membrane preparations from liver (6) and fat cells (3). There is very strong evidence that the specific insulin-binding structures of these particulate preparations are the biologically significant receptors for insulin (reviewed in References 24 and 25).

The present studies reveal striking similarities in the kinetic properties of the interaction of insulin with the soluble and with the particulate (2, 5, 6) structures. Thus, the interaction in both cases is saturable and reversible, and it does not involve inactivation of insulin. The rate constants of association and dissociation, and the dissociation constants (about $10^{-10}$ M) determined independently from equilibrium data, are very similar in the soluble and particulate states of the receptor. In addition to the kinetic homogeneous nature of the binding interaction, the inability to detect heterogeneous patterns on gel filtration and sucrose gradient centrifugation further suggests that the insulin receptor may be a single and unique receptor class. Furthermore, the nearly identical properties of the solubilized receptors from liver and from fat cell membrane points to the possible identity of the insulin receptors from these two tissues. This possibility has been suggested earlier (6) on the basis of the similarities of the binding interactions observed in the particulate state.

It has been shown that digestion of liver or fat cell membranes with phospholipases, and that extraction of these membranes with organic solvents, results in the exposure of a substantial (2- to 6-fold) number of new insulin-binding structures of similar kinetic behavior to those which are normally exposed (4). These effects are believed to result from the hydrolysis or displacement of membrane phospholipids which normally may mask or shield these receptors from large macromolecules (such as insulin and trypsin) in the solvent. A similar unmasking of insulin-binding sites in these membranes by high salt concentrations (2 M NaCl) suggests that the phospholipid polar head groups are important in this shielding effect (5). The studies on the solubilization of the insulin receptor are in agreement with these observations. Extraction of the membranes with increasing concentrations of detergents causes a progressive loss of insulin-binding activity of the residual membranes and the concomitant appearance in the soluble fraction of binding structures in progressively higher yield than were originally present in the membranes (1). Delipidation of the membrane phospholipase digestion or by extraction with organic solvents does not lead to increased yields of binding activity after subsequent detergent extraction. Also, as shown here, digestion of the solubilized proteins with phospholipase C does not appreciably increase insulin binding, and high concentrations of NaCl do not affect the binding of insulin to the solubilized proteins. These observations suggest that solubilization of the receptor by detergents is accompanied by gross displacement of membrane phospholipids from the insulin-binding structures. This is clearly confirmed in the present studies which show that phospholipase digestion or organic solvent extraction do not alter the molecular properties of the solubilized receptor. Furthermore, the sedimentation behavior of the receptor is compatible with the absence of significant lipid material. It therefore would have been surprising if NaCl or phospholipase digestion had significantly modified the binding process. It is also of some interest that the isolated and the membrane-bound receptor are not apparently denatured and their molecular parameters are not altered by such high concentrations of salt.

The extreme susceptibility of the solubilized receptor to tryptic digestion is also consistent with the removal of membrane phospholipids during the solubilization procedure. It has been shown (3, 4) that after digesting liver or fat cell membranes with phospholipase C the insulin receptor is rapidly and completely destroyed by concentrations of trypsin which are too low to measurably alter the receptor of normal cells. Furthermore, the primary early effect of tryptic digestion of normal cells or membranes is to decrease the affinity of the receptor for insulin without changing the maximal binding capacity (3). In contrast,
Absence of specific binding of insulin to cytosol and to intracellular membrane structures of isolated fat cells

