Properties of the Insulin Receptor of Isolated Fat Cell Membranes*

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SUMMARY

The interaction of 125I-insulin with crude membrane preparations from isolated fat cells has many properties in common with the interaction of insulin with biologically significant receptors in intact fat cells. Specific binding of 125I-insulin to the membranes is a saturable process with respect to insulin and to membranes, and native insulin competes for binding in a way expected from the biological identity of the 2 molecules. Reduced and desoctapeptide insulins do not compete with 125I-insulin for binding, and desalanine insulin is indistinguishable from native insulin. Proinsulin binds to the membranes with an affinity which is 20 times less than that of native insulin.

Insulin in the incubation medium containing fat cell membranes is not significantly destroyed under the conditions used in the binding assay. The 125I-insulin-membrane complex can be dissociated by acid, and the insulin thus released from this complex is similar to native insulin by several physical properties, by its ability to bind to membranes, and by its ability to stimulate glucose oxidation in isolated fat cells.

The rate constants of insulin-membrane association (8.5 X 10^7 mole^-1 sec^-1) and dissociation (4.2 X 10^-4 sec^-1) have been measured independently, and the dissociation constant (5 X 10^-11 M) based on these rate constants is similar to that (7.5 X 10^-11 M) calculated separately from equilibrium data. These constants are similar to those calculated for the interaction of insulin with intact fat cells. Measurements of the rate constants at various temperatures indicate that binding is much tighter at lower temperatures because the decrease in dissociation rate is disproportionately greater than the decrease in association rate. Thermodynamic calculations reveal a ΔF of -14 kcal mole^-1, a ΔH of -28 kcal mole^-1, and a ΔS of -45 cal mole^-1 deg^-1.

Binding of 125I-insulin to membranes is unaffected by the nature of the buffer used or by a number of different ionic species, heavy metals, or by metal-complexing agents. The optimum pH for binding occurs sharply at about 7.5, and the pH range over which binding occurs is rather narrow. A sharp thermal transition, with a midpoint at 53°, results in irreversible inactivation of binding activity.

Insulin binding to membranes is markedly affected by the ionic strength of the medium. Increasing concentrations of NaCl up to 2 M cause a dramatic (6-fold) increase in insulin binding. This probably results from the appearance (unmasking) on the membrane of new binding sites for insulin. The latter are kinetically identical with those normally exposed. These effects of 2 M NaCl, which appear to be qualitatively similar to the effects of digesting the membrane with phospholipase C, are readily reversible by decreasing the ionic strength of the medium.

Modification of the membranes with several protein agents suggests that tyrosyl and possibly histidyl residues may be important in the binding interaction. No evidence is present for the involvement of sulphydryl, tryptophanyl, or carboxyl groups of the membrane.

Techniques have been developed which permit measurement of the specific binding of insulin with the receptors of intact, metabolically active, isolated adipose tissue cells (1, 2). Many kinetic parameters of the insulin-receptor interaction have been studied directly, and the results have been correlated with the biological response of these cells to insulin (1). The binding of insulin and insulin responsiveness (glucose transport) have been studied in parallel after digesting isolated fat cells with sialidases (3), proteolytic enzymes (2), and phospholipases (4). In these studies on intact cells there is reasonable assurance that biologically significant receptor interactions are being studied by techniques which depend on measuring the physical binding of 125I-insulin to the cells.

Further characterization of the insulin receptor, which hopefully will lead toward its isolation and purification, must be performed on subcellular components. Since disruption of the cell results in the loss of all known biological effects of insulin, detection of insulin-specific structures must be based on measurements of physical interactions. The biological relevance of these interactions, and their relationship to the receptor interactions observed in the intact cell, can be surmised by comparing the detailed properties of the binding interaction in the intact cell and in the disrupted cell preparation.

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The capacity of intact fat cells to specifically bind insulin is recovered quantitatively in the particulate fraction (23,000 x g) of cell homogenates (1). Specific insulin binding, which in the intact cell is a kinetically homogeneous, reversible process which exhibits extraordinarily high affinity and specificity, is not recovered in the nuclear or mitochondrial subfractions of the particulate cell fraction (1). This and other independent data (4, 5) suggest that the insulin receptor is present in the plasma membrane of the cell. The present report presents detailed data on the properties of the binding interaction between insulin and the particulate membranes of isolated fat cells. The nearly identical nature of this interaction with that of the intact cell indicates that the plasma membranes used in these studies probably possess the full complement of biologically significant insulin receptors. These studies form a rational basis for the further characterization and isolation of the insulin-binding structures of plasma membranes from adipose tissue.

