

Ultrastructural localization of insulin receptors on adipocytes*

(ferritin-insulin/plasma membranes/cell structure/glycocalyx/vesicles)

LEONARD JARETT AND ROBERT M. SMITH

Division of Laboratory Medicine, Department of Pathology and Medicine, Washington University School of Medicine; and Barnes Hospital, St. Louis, Missouri 63110

Communicated by P. Roy Vagelos, June 2, 1975

ABSTRACT The method for preparing a stable, biologically active, covalently linked ferritin-insulin complex has been modified to provide a 25-fold increase in yield compared to the original procedure while reducing the molar ratio of ferritin to insulin to 1:1 from 40:1. Ultrastructural studies of isolated adipocytes revealed specific binding of ferritin-insulin to the cell surface in irregular clusters associated with the glycocalyx coating. The number of ferritin-insulin molecules observed was consistent with the number of receptors calculated from ^{125}I -labeled insulin binding studies. The ferritin-insulin was not observed in the cytoplasm of the cell but was found on the concave side of surface connected vesicles. These surface connected vesicles were part of an alveolar-like system of plasma membrane invaginations which project in various directions in the cytoplasm and by thin sectioning can appear as pinocytotic-like microvesicles. The morphological observations on ferritin-insulin binding were supported by the finding that ^{125}I -labeled insulin binding was almost exclusively localized to highly purified plasma membranes isolated by fractionation of adipocytes after incubation with ^{125}I -labeled insulin. These data supported the theory that insulin did not need to enter a cell to cause biological effects and was consistent with the negative cooperativity concept of insulin binding to cell receptors.

Insulin has been successfully coupled covalently to ferritin with the complex retaining equal biological and immunological activity (1). Electron micrographs showed the ferritin-insulin bound only to the outside of isolated plasma membranes of adipocytes but not to purified mitochondria. The present report describes a modification in the preparation of ferritin-insulin and results of ultrastructural and biochemical studies on intact adipocytes. The results directly show that insulin receptors are only on the plasma membrane and that the actions of the hormone do not require entry of insulin into the cell.

MATERIALS AND METHODS

All materials were obtained from commercial sources previously identified (1), except as noted.

Preparation and Purification of Ferritin-Insulin Conjugate. The previously published procedure (1) was used with only two modifications. After 1 hr of reacting 10 mg of porcine insulin and 270 mg of repurified ferritin (2) with 0.04% glutaraldehyde in 12 ml of 0.2 M NaAc pH 4.0 at room temperature, 100 μl of 0.1 M ammonium acetate was added directly to the solution and mixed for 5 min. Centrifugations, dialysis, and Sephadex G-75 chromatography were identical with the previous method. The final separation of free ferritin from ferritin-insulin was accomplished by absorption of the ferritin-insulin to talc tablets (Gold Leaf Pharmaceuti-

cal, Englewood, N.J.), as opposed to affinity chromatography performed previously. An aliquot of the ferritin, ferritin-insulin mixture obtained from the centrifugations and resuspension of the ferritin-rich Sephadex G-75 fraction was added to a 100-mg talc tablet. The talc was dispersed and stood with occasional mixing for 30 min at 4°. The suspension was diluted with 0.1 M NaAc pH 6.5 and centrifuged 10 min at 1000 $\times g$. The supernatant was discarded and the pellet was resuspended in 10 ml of 0.2 M NaAc pH 6.5. The centrifugation and resuspension was repeated twice with fresh buffer. The ferritin-insulin conjugate was removed from the talc by adding 2 ml of 0.37 M HCl containing 6% bovine serum albumin to the final talc pellet. The suspension was mixed and allowed to stand at 4° for 30 min. The talc was removed by centrifugation at 1000 $\times g$ for 10 min. The supernatant was collected and diluted to 12 ml with 0.1 M NaAc pH 6.5 and centrifuged at 100,000 $\times g$ 2 hr. The ferritin-insulin pellet was suspended in 2 ml of 0.1 M NaAc pH 6.5 and stored at 4°. The ferritin used as control material was processed in the absence of insulin through the above protocol and collected after the Sephadex G-75 column chromatography step.

Preparation of ^{125}I -Labeled Insulin and Binding to Subcellular Fractions. The method previously described (3) was used without modification.

