Antisera from rabbits injected with rat adipocyte plasma membranes or intrinsic proteins from such membranes, obtained by a dimethylmaleic anhydride extraction step, mimicked the action of insulin on both glucose transport and lipolysis in intact adipocytes. Biological activity in both types of antisera was mediated by immunoglobulin binding to one or more intrinsic proteins of the adipocyte plasma membrane since fat cells were unresponsive to all antisera absorbed with dimethylmaleic anhydride-extracted membranes. Acid treatment of immunoprecipitates released antibodies which activated glucose uptake and reacted with solubilized adipocyte membranes on immunodiffusion plates. The biologically active immunoglobulin preparations failed to form immunoprecipitin lines when tested against membranes from brain, liver, lung, muscle, kidney, and spleen. Insulin-sensitive glucose uptake in rat soleus muscle did not respond to the antisera. The antibodies activated hexose uptake into fat cells and reacted with solubilized adipocyte membrane proteins on immunodiffusion plates when rat or mouse adipocytes were studied, but not when monkey fat cells were used.

The anti-membrane antibody preparations readily activated hexose uptake in trypanized fat cells which had lost the capacity to bind or respond to insulin. These data are consistent with the concept previously proposed (Pillion, D. J., and Czech, M. P. (1978) J. Biol. Chem. 253, 3761-3764) that the anti-membrane immunoglobulins do not interact with the insulin binding site of the insulin receptor. Monovalent Fab fragments of the biologically active antisera, prepared by papain digestion of the native anti-membrane immunoglobulins, were ineffective in enhancing glucose uptake in adipocytes. However, biological activity of the anti-membraneFab fragments was restored by the addition of goat anti-rabbit Fab antisera to cells treated with the Fab fraction. Anti-rabbit Fab antisera alone or in combination with Fab fragments prepared from control rabbit sera exhibited no biological activity. These results demonstrate that the ability of anti-membrane antisera to mimic the biological activity of insulin on isolated fat cells is critically dependent on immunoglobulin binding to one or more intrinsic plasma membrane proteins and the multivalent nature of immunoglobulin structure.

The mammalian adipocyte has been studied extensively in recent years as a model system for hormone-sensitive processes. For example, D-glucose uptake can be rapidly stimulated severalfold by the addition of insulin to isolated fat cells (1-4). The mechanism whereby insulin activates sugar transport is not understood, although attempts have recently been made to partially purify and reconstitute the adipocyte membrane D-glucose transport system (5-7). Successful attempts have been made to treat intact adipocytes with insulin and partially purify the fat cell membrane hexose transport system in its activated state (8-10). These results suggest that insulin may cause some covalent or stable structural change in the membrane transport system which is retained by the membrane components during subsequent isolation procedures. We have attempted to raise antibodies against the adipocyte glucose transport system because available immunological techniques may provide a powerful tool in efforts to characterize membrane transport components and probe the mechanism by which insulin activates adipocyte hexose transport activity.

In order to simplify the task of raising antibodies against adipocyte membrane transport components, we first treated fat cell membrane vesicles with dimethylmaleic anhydride to remove extrinsic proteins. It has been shown (5-7) that treatment with dimethylmaleic anhydride will remove up to 80% of the total protein content of adipocyte membrane vesicles and that the residual membrane material retains stereospecific hexose transport activity. Extrinsic proteins not involved in transport are thus removed from the membrane preparation before injection into the rabbits in order to increase the titer of antibodies directed against intrinsic membrane proteins. We recently found (11-13) that antisera against rat adipocyte intrinsic membrane proteins causes cytolysis of intact rat fat cells and that this effect is eliminated by heat inactivation of complement. The addition of heat-inactivated antisera had no effect on insulin binding to fat cells but stimulated D-glucose transport into the cells to the same extent as maximal doses of insulin (11). These findings suggested that the antibodies against adipocyte intrinsic membrane proteins reacted with an antigenic site on the outer surface of intact fat cells and mimicked the action of insulin to stimulate hexose uptake without interacting with the insulin binding site of the adipocyte insulin receptor. The present studies were designed to
Characterize the biological effects of the antisera on various insulin-sensitive processes and to determine the species and tissue specificity of its action.