The fat cells isolated from 20 Sprague-Dawley rats (140 g) were suspended in 18 ml of Krebs-Ringer-bicarbonate buffer containing 0.5% (w/v) albumin. A fraction (8 ml) of this cell suspension was digested with trypsin (0.5 mg per ml) and another similar fraction with trypsin-agarose (1 mg per ml, Reference 4) for 10 min at 37° to which time soybean trypsin inhibitor (2 µg per ml) was added. The trypsin-agarose was then removed by centrifugation and washing with three changes of buffer (10 ml); the cells were suspended in 8 ml of buffer. Samples of these cells (intact cells) were assayed for specific 125I-insulin binding (2). Fractions (5 ml) of these cells were homogenized with a Polytron PT-10 (Brinkmann) for 30 sec at a setting of 3.0. Specific insulin binding (2, 5) was determined on samples of these whole homogenates. Other portions (3 ml) of the homogenates were centrifuged at 30,000 rpm for 30 min. The pellets were resuspended in 3 ml of the same buffer (particulate fraction), dispersed with the Polytron (10 sec), and tested for insulin binding by the same procedures. The specific binding of insulin to the supernantant (soluble) fraction was determined with the polyethylene glycol assay (1) described in the text. All incubations contained 0.25 ml of Krebs-Ringer-bicarbonate buffer, 0.5% (w/v) albumin, and 8 × 10^{-11} M 125I-insulin; control samples in addition contained native insulin (40 µg per ml) to correct for nonspecific binding. The samples were incubated for 45 min at 24°. Results are expressed as the mean ± standard error of the mean for three observations.

| Cells                        | Specific binding of 125I-insulin (cpm) |
|------------------------------|---------------------------------------|
| Normal cells                 |                                       |
| Intact cells                  | 8820 ± 530                            |
| Homogenate                   | 8360 ± 460                            |
| Whole homogenate             | 7830 ± 610                            |
| Soluble fraction             | 210 ± 80                              |
| Trypsin-treated cells        |                                       |
| Intact cells                  | 240 ± 140                             |
| Homogenate                   | 210 ± 60                              |
| Whole homogenate             | 150 ± 80                              |
| Soluble fraction             | 50 ± 110                              |
| Trypsin-agarose-treated cells|                                       |
| Intact cells                  | 160 ± 90                              |
| Homogenate                   | 190 ± 80                              |
| Whole homogenate             | 160 ± 70                              |
| Soluble fraction             | 110 ± 90                              |

This effect on binding affinity is not detectable by tryptic digestion of phospholipase-treated cells (4); the effects in this case are to totally abolish binding activity. This is consistent with the view that in its normal environment in the membrane the most critical or central portions of the insulin receptor are at least partially shielded from proteolytic attack by phospholipids. The present studies are also consistent with these views since the solubilized receptor is destroyed completely by tryptic digestion comparable to that required to destroy the receptor of phospholipase-treated membranes.

It is of considerable interest that the solubilized insulin receptor does not appear to be a lipoprotein. The lack of effects on the molecular parameters of the membrane-solubilized proteins by previous digestion of the membranes with phospholipases or by delipidation with organic solvents, and the sedimentation of the insulin-binding protein in cesium chloride solvents of densities as high as 1.298, strongly suggest a low content of lipid in this protein. The insulin-binding function of this receptor appears to be independent of membrane lipids since its detailed binding properties (affinity and rate constants) are so similar to those observed in the intact cells or membranes. It must be stressed, however, that the insulin receptor must have at least another distinct function, that of transmitting to other molecules the existence of "insulin binding." This function of the receptor, which at present is not understood and is not measurable in the isolated protein, may well be dependent on membrane lipids. It is very likely, furthermore, that membrane lipids are essential for the proper positioning and orientation of the insulin-binding protein within the cell membrane. It is of interest that Levey (26) has used Lubrol-PX to 'solubilize' from cat heart adenyl cyclase activity which on addition of exogenous phospholipids acquires responsiveness to glucagon. Pohl et al. (27) suggest that membrane lipids play an essential role in the process by which glucagon modulates the adenyl cyclase activity of liver membranes. Pastan et al. (28) have prepared from adrenal tumor nonsedimentable particles, presumably lipid-rich structures, which contain hormone-sensitive adenyl cyclase activity.