Determination of the Subcellular Distribution of ^{125}I -Labeled Insulin In Particulate Fractions from Adipocytes Treated with ^{125}I -Labeled Insulin. Isolated fat cells prepared by the technique of Rodbell (4) were suspended in Krebs-Ringer bicarbonate buffer with 0.1 mg/ml of dextrose and 10 mg/ml of bovine serum albumin at a protein concentration of about 2 mg/ml (2.2×10^6 cells per ml). Preincubation was for 15 min at 24° under 95% O_2 /5% CO_2 followed by the addition of 50 μU of ^{125}I -labeled insulin per ml and incubation for 30 min. The cells were centrifuged at 700 $\times g$ for 30 sec, the incubation medium was aspirated, and 10 ml of 0.25 M sucrose/10 mM Tris-HCl, pH 7.4/1 mM EDTA (Med I) at 10° was added to each tube. The cells were isolated by the dinonyl phthalate technique (5). Another 10 ml of chilled Med I was added, the cells were washed by centrifugation at 700 $\times g$ for 30 sec, and the wash medium was aspirated. The cells were resuspended in Med I at 10°, and various particulate fractions were prepared as described in detail previously (6, 7).

^{125}I -Labeled insulin binding to particulate fractions was determined by suspending the fractions in cold Med I and filtering and washing an aliquot on a Schleicher and Schuell B6A filter as described (3). Controls to correct for nonspecific ^{125}I -labeled insulin absorption consisted of a similar aliquot which was incubated with 500 mU of insulin per ml for 10 min at 24° prior to filtration.

Determination of Number of Insulin Receptors Per Unit Surface Area Based on ^{125}I -Labeled Insulin Binding. The ^{125}I -labeled insulin molecules bound per unit surface

Abbreviations: NaCac, sodium cacodylic acid; Con A, concanavalin A; Med I, 0.25 M sucrose/10 mM Tris-HCl, pH 7.4/1 mM EDTA.

* Presented at the Ninth Miles International Symposium, Johns Hopkins Medical Institutions, Baltimore, Md., June 4-6, 1975.

area was calculated from data obtained by incubating one mU of ^{125}I -labeled insulin per ml with 1×10^5 adipocytes in the presence and absence of 100 mU of unlabeled insulin in a total volume of 0.5 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, with 0.1 mg of dextrose per ml and 10 mg of bovine serum albumin per ml for 15 min at 24° . The cells were diluted with 1 ml of cold buffer and filtered on B6A filters followed by five 1-ml washes. Binding to filter blanks was subtracted from both conditions. The mean diameter of the fat cells was $50 \mu\text{m}$.

Incubation of Isolated Fat Cells with Ferritin-Insulin and Preparation of Cells for Electron Microscopy. Isolated cells prepared and preincubated as described above were incubated with no additions or 1 mU of ferritin-insulin per ml in the presence or absence of 100 mU of insulin per ml or ferritin equivalent to the ferritin concentration in 1 mU of ferritin-insulin per ml for 15 min at 24° . The cell suspensions (1×10^6 cells in 0.5 ml) were diluted to 10 ml with incubation buffer and separated by the dinonyl phthalate technique (5). The incubation medium and oil were aspirated. The entire separation phase required only 2–3 min, during which time negligible dissociation of insulin occurs (3, 8, 9). The carryover of ferritin-insulin was less than 0.2%, as determined by ^{3}H insulin studies. The cells were diluted to 1 ml with 0.25 M sucrose, 0.1 M Na cacodylate HCl (NaCac) buffer, pH 7.4, and 3% glutaraldehyde in the same buffer was added slowly with constant mixing until a final concentration of 2% glutaraldehyde was obtained.

The cells were allowed to stand at room temperature for 30 min with intermittent mixing; then they were washed with 0.25 M sucrose, 0.1 M NaCac buffer. The cells were suspended to two times the packed cell volume with the washing medium and brought to a final agar concentration of 2% with 3% purified agar. The suspension was spread in a petri dish and allowed to gel.

The agar was cut into cubes 1–2 mm on a side and transferred to 0.1 M NaCac in glass vials, post-fixed in 2% OsO_4 in 0.1 M NaCac for 15 min at room temperature, and washed with 0.1 M NaCac followed by deionized water. Staining *en bloc* was accomplished with 1% aqueous uranyl acetate for 30 min. The cubes were washed with deionized water, dehydrated through a graded ethanol series, infiltrated with propylene oxide, and embedded in Epon 812 with an A:B ratio (10) of 3:5.