MATERIALS AND METHODS

Crystalline pork zinc-insulin (24 units per mg) was obtained from Eli Lilly, [U-14C]glucose (10 μCi per μ mole) and carrier-free NaO18 in 0.1 M NaOH from New England Nuclear. Phospholipase C from Clostridium perfringens was obtained from Nutritional Biochemicals. Microfine silica, QUSO G-32, was obtained from the Philadelphia Quartz Co., and tale tablets (25 mg) from Gold Leaf Pharmacal Co., Inc. Adenosine triphosphate, cytidine triphosphate, uridine triphosphate, thymidine triphosphate, guanosine triphosphate, iodosacetamide, N-ethylmaleimide, and nystatin were obtained from Calbiochem. Pyruvate, D- and L-lactate, acetylcobalamin, and D-glucose were purchased from Sigma. Dexamethasone was obtained from Merck, Sharp and Dohme. 2-Hydroxy-5-nitrobenzyl (6, 7) and 2-methoxy-5-nitrobenzyl bromide (8) were from Nutritional Biochemicals. Tetranitromethane (9, 10) and 5-aminotetrazole (11, 12) were from K and K. Glycine-O-methyl ester was from Fox Chemical Co., 1,5-difluoro-2,4-dinitrobenzene sulfonate and 1-ethyl-3-(3-dimethylaminopropyl) carbo timidide (13) were purchased from Pierce Chemical Co. Prostaglandin were kindly provided by Dr. B. Pike, Upjohn Co., and the prostaglandin inhibitor, 7-oxa-13-prostynoic acid, was a gift from Dr. J. Fried. Desaline and deoctapeptide insulin derivatives, and porcine proinsulin were obtained from Eli Lilly.

125I-Insulin (1300 to 1600 mCi per μ mole) was prepared and purified as described previously (1, 2). Iodinated insulin derivatives prepared in this way have been shown to be biologically active (1). Furthermore, Freychet et al. (14) have recently shown that monoiodoinsulin separated from native insulin by ion exchange chromatography is fully active biologically. The assay for specific binding of 125I-insulin to membranes was a slight modification (4, 15) of that used to measure specific binding of insulin to intact fat cells (1, 2). Briefly, membranes are incubated at 24° to equilibrium in 0.2 ml of Krebs-Ringer-bicarbonate buffer containing 1% (w/v) albumin and 125I-insulin (10-18 to 10-12 M). Three millilitters of ice-cold Krebs-Ringer-bicarbonate buffer containing 0.5% (w/v) albumin is added and the contents are passed through cellulose acetate EGWP Millipore filters positioned with vacuum, and the filters are washed under vacuum with 10 ml of ice-cold Krebs-Ringer-bicarbonate buffer containing 0.1% (w/v) albumin. Every determination of binding is performed in triplicate, and for every such determination, parallel, triplicate samples are performed in the presence of native insulin (20 to 40 μg per ml) to determine and correct for nonspecific binding of insulin (1, 2). As stressed previously (2), it is imperative that such corrections be performed in order to accurately determine "specific" insulin binding. Determination of radioactivity has been described in detail (1).

Isolated fat cells, prepared by the method of Rodbell (6, 16), were suspended in Krebs-Ringer-bicarbonate buffer and homogenized with a Brinkmann Polytron PT-10 (setting 2.2) for 40 sec. The suspension was centrifuged at 24,000 × g for 30 min, and the pellet was washed in the same buffer used for homogenization. It has been shown (1) that the binding activity of the intact cells is present quantitatively in this pellet, and subsequent fractionation does not reveal significant binding in the nuclear or mitochondrial fractions. The present studies were performed on the unfractionated pellet (referred to as "membrane" preparation) since at this stage of study it is most important to determine the properties of the binding activity of the membranes in the broken cell with the least possible disruption or loss of potentially significant material. Further fractionation and purification must systematically follow careful characterization of the properties of the crude particulate material. Protein was determined by the method of Lowry et al. (17) after heating the membrane samples in a boiling water bath in 1.0 N NaOH for 30 min; bovine albumin was used as the standard.

RESULTS

Effects of 125I-Insulin Concentration—The specific binding of insulin to fat cell membranes is a saturable process with respect to insulin (Fig. 1). Specific binding of 125I-insulin is readily detected at 10-11 M insulin, and saturation is nearly complete at 4 × 10-10 M insulin. The concentration of insulin at which half-maximal saturation occurs is about 10-10 M, and the maximal binding capacity of the crude membrane preparation is about 0.2 pmole of insulin per mg of protein. The dissociation constant of the binding interaction, calculated (1) from the equilibrium data described in Fig. 1, is 7.5 (± 0.4) × 10-11 M.