**MATERIALS AND METHODS**

### Preparation of Fat Cells and Crude Plasma Membranes
Fat cells were isolated from adult female rats by digestion with collagenase (Clostridium histolyticum, Worthington) as previously described (6). Isolated fat cells were washed with Krebs-Ringer phosphate buffer, pH 7.4, containing 2% bovine serum albumin, resuspended in a solution consisting of 0.25 M sucrose, 3 mM Tris/HC1 (pH 7.4), and 1 mM EDTA and then homogenized by eight strokes with a Teflon pestle in a loose-fitting glass homogenizer. The homogenate was centrifuged at 8,000 x g for 10 min and, after removal of the triglyceride layer, the supernatant and a small portion of the fluffy white material on the surface of the pellet were centrifuged at 40,000 x g for 35 min; the pellet was resuspended in buffer and this material is referred to as "crude plasma membranes." When other rat tissues were studied, tissues were minced in the absence of collagenase and then homogenized and centrifuged in the same manner as described for fat cells.

### Preparation of Adipocyte Intrinsic Membrane Proteins
Crude plasma membranes were resuspended in 5 mM Tris/HC1 (pH 7.4), 1 mM EDTA and diluted with 15 volumes of H2O; 2 mg/ml of dimethylmaleic anhydride was then added slowly with constant stirring (6, 7, 14). The pH of the solution was maintained between 8.0 and 8.5 with 2 NaOH until acid evolution ceased (~40 min). The solution was then centrifuged at 40,000 x g for 35 min and the supernatant was diluted in 5 mM Tris/HC1 (pH 6.1). rabbit anti-rabbit immunoglobulin was added to release any bound dimethylmaleic anhydride. Before use, the sample was diluted with 5 mM Tris/HC1 (pH 7.4), 1 mM EDTA, centrifuged at 40,000 x g for 35 min and resuspended in this same buffer (~1 mg/ml).

### Production and Treatment of Antiserum
Dimethylmaleic anhydride-extracted membranes or crude plasma membranes (~1 mg/ml) were suspended in 5 mM Tris/HC1 (pH 8.0) and dialyzed against an equal volume of Freund's adjuvant (Cabilochrom). Adult albino New Zealand rabbits were injected in the scapular region at three sites with a total of 0.5 mg protein/rabbit. This procedure was repeated after 5 and 12 weeks for one rabbit, while the other rabbits received a single booster injection after 3 weeks. Four of five rabbits injected with membranes have produced antisera which give a positive immunoprecipitation reaction when tested against detergent-solubilized fat cell membranes on an Ouchterlony-type immunodiffusion plate. Blood was collected from an ear vein at 7 to 14-day intervals; after a 1-h incubation at 37°C, followed by 24 h at 4°C to promote clot formation, serum was isolated and stored at ~20°C. All antigens were heated at 56°C for 30 min to inactivate complement and used immediately after being tested for biological activity with fat cells.

### Immunoglobulin Purification
The immunoglobulin fraction of the rabbit serum was partially purified by the addition of 33% saturated ammonium sulfate (15); the pH of the saturated (NH4)2SO4 was adjusted to 7.4 just prior to use. The precipitate that formed upon addition of the precipitating agent was collected by centrifugation at 5,000 x g for 10 min and resuspended in H2O (in one-half of the original volume of serum). Twice more, the sample was brought to 33% saturated ammonium sulfate, centrifuged, and resuspended. Finally, the precipitate was resuspended in the original volume of serum in either 10 mM sodium phosphate buffer, pH 8.0, or phosphate-buffered saline (0.9% NaCl solution), pH 7.4, and dialyzed against the same buffer overnight at 4°C. The immunoglobulin fraction was then centrifuged at 5,000 x g to remove the precipitate and stored at ~20°C; repeated freezing and thawing of the immunoglobulin led to inactivation of the antibodies and was therefore avoided. The IgG fraction could be further purified by passage over a column of DEAE-cellulose (Cellex 1, Bio-Rad Laboratories) equilibrated with 10 mM sodium phosphate buffer, pH 8.0; the IgG came through the column with the phosphate buffer while other serum components were retained on the column and could be eluted with 0.5 M NaCl (16). The fractions containing IgG were then pooled and concentrated back to the original volume of the serum with a Millipore 47-mm stirred molecular filtration cell (PTGC, Millipore). The presence of Fab fragments in these fractions was determined by the use of the rabbit anti-rabbit immunoglobulin; the residual material was eluted from the column with 0.5 M acetic acid. The Fab fragments were dialyzed against phosphate-buffered saline (pH 7.4) before being used in any test of biological activity, in order to remove sodium azide. Commercial antisera were supplied with sodium azide and it was found that dialysis to remove this component was required to facilitate dialysis such samples resulted in strong inhibition of fat cell glucose oxidation.