The agar embedding technique provided mechanical stability which markedly reduced losses due to rupture of cells, prevented floatation of cells prior to osmification and packing of cells during repeated centrifugations in subsequent steps, and allowed even osmification without overosmification. The freely suspended cells in agar allowed better visualization of the plasma membrane surface. Unstained/sections were observed and photographed in a Philips EM200.

Biochemical Assays and Protein Determinations. Glucose oxidation by intact fat cells and adenylate cyclase activity of subcellular fractions were determined as described (1, 11). Immunological activity of insulin was determined by radioimmunoassay (1). Protein concentration was determined in fat cell suspensions as described (7) and in the isolated fractions by the method of Lowry *et al.* (12). Ferritin concentration was determined by the absorbance at 440 nm, assuming a value of 1 for $A_{440 \text{ nm}}^{0.65 \text{ mg/ml}}$ (13).

RESULTS

Purification of Ferritin-Insulin Complex. The recoveries of ferritin and insulin in the modified preparative pro-

Table 1. Purification of ferritin-insulin complex

Step	Recovery			
	Insulin		Ferritin	
	(Units)	(%)	(mg)	(%)
Conjugation	240	100	270	100
2400 × g centrifugation				
Supernatant	240	100	261	96.6
Pellet	0	0	9.0	3.4
Dialysis: 2400 × g centrifugation				
Supernatant	240	100	261	96.6
Pellet	0	0	0	0
Sephadex G-75 (ferritin-rich fraction)	1.8	0.8	260	96.4
Sephadex G-75 (free insulin fraction)	236	98.2	0	0
Talc eluate	1.2	0.5	4.3	1.6

Insulin concentration was based on biological activity as determined by glucose oxidation measurements. Ferritin concentration was determined by measuring A_{440} and assuming a value of 1 for $A_{440 \text{ nm}}^{0.65 \text{ mg/ml}}$.

cedure for ferritin-insulin are illustrated in Table 1. The direct addition of 0.1 M ammonium acetate resulted in an immediate cessation of the glutaraldehyde conjugation reaction and prevented the large amount of aggregation and precipitation of ferritin and insulin previously reported during the dialysis step. The Sephadex G-75 fractionation cleanly separated the free insulin from ferritin and ferritin-insulin, which eluted together in the void volume. The ferritin-insulin was absorbed to talc as originally described by Cuatrecasas (14) for purifying biologically active ^{125}I -labeled insulin. Ferritin did not absorb to the talc and was easily removed by extensive washing of the talc with 0.1 M sodium acetate, pH 6.5. The ferritin-insulin was removed from the talc using the same HCl/albumin mixture used to remove ^{125}I -labeled insulin. The ferritin-insulin was concentrated by centrifugation of the eluate at $100,000 \times g$ for 2 hr, which removed any remaining free insulin along with the bovine serum albumin. The final ferritin-insulin complex contained 0.5% of the original insulin and 1.6% of the original ferritin, for a molar ratio of about 1:1. This was a 25 times better yield of ferritin-insulin than the original procedure and a reduction of the molar ratio of ferritin to insulin from 40:1.

The complex produced by the current modification showed the same characteristics of equipotent biologic and immunologic activity and stability as the previous material. Various size aliquots of ferritin-insulin were assayed by both biological (glucose oxidation) assay and immunoassay and yielded identical quantities of insulin by both methods (Table 2). Storage up to 4 months at 4° has not resulted in any change in insulin activity or separation of insulin from ferritin. The bulk of the complex has been stored as the ferritin-rich Sephadex G-75 eluate and purified by talc absorption as needed.

Morphological Observations With Ferritin-Insulin. Isolated fat cells incubated with 1.0 mU of ferritin-insulin per ml showed ferritin cores irregularly distributed on the sur-

Table 2. Comparison of the biological and immunological activity of talc-purified ferritin-insulin

Ferritin (ng/ml)	Insulin (μ U/ml)	
	Bioassay	Immunoassay
229	71	60
114	36	37
57	18	15
30	9.0	8.6
5	1.5	0.9

The ferritin concentrations were calculated from the observed absorbance of the talc-purified ferritin-insulin. The biological activity was determined by comparing the effect of ferritin-insulin to native insulin in the glucose oxidation assay. Immunological activity was measured by insulin radioimmunoassay on the same aliquots.