Displacement of Binding by Insulin Derivatives—Very low concentrations of native insulin readily displace 125I-insulin specifically bound to the fat cell membranes (Fig. 2). Derivatives of insulin were tested for their ability to interfere with the
specific binding of \textsuperscript{125}I-insulin to membranes (Fig. 2). Removal of the carboxyl-terminal alanine residue of the B chain does not appreciably modify insulin binding, consistent with the full biological activity of this insulin derivative (18-20). Desoctapeptide insulin, which is devoid of biological activity (21, 22), does not compete with \textsuperscript{125}I-insulin for binding to the membranes. Proinsulin can compete for binding but only at higher concentrations than native insulin. From such data the affinity of proinsulin for the fat cell membranes appears to be about 20 times less than that of native insulin. Nearly identical results are observed in studies of \textsuperscript{125}I-insulin binding to intact fat cells.\textsuperscript{1} Reduced insulin, prepared by incubating native insulin (1 mg per ml) with 0.2 \text{m} dithiothreitol in \text{0.1 m NaHCO}\textsubscript{3} buffer, \text{pH} 8.2, for 2 hours at 37\textdegree, does not cause significant displacement at concentrations as high as 20 milliunits per ml (not shown in figure).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Effect of native insulin (\textbullet{}), desalanine insulin (\textsquare{}), desoctapeptide insulin (\textdiamond{}), and proinsulin (\texttriangle{}), on the displacement of specific \textsuperscript{125}I-insulin binding to fat cell membranes. Suspensions containing 145 \textmu{g} of membrane protein in 0.2 ml of Krebs-Ringer-bicarbonate buffer with 1\% (w/v) albumin were incubated with 2.3 \times 10^{-11} \text{M} \textsuperscript{125}I-insulin and the indicated insulin derivative for 40 min at 24\textdegree. Specific insulin binding was then determined by membrane filtration procedures as described in the text. The concentrations were determined on the basis of 24 microunits per mg of insulin, desalanine insulin, and desoctapeptide insulin, and 24 units per 1.5 mg of proinsulin (molecular weight 9,000). Reduced insulin, prepared by incubating native insulin (1 mg per ml) with 0.2 \text{m} dithiothreitol in \text{0.1 m NaHCO}\textsubscript{3} buffer, \text{pH} 8.2, for 2 hours at 37\textdegree, does not cause significant displacement at concentrations as high as 20 milliunits per ml (not shown in figure).
}
\end{figure}

FIG. 2. Effect of native insulin (\textbullet{}), desalanine insulin (\textsquare{}), desoctapeptide insulin (\textdiamond{}), and proinsulin (\texttriangle{}), on the displacement of specific \textsuperscript{125}I-insulin binding to fat cell membranes. Suspensions containing 145 \textmu{g} of membrane protein in 0.2 ml of Krebs-Ringer-bicarbonate buffer with 1\% (w/v) albumin were incubated with 2.3 \times 10^{-11} \text{M} \textsuperscript{125}I-insulin and the indicated insulin derivative for 40 min at 24\textdegree. Specific insulin binding was then determined by membrane filtration procedures as described in the text. The concentrations were determined on the basis of 24 microunits per mg of insulin, desalanine insulin, and desoctapeptide insulin, and 24 units per 1.5 mg of proinsulin (molecular weight 9,000). Reduced insulin, prepared by incubating native insulin (1 mg per ml) with 0.2 \text{m} dithiothreitol in \text{0.1 m NaHCO}\textsubscript{3} buffer, \text{pH} 8.2, for 2 hours at 37\textdegree, does not cause significant displacement at concentrations as high as 20 milliunits per ml (not shown in figure).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Specific binding of \textsuperscript{125}I-insulin to fat cell membranes as a function of the concentration of membrane protein in the medium. Incubation media contained the indicated amount of membrane in 0.2 ml of Krebs-Ringer-bicarbonate buffer containing 1\% (w/v) albumin and 4.5 \times 10^{-11} \text{M} \textsuperscript{125}I-insulin. After incubating for 30 min at 24\textdegree specific binding was determined as described in the text.
}
\end{figure}

\textbf{Nature of \textsuperscript{125}I-Insulin Incubated with Membranes—}No significant destruction of medium \textsuperscript{125}I-insulin could be detected under the usual conditions of incubation utilized in these studies. Samples containing 0.4 \textmu{g} of membrane protein in 0.5 ml of Krebs-Ringer-bicarbonate buffer containing 1\% (w/v) albumin, 9 \times 10^{-11} \text{M} \textsuperscript{125}I-insulin, were incubated for 5, 10, 20, and 40 min at 24\textdegree. After Millipore filtration, the \textsuperscript{125}I-insulin present in the filtrate was virtually indistinguishable from that not exposed to fat cell membranes in terms of binding to fresh membranes. No significant inactivation of insulin occurs with these crude membrane preparations under the conditions used here.

The \textsuperscript{125}I-insulin in tight complex with the membranes can be eluted from the complex with an acidic buffer. This \textsuperscript{125}I-insulin is nearly identical with the \textsuperscript{125}I-insulin which has not been exposed to membranes with respect to several physical properties, ability to bind to fresh fat cell membranes, and capacity to stimulate glucose transport in intact fat cells (Table I). Thus, formation of the insulin-membrane complex does not seem to result in significant or irreversible chemical alterations of the insulin molecule. Furthermore, binding does not involve formation of insulin-membrane disulfide bridges. The absence of significant purification of \textsuperscript{125}I-insulin by specific adsorption to membranes in this and other similar experiments provides further evidence for the identity of native and \textsuperscript{125}I-insulins.