### Double Immunodiffusion
Immunodiffusion studies were carried out in plastic Petri dishes (9 x 50 mm) containing 3 ml of 1% agarose dissolved in phosphate-buffered saline (10 mM phosphate buffer, pH 7.4) containing 0.02% sodium azide. The solution was applied to the wells of a fusion plate. Blood was collected from an ear vein at 7 to 14-day intervals; after a 1-h incubation at 37°C, followed by 24 h at 4°C to promote clot formation, serum was isolated and stored at ~20°C. All antigens were heated at 56°C for 30 min to inactivate complement and used immediately after being tested for biological activity with fat cells. Occasionally, immunoprecipitation lines could be seen so clearly that it was deemed unnecessary to stain with Coomassie blue.

### Glucose Oxidation in Adipocytes
Production of CO2 from d-glucose was determined according to the procedure of Rain and others (17). Fat cells (10 cells/ml) were suspended in 1 ml of Krebs-Ringer phosphate buffer, pH 7.4, containing 0.2% (1-C)carbonate (0.2 MM, 0.5 MCl/m) and 4% bovine serum albumin in plastic test tubes; 0.1 ml of various buffers, sera or reagents were added to the cells and the tubes were sealed for an incubation of 1 h at 37°C. The incubation was ended by the addition of 0.2 ml of phenethylamine to pieces of Whatman filter paper suspended in plastic test tubes about the fat cells. Sulphuric acid (0.2 ml) was added followed by additional buffer and the tubes were incubated at 37°C for 30 min. The liberated [1(4)C]CO2 precipitated on the filter papers and then stained with Coomassie brilliant blue R-250 (1.25 g/450 ml of 50% methanol plus 45 ml of glacial acetic acid) for 5 min. Destaining was performed in 5% methanol containing 7.5% acetic acid for several hours. The addition of 0.5% to 2% sodium cholate in addition to Triton X-100 to increase membrane solubilization had no effect on the appearance of immunoprecipitation lines. Occasionally, immunoprecipitation lines could be seen so clearly that it was deemed unnecessary to stain with Coomassie blue.

**Antiserum Utilization**
Solutions of antisera were removed from decapitated rats as previously described (18, 19). The muscle was tied tightly across a miniature device shaped like a horseshoe constructed with stainless steel wire. The tied muscle was incubated

The abbreviation used is IgG, immunoglobulin G.
in the bottom of flat-bottomed vessels containing 2 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) containing 2% bovine serum albumin and continually gassed (O_2:CO_2, 96:4). Muscles were incubated for 10 min at 37°C with or without the addition of insulin or antisera. D-[5^-H]glucose (5 mm) was then added to the samples for 15 min at 37°C and an aliquot of the incubation medium was then analyzed for labeled H_2O as an indication of glycolytic flux. In addition, the muscles were digested in boiling ethanol containing KOH (30%) for the determination of labeled glycogen levels (20). Paired muscles were used in each experiment, one of which served as a control.

Lipolysis—Lipolysis was measured in fat cells as glycerol release and fatty acid release according to Fain et al. (21). Fat cells (10^6 cells/ml, 0.1 ml) were incubated in 0.5 ml of Krebs-Ringer phosphate buffer containing 4% bovine serum albumin (pH 7.4) plus 0.1 ml of buffer, sera, or insulin for 5 min at 37°C. There was no exogenous D-glucose present in these studies. At the start of the 60-min incubation, half of the cells were exposed to 2 µM norepinephrine to stimulate the rate of lipolysis. At the end of the incubation, an aliquot (0.1 ml) of the incubation medium was used to measure the release of glycerol by a standard fluorometric technique described previously (21). The remaining cells and incubation medium were assayed for the presence of free fatty acids by a titrometric method (21). All determinations were performed in triplicate.

Trypsin Digestion—Fat cells (10^6 cells/ml) were incubated in the presence or absence of trypsin (2 µg/ml) at 15 min at 37°C. Trypsin inhibitor (2.2 mg/ml) was then added to the cells for 5 min at 37°C, washed three times and resuspended in Krebs-Ringer phosphate buffer containing 4% bovine serum albumin (pH 7.4), and D-glucose utilization was determined as described above.