face of the adipocyte (Fig. 1A-D), frequently in clusters of two or more, and almost invariably associated with the glycocalyx material. This coating on the surface of the plasma membrane was fuzzy in appearance and patchy in distribution and not all contained visible ferritin-insulin. The only other portion of the cell found to contain ferritin-insulin was on the concave surface of vesicular structures in the cytoplasm (Fig. 1B-D) and again associated with the fuzzy coat. Most of these vesicular structures containing ferritin-insulin were seen connecting to one another and/or to the surface of the cell. The ferritin-insulin did not appear to ever cross the membrane structure of the cell and enter the cytosol or attach to cytoplasmic organelles. No ferritin cores were observed with mitochondria, nuclei, endoplasmic reticulum, or the fenestrated envelope (15) of membrane sacs surrounding the larger lipid storage depots.

125 I-Labeled insulin binding to adipocytes was carried out under incubation conditions similar to those for the ferritin-insulin studies in order to compare the molecules of 125 I-labeled insulin bound per cell to the number of ferritin-insulin molecules observed by electron microscopy. It was found that at 1 mU of 125 I-labeled insulin per ml there were 170,000 molecules bound per cell, equivalent to 22.4 molecules per μm^2 of surface area. The number of ferritin-insulin molecules observed in electron micrographs along 100 randomly selected lengths of plasma membrane corresponded to approximately 100 molecules per μm^2 , which compares favorably to the data obtained by 125 I-labeled insulin measurements. This is in contrast to our earlier study with highly purified subcellular fractions of adipocytes (1) and to that of Siess *et al.* (16), using fat cell ghosts where almost a 100 times greater number of ferritin particles was found on the plasma membranes than expected from 125 I-labeled insulin binding studies.

Adipocytes incubated with ferritin-insulin plus an excess of free insulin (Fig. 1E) or with the same amount of ferritin as contained in the ferritin-insulin (not shown) did not contain ferritin cores either on the cell surface or inside the vesicular structures. None of the incubation conditions used resulted in any detectable structural changes compared to the control adipocytes.

These morphological studies have provided new insight into the structural relationships among the plasma membrane invaginations, the rosettes or flask-like vesicles associated with the plasma membrane, and the so-called "free" cytoplasmic microvesicles. These structures have been described before, and it has been suggested that they provide a

Table 3. Subcellular distribution of 125 I-labeled insulin and adenylate cyclase in particulate fractions of adipocytes with treated 125 I-labeled insulin

Frac- tion	125 I-Labeled insulin		Adenylate cyclase	
	Binding (fmol/mg of protein)	Recovery (% P_1 + Mic)	Specific activity (pmol/mg of pro- tein per 10 min)	Recovery (% P_1 + Mic)
P_1	14.5	91.9	1220 \pm 15	97.1
P.M.	26.0	36.0	2709 \pm 11	35.7
Mit	4.0	3.3	138 \pm 7	2.1
Mic	6.0	8.1	157 \pm 4	2.9

The distribution of 125 I-labeled insulin among particulate subcellular fractions of adipocytes was determined by incubating adipocytes for 30 min at 24° with 50 μ U of 125 I-labeled insulin per ml, washing with chilled (10°) buffer, and fractionating as described in *Materials and Methods* to yield the following particulate fractions: P_1 , 16,000 \times g pellet from homogenate; P.M., plasma membranes; Mit, mitochondria; Mic, microsomes. Binding was determined by membrane filtration with nonspecific binding subtracted. The values for binding are means of duplicates. Adenylate cyclase activity was determined on identical fractions from a separate experiment handled in the same manner except for the omission of 125 I-labeled insulin. The specific activity represents the mean \pm SEM of triplicate measurements.