\textbf{Rate Constants of Insulin-Membrane Interaction—}The binding of \textsuperscript{125}I-insulin to fat cell membranes is a time- and temperature-dependent process (Fig. 4). The rate constants of association can be calculated from this data, since they satisfy second order kinetics in a manner described earlier for insulin-cell association kinetics. At 25\textdegree, the rate of association is 8.5 (\pm 0.4) \times 10^{4} \text{ mole}^{-1} \text{sec}^{-1}.

The \textsuperscript{125}I-insulin-membrane complex dissociates spontaneously by a process which is very sensitive to temperature (Fig. 5). The dissociation data obey first order kinetics in a manner similar to the dissociation of the insulin-cell (1) and insulin-liver

\footnotesize
\textsuperscript{1} P. Cuatrecasas, unpublished observations.
TABLE I
Characterization of \(^{125}\)I-insulin eluted from insulin-fat cell membrane complex

| Properties                              | Native                              | Eluted from membranes |
|-----------------------------------------|-------------------------------------|------------------------|
| Percentage precipitable by 8\% trichloroacetic acid | 97                                  | 98                     |
| Percentage adsorbed to talc             | 96                                  | 97                     |
| Percentage adsorbed to microsilica (QUSO G-32) | 98                                  | 98                     |
| Specific binding to membranes\(a\)      | 5.3 \(\times\) \(10^{-12}\) M \(^{125}\)I-insulin | 1.2 \(\pm\) 0.3 \(\times\) \(10^{-7}\) nmoles |
|                                         | 1.4 \(\times\) \(10^{-11}\) M \(^{125}\)I-insulin | 2.1 \(\pm\) 0.1 \(\times\) \(10^{-7}\) nmoles |
| Conversion of \[^{14}\]C-glucose to \[^{14}\]CO\(_{2}\) | 6 microunits per ml                 | 310%                   |
|                                         | 83 microunits per ml                | 760%                   |

\(a\) Amount of \(^{125}\)I-insulin specifically bound to native membranes with two different concentrations of native and eluted insulin, expressed as the mean value \(\pm\) standard error of the mean (three observations).

\(b\) Expressed as the percentage increase of the basal rate of glucose oxidation of isolated fat cells (2, 5).

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**Fig. 4.** Time dependence of specific binding of \(^{125}\)I-insulin to fat cell membranes at various temperatures. Membranes (60 
\(\mu\)g of protein) were incubated at the indicated temperature in 0.2 ml of Krebs-Ringer-bicarbonate buffer containing 1% (w/v) albumin and 1.2 \(\times\) \(10^{-20}\) M \(^{125}\)I-insulin. At various times specific binding was determined as described in the text.

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**Fig. 5.** Time dependence at various temperatures of the dissociation of \(^{125}\)I-insulin bound to fat cell membranes. Membranes (60 
\(\mu\)g of protein) were incubated at 24\(^\circ\) for 30 min in 0.2 ml of Krebs-Ringer-bicarbonate buffer containing 1% (w/v) albumin and 1.2 \(\times\) \(10^{-20}\) M \(^{125}\)I-insulin. Three milliliters of Krebs-Ringer-bicarbonate buffer containing 1% (w/v) albumin and native insulin (50 \(\mu\)g per ml), and adjusted to a specified temperature, were added, and the suspensions were incubated further at that temperature. At the indicated times the samples were rapidly filtered on Millipore membranes and washed with 10 ml of ice-cold Krebs-Ringer-bicarbonate buffer containing 1% (w/v) albumin. The rate constants for dissociation at 5 and 15\(^\circ\) were obtained on samples which were similarly examined during a 5-hour incubation period.

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At 25\(^\circ\) the rate of dissociation is 4.2 \(\times\) \(10^{-4}\) sec\(^{-1}\). At 24\(^\circ\) the dissociation constant obtained from the ratio of the rate constants is 5 \(\times\) \(10^{-11}\) M. This compares favorably with the value (7.5 \(\times\) \(10^{-11}\) M) calculated from equilibrium data (Fig. 1), and indicates that the kinetic experiments are almost certainly giving true rate constants.

The rate of dissociation is more sensitive to temperature than is the rate of association (Fig. 6). This nonparallel change of rate constants with temperature results in a dissociation constant which is very temperature dependent. Thus, the binding of insulin to the membranes is about 10 times stronger at 15\(^\circ\) than at 37\(^\circ\). This dependence on temperature is clearly seen in an Arrhenius plot of the equilibrium constant as a function of temperature (Fig. 7). These studies must be qualified by the possibility that the number of insulin-binding sites may be different or not constant at the various temperatures studied.
Effect of buffers and salts on specific binding of 125I-insulin to fat cell membranes

Fat cell membrane were suspended in a small volume of distilled water. Aliquot (0.2 ml, containing 100 ug of protein) was added to 2.5 ml of the indicated buffer containing 1% (w/v) albumin. Samples were then incubated at 24° for 30 min with 2.1 X 10^-11 M 125I-insulin to determine specific binding, as described in the text. The pH of all the buffers was adjusted to 7.4.