The present studies serve as a rational basis for the purification of the insulin receptor, since this structure has been separated from most of the components of the membrane without severely compromising its insulin-binding function. The possible contribution of other membrane components to the integrated biological function of the receptor will depend on the identification of the processes which immediately follow the initial insulin-receptor interaction. It has recently been shown that insulin at physiological concentrations (10^{-9} to 10^{-11} M) can inhibit the stimulated adeny cyclase activity of isolated liver and fat cell membranes (7). This biological effect at a subcellular level suggests an experimental system which should be useful in elucidating the manner by which the insulin receptor is coupled to subsequent processes. It will be important, for example, to determine whether the effect of insulin on the adeny cyclase activity of these membranes occurs directly or through chemical mediators present in the membrane. It will also be of interest to determine whether the purified receptor complex for insulin, which is of substantial size, contains some or all of the catalytic components of the adeny cyclase enzyme.

It has not yet been possible by physical methods to separate the insulin receptor (molecular weight about 300,000) into smaller components which retain the capacity to bind insulin by separation procedures which utilize denaturants, such as sodium dodecyl sulfate, urea, or guanidine-HCl. The extremely small quantities of the receptor and the very crude nature of the mixture in which it is found complicate such studies. It will probably be necessary to purify the receptor before its possible subunit structure is resolved. Furthermore, it will be important to substantiate the molecular parameters described in this report on the purified

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TABLE XIII

Absence of specific binding of insulin to cytosol and to intracellular membrane structures of isolated fat cells

| Cells                        | Specific binding of 125I-insulin (cpm) |
|------------------------------|---------------------------------------|
| Normal cells                 |                                       |
| Intact cells                  | 8820 ± 530                            |
| Homogenate                   | 8360 ± 460                            |
| Whole homogenate             | 7830 ± 610                            |
| Soluble fraction             | 210 ± 80                              |
| Trypsin-treated cells        |                                       |
| Intact cells                  | 240 ± 140                             |
| Homogenate                   | 210 ± 60                              |
| Whole homogenate             | 150 ± 80                              |
| Soluble fraction             | 50 ± 110                              |
| Trypsin-agarose-treated cells|                                       |
| Intact cells                  | 160 ± 90                              |
| Homogenate                   | 190 ± 80                              |
| Whole homogenate             | 160 ± 70                              |
| Soluble fraction             | 110 ± 90                              |
material. The need for the continued presence of detergents in low concentration to maintain the receptor in soluble form should not in itself preclude or invalidate further and complete purification.

There are some interesting similarities between the molecular properties of the membrane-solubilized insulin receptor described here and the properties of steroid hormone receptors (20–22) present in the soluble fraction of various tissues which have been characterized in crude form by procedures similar to those described here. These are also large, asymmetrical, and lipid-free proteins which exhibit the unique combination of large Stokes radii and low sedimentation constants.

The availability of a sensitive and reliable assay (1) for detecting soluble insulin-receptor complexes offers an opportunity to test the view (2, 4, 5, 29) that insulin receptors are present exclusively in particulate structures of the cell. With the polyethylene glycol assay used in this report it is not possible to show significant binding of 125I-insulin to the soluble fraction of a fat cell homogenate (Table XIII). Furthermore, such experiments confirm the contention that, at least in isolated fat cells, the insulin receptor is confined exclusively to cell surface or to cytoplasmic membranes. After modification of the fat cell surface with trypsin or trypsin-agarose breakage of the cell does not unveil new insulin-binding structures in the particulate or soluble fractions, thus excluding specific binding of insulin to intracellular membrane structures or to the cytosol. It is of importance, furthermore, that the insulin-binding activity of normal intact fat cells is recovered quantitatively in the particulate cell fraction after homogenization (Table XIII and Reference 2), and that insoluble and bulky polymers of insulin retain the varied biological activity of the native hormone when tested on isolated fat cells (29), mammary gland cells (30, 31), and liver cells (32).

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