transportation system back and forth across the cytoplasm for the storage and mobilization of lipid (17, 18). The present study would suggest that these structures are contiguous with and do not separate from the plasma membrane and certainly are not moving back and forth across the cytoplasm. This is supported by several pieces of data. The vesicles are frequently seen making connections to each other and to the cell surface (Fig. 1C and D). It has been shown previously by tilt angle photography that many sub-plasma membrane vesicles are actually open to the surface at another plane or angle of the specimen (19). The material lining these structures is similar in appearance to the glycocalyx, and the vesicles are usually less dense internally than the fenestrated envelope around the lipid storage depot. In sections revealing wider portions of the cytoplasm, as in Fig. 1B, these surface connected vesicles are never found more than half to two-thirds of the way across the cytoplasm and are easily distinguished from the endoplasmic reticulum and the fenestrated envelope usually found in the lower half of the cell. The lack of ferritin cores free inside the vesicles in ferritin-insulin incubated cells and the absence of any ferritin in ferritin incubated cells support the concept that these vesicles are connected to the surface. If they did form pinocytotic vesicles, ferritin should be trapped and carried into the cells during incubation. If they are open to the surface as suggested, free ferritin-insulin or ferritin would be washed out during processing of the cells after incubation. The resulting structural picture of the adipocyte surface is that of multiple invaginations of the plasma membrane forming an alveolar-like subsurface structure. It is proposed that this network of membrane invaginations be termed "surface connected vesicles" (SCV).

Subcellular Distribution of 125 I-Labeled Insulin Binding. The distribution of 125 I-labeled insulin among highly purified subcellular fractions of adipocytes after incubation of the intact cells with the 125 I-labeled insulin confirmed the morphological observation that insulin binding was restricted to the plasma membrane and its contiguous components. The data in Table 3 show that the vast majority of the bound