| Buffer conditions | Specific binding of 125I-insulin |
|-------------------|--------------------------------|
| Krebs-Ringer-bicarbonate | 8.6 ± 1.1 |
| Tris-HCl, 0.1 M | 8.1 ± 1.0 |
| Tris-HCl, 0.1 M + CaCl_2, 2 mM + MgCl_2, 10 mM | 9.2 ± 0.9 |
| Tris-HCl, 0.1 M + EDTA, 5 mM | 8.2 ± 1.0 |
| Sodium phosphate, 0.1 M | 8.8 ± 0.8 |
| Sodium phosphate, 0.1 M + KCl, 50 mM | 8.7 ± 0.7 |
| Potassium phosphate, 0.1 M | 8.5 ± 0.5 |
| Potassium phosphate, 0.1 M + EDTA, 5 mM | 8.4 ± 0.9 |
| Krebs-Ringer-bicarbonate, no albumin | 8.7 ± 0.9 |
| Krebs-Ringer-bicarbonate, 3 M urea | 1.1 ± 0.3 |

*Mean value ± standard error of the mean (three observations).

It appears that binding of insulin to fat cell membranes does not depend on the presence in the medium of specific salts or divalent cations.

It is also apparent from the data on Table II that the presence of albumin in the medium is not necessary for binding. Albumin, nevertheless, is almost always used in the studies of binding since in its presence the nonspecific binding of 125I-insulin is considerably lower. Urea at a concentration of 3 M decreases specific binding by about 90% (Table II). Virtually no binding is detected in the presence of 5 M urea or 4 M guanidine-HCl.

Dependence of Insulin Binding on pH—The specific binding of 125I-insulin to fat cell membranes occurs over a relatively narrow range of buffer pH (Fig. 8). Maximum binding occurs quite sharply at a pII of 7.5. It can not yet be determined whether this profile of pH dependency is a valid expression of molecular ionization constants of groups directly involved in the insulin-receptor interaction, or whether other molecular transitions are occurring in the membrane which secondarily affect the structure and thus the binding of insulin to the receptor. If such generalized structural alterations occur in the membrane, they are fully reversible in terms of insulin binding within the pH range of 5 to 9. Membranes suspended in buffers having these pH values, and then diluted with another buffer to neutrality, do not display diminished capacity for binding 125I-insulin.

Ionic Strength and Unmasking of Insulin-binding Structures—The specific binding of insulin to fat cell membranes depends quite critically on the ionic strength of the medium (Table III). The binding of insulin can be dramatically increased by raising the concentration of NaCl in the medium. Maximal binding occurs with 2 M NaCl. Raising the concentration of NaCl further results in a decrease of specific binding. Nearly the same results are obtained with KCl as with NaCl. A similar dependence on ionic strength is observed with LiCl, although with this salt maximal binding is observed at 0.5 M. Relatively
7270 Insulin Receptors of Fat Cell Membranes Vol. 246, No. 23

Fat cell membranes (40 μg of protein in 0.1 ml of 0.1 M NaCl) were added to 0.4 ml of a buffer containing 1% (w/v) albumin and 6 × 10⁻¹¹ M ¹²⁵I-insulin. Specific binding was determined after incubating at 24° for 40 min. The buffers used were: 0.1 M sodium acetate (pH 4.2, 5.0, and 6.0), 0.1 M sodium phosphate (pH 6.8 and 7.5), and 0.1 M sodium bicarbonate (pH 8.2 and 9.0).

**TABLE III**

*Enhancement of specific binding of ¹²⁵I-insulin to fat cell membranes by NaCl, LiCl, MgCl₂, and CaCl₂*

Fat cell membranes (48 μg of protein) were incubated at 24° for 30 min in 0.5 ml of Krebs-Ringer-bicarbonate buffer containing 1% (w/v) albumin, the indicated salts, and ¹²⁵I-insulin (2.1 × 10⁻¹¹ M). Specific binding was determined as indicated in the text.

| Salt                  | Specific binding of ¹²⁵I-insulin (nmoles X 10⁵/mg protein) |
|-----------------------|------------------------------------------------------------|
| No additions          | 11.2 ± 0.4                                                |
| NaCl, 0.5 M           | 30.7 ± 0.9                                                |
| NaCl, 1 M             | 35.4 ± 1.2                                                |
| NaCl, 2 M             | 59.8 ± 1.8                                                |
| NaCl, 4 M             | 18.4 ± 0.9                                                |
| LiCl, 0.5 M           | 42.6 ± 1.3                                                |
| LiCl, 1 M             | 29.7 ± 0.8                                                |
| LiCl, 2 M             | 16.3 ± 1.1                                                |
| LiCl, 4 M             | 0.9 ± 0.2                                                 |
| CaCl₂, 10 mM          | 22.2 ± 0.6                                                |
| MgCl₂, 10 mM          | 17.3 ± 1.2                                                |
| CaCl₂, 10 mM + MgCl₂, 10 mM | 27.4 ± 0.9                               |

* Mean value ± standard error of the mean (three observations).