Absorption and Release of Antibodies—Antisera or the immunoglobulin fraction (0.25 to 0.5 ml) were incubated with crude plasma membrane vesicles (2 µg/ml, 0.02 to 0.5 ml) overnight at 4°C and the precipitates were collected by centrifugation at 40,000 × g for 35 min; the supernatants were carefully decanted and this fraction was called the "absorbed sera or Ig." The precipitated membrane-antibody complexes were resuspended in 5 mM Tris/HCl (pH 7.4), 1 mM EDTA and again centrifuged at 40,000 × g to remove any nonspecifically bound antibodies. The precipitates were then resuspended in 0.5 ml of glycerine buffer (0.1 M, pH 2.8), incubated on ice for 60 min with occasional stirring, and then centrifuged at 40,000 × g for 35 min. The supernatant solutions, containing antibodies released from membrane vesicles, were neutralized with 1 N NaOH and then used in various biological experiments, this fraction was labeled "released sera or Ig."

Reagents—Bovine serum albumin was obtained from Armour and cytochalasin B from Aldrich. Goat antisera against rabbit γ-globulins and Fab fragments were obtained from Calbiochem and Miles Laboratories, respectively. Coomassie brilliant blue R-250 was obtained from Pierce Chemical Co. and D-[1-^14C]glucose (0.2 mM, 0.5 µCi/ml) plus 0.1 ml of buffer, insulin, or heat-inactivated serum. Three rabbits were injected with rat adipocyte membranes after extraction with dimethylmaleic anhydride as described under "Materials and Methods." Antisera were diluted with various amounts of phosphate-buffered saline (pH 7.4); insulin was added to other tubes, at a final concentration of 2.4 milliumilits/ml. [1^-14C]CO_2 production was determined at the end of a 60-min incubation and the relative increase in glucose oxidation caused by insulin or antisera was then calculated; the data presented are the mean of three experiments with triplicate determinations.

RESULTS

Antisera were collected from three different rabbits immunized with intrinsic membrane proteins from rat fat cells. The data in Fig. 1 indicate that all three antisera caused an increase in the rate of glucose oxidation to CO_2 when added to rat adipocytes. Previous studies (11-13) had shown that these antisera can cause complement-mediated cytolyis of fat cells. Therefore, the sera were heated for 30 min at 56°C to inactivate complement before being used in the studies depicted in Fig. 1 or in any of the subsequent determinations of biological activity. The dose-response relationships for the three rabbit antisera were similar, with no effect seen at a dilution of 1:160 and maximal effects at a dilution of 1:10 to 1:5. The antisera were less potent at stimulating glucose oxidation than insulin in the experiments depicted in Fig. 1, but in some cases (Fig. 5), the antisera enhanced glucose oxidation in fat cells more than maximal doses of insulin. That the increased glucose conversion to CO_2 due to antisera reflects activation of the hexose transport system has previously been documented using 3-O-methylglucose uptake rates in these cells (11). Normal rabbit serum occasionally produced a stimulation of glucose oxidation to CO_2 due to antiserum reflects activation of the hexose transport system. The data in Fig. 2 show that, in the absence of norepinephrine, the rate of lipolysis was quite low in the presence or absence of either insulin or antisera against intrinsic membrane proteins. Norepinephrine at 2 µM stimulated both glycerol release and free fatty acid release by untreated fat cells, while insulin inhibited this effect of the catecholamine on lipolysis. Serum from untreated rabbits had no effect on the rate of fat cell lipolysis, while antisera against intrinsic membrane proteins mimicked insulin action and caused an inhibition of norepinephrine-stimulated lipolysis.

Previous data (11) had shown that the anti-membrane antibodies did not compete with insulin for binding sites on the surface of fat cells. However, the question remains as to whether or not the antibodies are interacting with portions of the insulin receptor not involving the insulin binding sites, or with other membrane components related to insulin-sensitive
processes. One approach to this question is to alter chemically the insulin receptor and to determine what effect such treatment would have on the ability of the antibodies to mimic insulin action. Rat cells, therefore, were incubated with trypsin (2 mg/ml) long enough to destroy the capacity of the cells to bind and respond to insulin. Basal hexose transport activity was disrupted only slightly under these conditions, as shown in Fig. 3. The data indicate that the adipocyte response to insulin was completely eliminated in such trypsin-digested cells. In contrast, the immunoglobulin fraction from antiserum against intrinsic membrane proteins was still capable of stimulating hexose transport into trypsin-digested cells (Fig. 3). No marked change in the dose-response relationship for the antibody preparation was apparent due to trypsinization of fat cells.