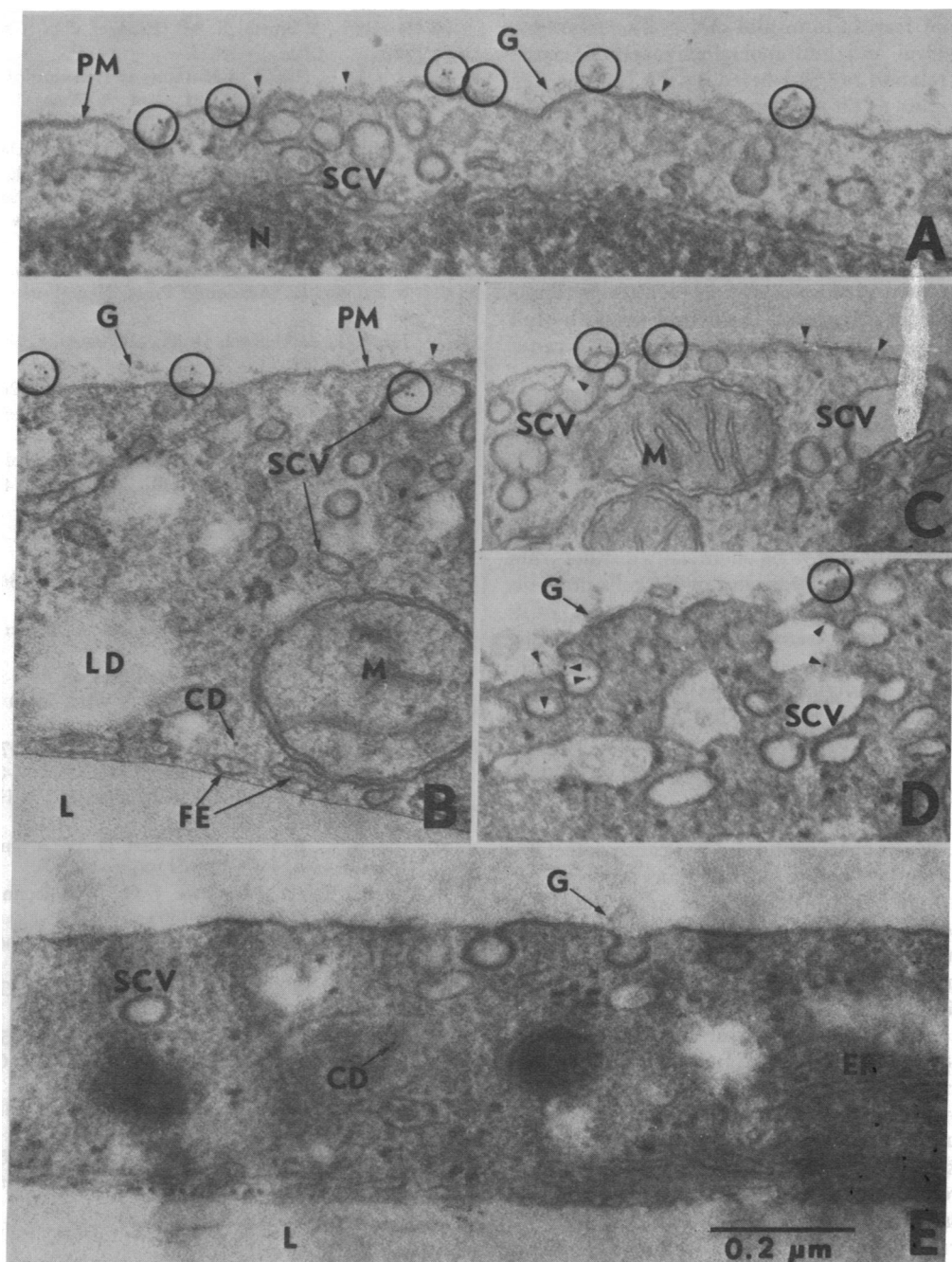


FIG. 1. Intact adipocytes were isolated, incubated, and prepared for electron microscopy as described in *Materials and Methods*. In order to allow better visualization of ferritin-insulin molecules, sections were examined without staining. All micrographs were oriented with the plasma membrane toward the top of the figure, the central lipid depot to the bottom. Micrographs $\times 100,000$. Scale bar equals $0.2 \mu\text{m}$. PM, plasma membrane; G, glycocalyx; SCV, surface connected vesicles; N, nucleus; LD, cytoplasmic lipid droplet; CD, cytoplasmic density; M, mitochondria; FE, fenestrated envelope; L, central lipid depot; ER, endoplasmic reticulum.

^{125}I -labeled insulin was associated with the first $16,000 \times g$ pellet (P_1) from the fat cell homogenate. Further fractionation of P_1 to yield plasma membranes and mitochondria clearly demonstrated the association of the ^{125}I -labeled insulin with the former fraction (Table 3). The loss of bound ^{125}I -labeled insulin between P_1 and the plasma membranes was consistent with losses of the plasma membrane protein during the fractionation procedure (7), with the dissociation of hormone occurring during the fractionation (data not published), and with the loss of adenylate cyclase during the fractionation (ref. 20 and Table 3). The ^{125}I -labeled insulin

binding and adenylate cyclase copurified in a similar pattern in separate but identically handled experiments (Table 3). Adenylate cyclase was used as a plasma membrane marker and showed that the small amount of ^{125}I -labeled insulin binding found in the mitochondrial and microsomal fractions could be accounted for by plasma membrane contamination.

DISCUSSION

The modifications reported for preparing ferritin-insulin resulted in a marked increase in yield and a final product with

a molecular ratio of ferritin to insulin of 1:1. The retention of identical biological and immunological reactivity, coupled with the correlation to ^{125}I -labeled insulin binding, establishes this complex as an excellent electron microscopic marker for the insulin receptor.

The insulin receptor on intact adipocytes is located in association with the glycocalyx coating on the external surface of the plasma membrane. This is in agreement with previous morphological observations on purified adipocyte plasma membranes (1) and fat cell ghosts (16). The finding that ferritin-insulin stimulated glucose oxidation identically to native insulin but was not observed in the cytoplasm of the cell indicates that the hormone need not enter the cell to cause its biological effects.

The association of the insulin receptor with the cell surface coat may explain in part the ability of concanavalin A (Con A) to mimic certain insulin action, including stimulation of glucose transport (21, 22) and of Mg^{++} -dependent ATPase (23), and to block insulin binding to the cell (24). It has been established that Con A binding sites are in the glycocalyx (25, 26), but appeared to be chemically distinct from the insulin receptor. Trypsin treatment of fat cells prevents insulin binding (24, 27) but not the Con A binding (24) or its ability to stimulate glucose transport (21). Con A binding and stimulation of glucose transport is prevented by α -methylglucoside, which only slightly inhibits insulin-stimulated glucose oxidation (28). The ability of Con A to block insulin binding may only be due to the close association of the two receptors and the large size of Con A, its charge, or induced conformational changes in the glycocalyx.

These studies with improved morphological techniques for fat cells have shown that insulin did not cause detectable morphological alterations of the adipocyte, as reported earlier by Barnett and Ball (29) and Soifer *et al.* (30). The structural characteristics of the fat cell have been further clarified with the demonstration that plasma membrane invaginations do not form pinocytotic microvesicles in the cytoplasm as previously suggested (17, 18) but are actually an alveolar-like interconnecting system of surface connecting vesicles. It is possible that this system functions by providing the adipocyte with more surface area and by facilitating the interaction of cytoplasmic elements with the cell surface.