High concentrations of MgCl₂ and CaCl₂ also result in an enhancement of binding. In view of the results described in Table II, and the effects of NaCl, LiCl, and KCl, the effects of Mg²⁺ and Ca²⁺ are almost certainly related to similar types of changes in the ionic environment rather than to a requirement of specific divalent cations for the binding interaction.

It has been reported that digestion of isolated fat cells, fat cell membranes, and liver cell membranes with phospholipase C results in a marked (3- to 4-fold) enhancement of specific binding of ¹²⁵I-insulin (4, 15). These effects result from the exposure of new insulin receptor sites rather than from changes in the affinity or character of those binding sites which are normally exposed to the medium in the native membranes (4). It was important to determine whether the effects of NaCl occurred by a similar process. Studies on the effects of 2 M NaCl on the specific binding of insulin to fat cell membranes as a function of the concentration of ¹²⁵I-insulin (Table IV) indicate that the predominant effect of this salt is to increase the maximal binding capacity for insulin. From equilibrium data very little, if any, effect can be detected on the affinity of the membranes for insulin. Furthermore, although 2 M NaCl can further enhance the binding of ¹²⁵I-insulin to membranes previously digested with phospholipase C, the effects are much less dramatic than those of NaCl on undigested membranes (Table IV). These data suggest that very high ionic strengths can enhance insulin binding by unmasking new insulin-binding structures by a mechanism similar to that operative during digestion of the membranes or of intact cells with phospholipase C.

The rates of association and dissociation of the insulin-membrane complex, measured at 24°, are virtually the same in the presence or absence of 2 M NaCl. Similar observations were recorded on the rate constants of the interaction between insulin and native or phospholipase C-treated fat cells and membranes (4). These studies suggest, then, that conditions of high ionic strength, like phospholipase digestion, result in the exposure of new insulin receptor sites which are kinetically indistinguishable from those normally accessible to insulin in the medium.

Since the effects of phospholipase digestion described above appear to be mediated by perturbations of membrane phospholipid structures (4), it seemed possible that 2 M NaCl was similarly acting by disrupting the normal array of membrane phospholipids. Studies were, therefore, performed to determine if the effects of 2 M NaCl on insulin binding were reversible (Table V). The results of these studies indicate that the effects of ionic strength are fully reversible. If distortion of membrane phospholipid structure is the basis of the enhanced insulin bind-
membranes kept in the absence of additional salt. These sus-
suspensions were centrifuged for 20 min at 24,000 × g for 30 min, and
pellets were suspended in 2 ml of Krebs-Ringer-bicarbonate buffer containing 1% (w/v) albumin. The binding of ¹²⁵I-insulin
(7 × 10⁻¹¹ M) to the various membrane suspensions was determined as described in the text.

### Table V

Reversibility of NaCl-produced enhancement of binding of ¹²⁵I-insulin to fat cell membranes

| Buffer conditions                                      | Specific binding of ¹²⁵I-insulin (nmol x 10⁶/mg protein) |
|--------------------------------------------------------|----------------------------------------------------------|
| Krebs-Ringer-bicarbonate, 1% albumin                   | 1.8 ± 0.2                                                 |
| Krebs-Ringer-bicarbonate, washed and centrifuged      | 5.1 ± 0.2                                                 |
| NaCl                                                    | 22.0 ± 0.3                                               |
| Krebs-Ringer-bicarbonate, washed and centrifuged      | 5.8 ± 0.3                                                 |

* Mean value ± standard error of the mean (three observations).

Effect of Various Compounds on Insulin Binding—Certain compounds, such as digitonin, poylene antibiotics, and vitamin K₃, which can interact with membrane lipids, have been shown to modify (enhance) the specific binding of ¹²⁵I-insulin to intact fat cells and fat cell membranes (4). A number of other compounds have been tested for their ability to modify the binding interaction (Table VI). Nucleoside triphosphate derivatives of adenine, cytosine, and uracil cause a slight depression of binding at concentrations of 3 mM, but not at concentrations below 0.2 mM. Comparable derivatives of thymidine and guanosine have no effects. Several cyclic phosphate nucleotides, as well as prostaglandins, do not modify the binding of insulin to the membranes.

Effects of Chemical Modifications of Membrane Proteins—Membranes were treated with several protein-modifying re-

![Fig. 9. Effect of heating fat cell membranes on the specific binding of ¹²⁵I-insulin. Fat cell membranes (containing 0.6 mg of membrane protein per ml) were suspended in Krebs-Ringer-bicarbonate buffer containing 1% (w/v) albumin and kept in an ice bath. Samples were incubated for 10 min at the indicated temperature and returned to the ice bath. Samples were then incubated at 24°C for 20 min in the above buffer, containing 1.8 × 10⁻¹⁰ M ¹²⁵I-insulin, to determine specific binding, as described in the text.](http://www.jbc.org/)