Rabbits have also been injected with intact adipocyte plasma membranes which had not yet undergone extraction with dimethylmaleic anhydride. The results of double immunodiffusion studies, in which rat adipocyte plasma membranes were solubilized in Triton X-100 (1%) and placed in the central well of an agarose plate which contained Triton X-100 (0.1%), are depicted in Fig. 4. Serum from rabbits which had been injected with either plasma membranes or intrinsic membrane proteins from rat adipocytes were placed in the outer wells. Immunoprecipitin lines formed between the detergent-solubilized membranes and each of the antisera except the one from an untreated rabbit. The reaction between membranes and antisera showed a single immunoprecipitin line for two rabbits injected with intrinsic membrane proteins, while a third antiserum (anti-dimethylmaleic anhydride serum 2) gave a faint second line of immunoprecipitation. The antiserum against plasma membranes presented an immunoprecipitin line which had spurs of partial identity with each of the neighboring antisera against intrinsic membrane proteins. These spurs apparently represent the reaction between extrinsic membrane proteins in the detergent-solubilized plasma membrane preparation and the corresponding antibodies against these extrinsic proteins which are present only in the antiserum from the rabbit immunized with plasma membranes. Preliminary experiments indicate that crossed immu-

![Fig. 2. Antilipolytic effect of antibodies to intrinsic membrane proteins in norepinephrine-stimulated rat adipocytes. Rat fat cells (10⁶ cells/ml, 0.1 ml) were incubated for 10 min at 37°C in the presence of 0.3 ml of Krebs-Ringer phosphate buffer (pH 7.4) containing 4% bovine serum albumin plus 0.1 ml of buffer, insulin, or the immunoglobulin fraction of antiserum from normal rabbits or rabbits injected with rat adipocyte intrinsic membrane proteins. Norepinephrine (2 μM) was then added to half of the samples for 60 min at 37°C, at which time 0.1 ml of incubation medium was analyzed for the presence of glycerol by standard fluorometric techniques. The remaining cells and medium were assayed for free fatty acid levels by a titrometric method. The data represent the mean of two experiments with triplicate determinations.](http://www.jbc.org/)

![Fig. 3. Stimulation of rat adipocyte glucose oxidation by antibodies to intrinsic membrane proteins after trypsin digestion of fat cells. Rat adipocytes (10⁶ cells/ml) were incubated with or without trypsin (2 mg/ml) for 15 min at 37°C and then trypsin inhibitor (2.2 mg/ml) was added and the cells were washed thoroughly. Glucose oxidation was then measured, as described in Fig. 1, with 0.1 ml of fat cells plus various concentrations of insulin or the immunoglobulin fraction of antiserum from rabbits injected with intrinsic membrane proteins from rat adipocytes. Data is expressed as nanomoles of CO₂/10⁵ cells/60 min and represent the mean of two separate experiments with triplicate determinations.](http://www.jbc.org/)

![Fig. 4. Double immunodiffusion analysis of the reaction between rat adipocyte plasma membranes (PM) and rabbit antiserum against either rat plasma membranes or membrane proteins which have been retained after extraction with dimethylmaleic anhydride (DMMA). Sera from five different rabbits injected with dimethylmaleic anhydride-extracted membranes or simply with Freund's complete adjuvant (normal rabbit serum) are collected and placed in the outer wells of an immunodiffusion plate which contained 0.1% Triton X-100 in 1% agarose. Rat plasma membranes were then solubilized in Triton X-100 (1%) and placed in the central well; after immunodiffusion for 48 h, the plate was washed thoroughly and stained with Coomassie blue.](http://www.jbc.org/)
noelectrophoretic patterns obtained with solubilised adipocyte membrane proteins or fat cell intrinsic membrane proteins. Rat fat cells were isolated and used to determine glucose oxidation as described in Fig. 1 with or without the addition of insulin (2.4 milliunits/ml) or various concentrations of heat-inactivated antisera from a rabbit injected with either rat plasma membrane vesicles (Rabbit 4) or rat adipocyte intrinsic membrane proteins (Rabbit 2). The data are expressed relative to the values found for glucose oxidation in samples which received only buffer and represent the mean for two experiments with triplicate determinations.