DeMeyts *et al.* (31) have reported evidence that insulin binding with its receptor shows negative cooperative interactions. A theoretical explanation for negative cooperativity involves the clustering of receptors (32). The observed clustering of the ferritin-insulin on the glycocalyx, in contrast to a random distribution, is consistent with such a theory. Further morphological studies will be necessary to confirm this.

This work was supported by U.S. Public Health Service Grant AM-11892.

1. Jarett, L. & Smith, R. M. (1974) *J. Biol. Chem.* **249**, 7024-7031.
2. Hsu, K. C. (1967) in *Methods in Immunology and Immunochimistry*, eds. Williams, C. A. & Chase, M. W. (Academic Press, New York), Vol. I, pp. 397-401.
3. Hammond, J. M., Jarett, L., Mariz, I. K. & Daughaday, W. H. (1972) *Biochem. Biophys. Res. Commun.* **49**, 1122-1127.
4. Rodbell, M. (1964) *J. Biol. Chem.* **239**, 375-380.
5. Gliemann, J., Osterland, K., Vinten, J. & Gammeltoft, S. (1972) *Biochim. Biophys. Acta* **286**, 1-9.
6. Jarett, L. (1974) in *Methods in Enzymology*, eds. Fleischer, S. & Packer, L. (Academic Press, New York), Vol. XXXI, pp. 60-71.
7. Jarett, L. & McKeel, D. W. (1970) *Arch. Biochem. Biophys.* **140**, 362-370.
8. Cuatrecasas, P. (1971) *J. Biol. Chem.* **246**, 7265-7274.
9. Kahn, C. R., Freychet, P., Roth, J. & Neville, D. M., Jr. (1974) *J. Biol. Chem.* **249**, 2249-2257.
10. Luft, J. H. (1961) *J. Biophys. Biochem. Cytol.* **9**, 409-414.
11. Salomon, Y., Londos, C. & Rodbell, M. (1974) *Anal. Biochem.* **58**, 541-548.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275.
13. Singer, S. J. & Schick, A. F. (1961) *J. Biophys. Biochem. Cytol.* **9**, 519-537.
14. Cuatrecasas, P. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1264-1268.
15. Cushman, S. W. (1970) *J. Cell Biol.* **46**, 326-341.
16. Siess, E. A., Nestorescu, M.-L. & Wieland, O. H. (1974) *Diabetologia* **10**, 439-447.
17. Williamson, J. R. (1964) *J. Cell Biol.* **20**, 57-74.
18. Slavin, B. G. (1972) *Int. Rev. Cytol.* **33**, 297-334.
19. Hales, C. N., Luzio, J. P., Chandler, J. A. & Herman, L. (1974) *J. Cell Sci.* **15**, 1-15.
20. Jarett, L., Reuter, M., McKeel, D. W. & Smith, R. M. (1971) *Endocrinology* **89**, 1186-1190.
21. Czech, M. P., Lawrence, J. C., Jr. & Lynn, W. S. (1974) *J. Biol. Chem.* **249**, 7499-7505.
22. Cuatrecasas, P. & Tell, G. P. E. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 485-489.
23. Jarett, L. & Smith, R. M. (1974) *J. Biol. Chem.* **249**, 5195-5199.
24. Cuatrecasas, P. (1973) *J. Biol. Chem.* **248**, 3528-3534.
25. Bernhard, W. & Avrameas, S. (1971) *Exp. Cell Res.* **64**, 232-236.
26. Oseroff, A. R., Robbins, P. W. & Burger, M. M. (1973) *Annu. Rev. Biochem.* **42**, 647-682.
27. Cuatrecasas, P. (1971) *J. Biol. Chem.* **246**, 6522-6531.
28. Czech, M. P. & Lynn, W. S. (1973) *Biochim. Biophys. Acta* **297**, 368-377.
29. Barnett, R. J. & Ball, E. G. (1960) *J. Biophys. Biochem. Cytol.* **8**, 83-101.
30. Soifer, D., Braun, T. & Hechter, O. (1971) *Science* **172**, 269-271.
31. DeMeyts, P., Roth, J., Neville, D. M., Jr., Gavin, J. R., III & Lesniak, M. A. (1973) *Biochem. Biophys. Res. Commun.* **55**, 154-161.
32. Levitzki, A. (1974) *J. Theor. Biol.* **44**, 367-372.