### Table VI

Effect of various compounds on specific binding of ¹²⁵I-insulin to fat cell membranes

| Addition                                      | Specific binding of ¹²⁵I-insulin (nmol x 10⁶/mg protein) |
|-----------------------------------------------|----------------------------------------------------------|
| No additions                                  | 8.7 ± 0.7                                                 |
| Nucleoside triphosphates                      |                                                           |
| ATP, 3 mM                                     | 6.1 ± 0.6                                                 |
| ATP, 0.6 mM                                   | 8.8 ± 0.7                                                 |
| CTP, 3 mM                                     | 5.7 ± 0.6                                                 |
| CTP, 0.2 mM                                   | 8.6 ± 0.6                                                 |
| UTP, 3 mM                                     | 5.9 ± 0.5                                                 |
| UTP, 0.7 mM                                   | 7.0 ± 0.5                                                 |
| UTP, 0.1 mM                                   | 7.9 ± 0.7                                                 |
| TTP, 3 mM                                     | 7.9 ± 0.8                                                 |
| GTP, 3 mM                                     | 8.1 ± 0.7                                                 |
| Nucleoside monophosphates, 3 mM (GMP, AMP, UMP, TMP) | No effect                                               |
| Cyclic nucleoside 3',5'-monophosphates, 3 mm (cAMP, cGMP, cCMP) | No effect                                               |
| Pyruvate, 5 mM                                | 8.8 ± 0.9                                                 |
| d-Lactate, 5 mM                               | 9.1 ± 0.7                                                 |
| L-Lactate, 5 mM                               | 9.3 ± 0.7                                                 |
| Acetylcholine, 100 μg per ml                   | 8.8 ± 0.5                                                 |
| Dexamethasone, 200 μg per ml                   | 9.6 ± 0.7                                                 |
| Glucose, 10 mM                                | 9.4 ± 0.8                                                 |
| Prostaglandins (P₄G), 40 μg per ml (P₂G₄, PGE₂, PGF₃, PGA₂, PGB) | No effect                                               |
| 7-Oxa-13-prostynol acid                      | 9.5 ± 0.6                                                 |

* Mean value ± standard error of the mean (three observations).
agents to determine the possible contribution of functional groups to the binding interaction (Table VII). Relatively drastic reaction of the membranes with N-ethylmaleimide and iodoacetamide does not decrease the ability of these membranes to bind insulin. Thus, there is no evidence that membrane sulfhydryl groups participate in the binding interaction. The water-soluble carbodiimide reagent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, which can react with protein carboxyl groups (13), also does not modify insulin binding after reaction with membranes in the absence or presence of glycine-O-methyl ester. Reaction of the membranes with high concentrations of the tryptophan-modifying reagents, 2-hydroxy-5-nitrobenzyl bromide (6, 7) and 2-methoxy-5-nitrobenzyl bromide (8), does not affect insulin binding.

Dramatic reduction of insulin binding was observed after treating the membranes with tetranitromethane (Table VII), a reagent which in the absence of sulfhydryl groups is relatively specific for tyrosyl residues (9), although reaction with tryptophan has also been documented (10). Diazenium-1-H tetrazole, which reacts primarily with histidyl or tyrosyl groups (11) and to a lesser degree with tryptophanyl residues (12), totally abolished the ability of the membranes to bind insulin. These results suggest that tyrosyl and possibly histidyl residues of membrane proteins may be involved in the specific recognition of insulin.

Difluorodinitrobenzene, which can modify virtually any protein functional group, also decreases the binding of insulin (Table VII). Very high concentrations (50 mM) of acetic anhydride cause only a moderate fall in the binding activity. The latter reagent reacts primarily with free amino groups and with tyrosyl groups of proteins.

The studies described earlier in this report indicate that high concentrations of NaCl or digestion of the membranes with phospholipase C appear to unmask insulin receptors which are ordinarily not freely accessible to insulin in the medium. It was of interest to determine whether these "masked" receptor structures were also hindered from reaction with small molecular weight compounds, such as tetranitromethane. Studies designed to test this question reveal that treatment of membranes with this reagent drastically diminishes the subsequent effects of NaCl or phospholipase C digestion on insulin binding (Table VIII). Tetranitromethane, therefore, affects the normally exposed insulin-binding sites of the membranes as well as those which are normally "buried" with respect to insulin in the medium.

**DISCUSSION**

Several recent studies have measured the binding of radioactive polypeptide hormones, such as adrenocorticotropin (26-28), angiotensin (29, 30), glucagon (31, 32), luteinizing hormone (33), and chorionic gonadotropin (33), to various membrane fractions of tissue homogenates. Freychet et al. (14, 34), and House and Weidermann (35), and Custrecessas et al. (15) have recently measured the binding of $^{125}$I-insulin to isolated liver cell membranes.