The biological activity of antisera against the rat adipocyte plasma membrane was also tested. It can be seen in Fig. 5 that glucose oxidation in rat fat cells was stimulated by insulin (2.4 milliunits/ml) almost 5-fold and that, in these experiments, antiserum against rat adipocyte intrinsic membrane proteins was even more effective in accelerating this process. In addition, it was found that antisera against the rat adipocyte plasma membrane also stimulated glucose oxidation in a concentration-dependent fashion and caused higher rates of glucose oxidation than those seen in the presence of either insulin or antibodies against intrinsic membrane proteins. Once again, maximal rates of glucose oxidation were observed when antisera were used at a dilution of 1:5 or 1:10.

It is most probable that the biologically active component of antiserum serum is an immunoglobulin since this component is retained in serum fractions treated with ammonium sulfate or DEAE-cellulose, or both, for immunoglobulin purification. There is evidence, however, that serum proteases co-purify with immunoglobulins during ammonium sulfate precipitation and DEAE-cellulose chromatography (22). This observation, coupled to the fact (23) that limited proteolysis can mimic the action of insulin on fat cell glucose transport, made it necessary to rigorously exclude the possibility that serum proteases were causing the biological effects seen with these antisera. This question can be answered experimentally by antibody absorption studies. Antibodies can be isolated from sera by incubation with membranes followed by centrifugation of insoluble membrane protein-antibody complexes and release of the antibodies from these complexes by acid treatment. Antibodies released from immune complexes by acid treatment are very unlikely to contain contaminating proteases. It can be seen in Fig. 6 that incubation of the immunoglobulin fraction of antisera against intrinsic membrane proteins with rat adipocyte plasma membranes followed by centrifugation removes almost all of the capacity of the antiserum to form an immunoprecipitation line against plasma membrane proteins. We have found that complete elimination of immunoprecipitation could be achieved with higher concentrations of plasma membranes (not shown). In addition, acid treatment of precipitated membrane-antibody complexes resulted in the release of antibodies which were still capable of an immunoprecipitation reaction with detergent-solubilized plasma membranes (Fig. 6). Similar results are obtained in absorption experiments with intact rat fat cells or intrinsic membrane proteins or with mouse adipocyte plasma membranes. The immunoglobulin fraction from normal rabbit serum does not react with solubilized membranes under any of these conditions.

The absorption and release experiments described above involve the use of immunoprecipitation as an assay for the presence or absence of antibodies and additional studies must be conducted to confirm the absence or presence of biologically active antibodies in these preparations. The data in Table I depict the results from experiments in which antiserum against either whole plasma membranes or intrinsic membrane proteins were incubated with intrinsic membrane proteins. Absorption took place overnight and after centrifugation to isolate antigen-antibody complexes, antibodies were re-

![Fig. 5](http://www.jbc.org/)

**Fig. 5.** Stimulation of rat adipocyte glucose oxidation by antiserum against either fat cell plasma membranes or fat cell intrinsic membrane proteins. Rat fat cells were isolated and used to determine glucose oxidation as described in Fig. 1 with or without the addition of insulin (2.4 milliunits/ml) or various concentrations of heat-inactivated antiserum from a rabbit injected with either rat plasma membrane vesicles (Rabbit 4) or rat adipocyte intrinsic membrane proteins (Rabbit 2). The data are expressed relative to the values found for glucose oxidation in samples which received only buffer and represent the mean for two experiments with triplicate determinations.

![Fig. 6](http://www.jbc.org/)

**Fig. 6.** Double immunodiffusion analysis of the reaction between rat fat cell plasma membrane proteins and antibodies against intrinsic membrane proteins after absorption and release from rat adipocyte plasma membranes. Absorption was performed by mixing 0.45 ml of adipocyte plasma membrane vesicles (1 mg/ml) with 0.45 ml of the immunoglobulin fraction from normal or anti-intrinsic membrane protein serum and incubating for 60 min at 37°C followed by storage overnight at 4°C. After centrifugation at 40,000 x g for 35 min, the supernatant and labeled "Ig after Absorption" precipitated membrane-antibody complexes were washed thoroughly and resuspended in glycerine buffer (0.1 M, pH 2.8) to release antibodies. After 60 min on ice, membranes were removed by centrifugation at 40,000 x g; the supernatants were neutralized with NaOH and labeled "Ig released." Adipocyte plasma membranes were then solubilized in 0.1% Triton X-100 and placed in 1% agarose plates containing 0.1% Triton X-100 opposite wells containing the various immunoglobulins. Immunodiffusion took place for 24 h, after which the plates were washed and stained with Coomassie blue.