The binding interactions measured in the present studies...
almost certainly represent interactions of the biologically significant insulin receptors of adipose tissue cells. In the intact fat cells, where the biological response to insulin can be monitored simultaneously with the binding of 125I-insulin, there is strong evidence that the binding process as measured by these techniques represents the initial interaction that leads to biological activation (1). Further support for this comes from the effects of enzymic modifications of intact cells on the binding of insulin and on the biological responsiveness to insulin (2–4). All of the binding activity present in the intact cell is recovered in the membrane fraction after homogenization of the cell (1, 4). The present studies reveal that the properties of insulin binding in the membrane fraction are very similar to those of the intact cell. Thus, despite the loss of the biological response in the broken cell preparation, it is reasonably certain that specific binding in the particulate cell fraction reflects a relatively unaltered receptor interaction.

As in intact cells (1), the interaction of insulin with fat cell membranes is a saturable and reversible process which does not involve significant chemical alteration or inactivation of insulin. Displacement of binding does not occur with biologically inactive insulin derivatives, such as reduced, carboxymethylated, or desoctapeptide insulin. The displacement of 125I-insulin binding by proinsulin indicates a 20-fold lower affinity than native insulin, which is in harmony with the 20-fold decrease in affinity of this derivative when its biological activity is determined (29–35).

The kinetics of association and of dissociation suggest that relatively homogeneous binding structures are involved. The kinetic constants obtained in studies on intact cells are very similar to those observed with the membrane preparations (Table IX). In the fat cell membranes the dissociation constant calculated from equilibrium kinetics (7.5 X 10^-14 M) is nearly the same as that (5 X 10^-11 M) calculated from the ratio of the rate constants, and both of these are very similar to the values reported for the insulin-receptor interaction in intact fat cells (1). Digestion of intact fat cells (4) or fat cell membranes with phospholipase C produces a nearly identical enhancement of insulin binding, and the kinetic rate constants in both preparations are unchanged by such enzymic digestion.

It is of interest that the kinetic constants (Table IX) of intact fat cells or membranes are also very similar to those obtained in studies with liver cell membranes (15). The unique effect of enhanced insulin binding observed with high ionic strength and with digestion with phospholipase C is also observed in liver membranes (15). These observations suggest that the insulin receptor structures of liver and of adipose tissues may be similar or identical structures. It is significant that the properties of the receptor for another polypeptide hormone, glucagon, are very different from those of insulin. The binding of glucagon is abolished by digesting liver (31) or fat cell (4) membranes by phospholipase C, and high ionic strength markedly reduces the binding of glucagon to both of these tissue preparations.

The maximal insulin binding capacity of fat cell membranes is markedly increased in the presence of 2 mM NaCl. The equilibrium and rate constants of complex formation are not appreciably altered. The rate of dissociation of the insulin-membrane complex is studied after diluting the membrane suspension, and therefore the NaCl in the medium, more than 10-fold. Since the effects of NaCl on the membranes are known to be reversible (Table V), it is notable that the rate of dissociation is unchanged from that of the complex which is originally formed in the absence of high concentrations of NaCl. The normally "masked" state of certain insulin receptors (4) must, therefore, refer only to the process of complex formation. Once the complex has formed with these apparently "buried" receptors there is no apparent hindrance from dissociation even when the original conditions of unmasking (2 mM NaCl) have been removed.

The explanation for this is not clear but may be related to a change in the conformational state of the receptor or membrane attendant to binding.

The normally inaccessible binding sites for insulin, which are exposed with 2 mM NaCl or by phospholipase digestion of the membrane, must be considered as "masked" with respect to insulin molecules but not to solvent molecules. Tetranitromethane appears to alter these structures quite readily in the absence of high ionic strength or of phospholipase digestion (Table VIII). It is not yet known whether modification with tetranitromethane under milder conditions, or modification with a diazonium reagent (Table VIII), can result in discriminative reaction of buried and exposed receptors.

The binding of 125I-insulin to the fat cell membranes is considerably stronger at lower temperatures (Fig. 3). This is due to a greater effect of temperature on the rate of dissociation than on the rate of association (Fig. 7). The ΔH calculated from the equilibrium constant (2 X 10^6 M) at 24°C is -14 kcal mole^-1. From the dependence of the equilibrium constant on temperature (Fig. 8) the ΔH is about -28 kcal mole^-1. The heat energy for binding of insulin to membrane receptors is therefore quite favorable and is probably the major driving force for the reaction. The entropy change at 24°C, -45 cal mole^-1 deg^-1, is energetically unfavorable. It is not yet possible to speculate on how these energy changes depend on the nature of specific bond formation or on the role of the various chemical components of the membrane.

Furthermore, at least some of the effects of temperature may be related to alterations in the number of binding sites. It will be interesting to determine the thermodynamic data after the receptor structures have been isolated and separated from other components of the membrane.

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2 B. Desbuquois, F. Krug, and P. Cuatrecasas, unpublished observations.
The present studies indicate that membrane fractions derived from isolated fat cells are valid structures for study of insulin-receptor interactions. These studies should provide a rational basis for further characterization and isolation of the insulin-receptor structures. To gain confidence on the biologically important nature of the interaction being studied, and to avoid studying binding artifacts, subsequent fractionation and purification must involve systematic quantitation, characterization of properties, and comparison with the data obtained on crude membranes and on intact cells.

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