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2 D. J. Pillon, J. Grantham, D. Seidman, and M. P. Czech, manuscript in preparation.
leased by acid treatment. Both types of antisera were capable of stimulating rat adipocyte glucose transport activity compared to the rate obtained in the presence of normal rabbit serum (Table I). After absorption with intrinsic membrane proteins, antisera against either intrinsic membrane proteins or whole rat adipocyte plasma membranes were no longer capable of stimulating hexose uptake. The conclusion from these experiments is that the membrane component(s) to which biologically active antibodies in the anti-plasma membrane antibody preparation are binding must be present in the intrinsic membrane protein preparation. Biological activity of the preparation is mediated solely by binding to an intrinsic membrane protein or proteins.

Another possible mechanism whereby these antisera could stimulate fat cell glucose uptake is by the release of endogenous fat cell proteases. It has been shown (24) that antibodies from pemphigus patients bind to the surface of mouse epidermal cells in culture and cause the release of epidermal proteases. These proteases disrupt adherence between viable epidermal cells by catalyzing the breakdown of the extracellular matrix of the cell culture. The addition of trypsin inhibitor to these cells prevents the breakdown of the extracellular matrix caused by the antibody. It is possible that our antisera bind to fat cells and promote the release of cellular proteases, which subsequently cause an accelerated rate of glucose oxidation by limited digestion of sensitive membrane components. This hypothesis has been tested by prior incubation of fat cells with trypsin inhibitor and then measuring the rate of glucose oxidation which occurs in the presence and absence of insulin or the various antisera (not shown). The data demonstrated that trypsin inhibitor had no effect on the ability of insulin or the antisera to stimulate glucose oxidation in rat adipocytes.

In order to characterize further the specificity of the reaction between membranes and the antibodies which we have raised, various tissues from the rat were homogenized and plasma membrane vesicles were prepared. Triton X-100 (1%) was used to solubilize the membranes from spleen, heart, brain, kidney, liver, lung, muscle, and fat cells. The protein content of each sample was normalized to ~1 mg of protein/ml before solubilization. Double immunodiffusion studies were performed in agarose plates containing Triton X-100 (1%). As seen in Fig. 7, adipocyte membranes react with the immunoglobulin fraction from antiserum against fat cell intrinsic membrane proteins. Despite the clear immunoprecipitation line seen between fat cell membranes and the immunoglobulin fraction, no lines of immunoprecipitation between the antiserum and other membrane sources could be observed. This

| Sera                          | Treatment | n-[1-14C]Glucose oxidation to CO₂ (nmol/10^6 cells/80 min) |
|-------------------------------|-----------|----------------------------------------------------------|
| Control                       | None      | 0.41 ± 0.10                                              |
| Anti-dimethylmaleic anhydride | None      | 0.87 ± 0.09                                              |
|                               | Absorption| 0.24 ± 0.04                                              |
|                               | Release   | 0.70 ± 0.03                                              |
| Anti-plasma membrane          | None      | 1.46 ± 0.14                                              |
|                               | Absorption| 0.24 ± 0.05                                              |
|                               | Release   | 0.84 ± 0.01                                              |

**FIG. 7 (left).** Double immunodiffusion analysis of the reaction between detergent-solubilized plasma membranes from various rat tissues and antibodies against rat fat cell intrinsic membrane proteins. Various rat tissues were homogenized in 0.25 M sucrose, 5 mM Tris/HCl (pH 7.4), 1 mM EDTA and a crude plasma membrane fraction was prepared by differential centrifugation. Triton X-100 (1%) was added to the membrane fraction to solubilize membrane proteins and then the samples (0.015 ml) were applied to an immunodiffusion plate composed of 1% agarose, 0.1% Triton X-100 in phosphate-buffered saline (pH 7.4). The immunoglobulin fraction from rabbit antiserum against rat fat cell membranes which had been extracted with dimethylmaleic anhydride was then added to the center wells (0.015 ml) and after 48 h at 25°C, the plate was washed thoroughly and stained with Coomassie blue.

**FIG. 8 (right).** Double immunodiffusion analysis of the reaction between fat cell plasma membranes from various species and antibodies against rat fat cell intrinsic membrane proteins. Adipocytes were isolated from rat, mouse, or monkey parametrial fat pads by collagenase digestion and a crude plasma membrane fraction was prepared by homogenization and differential centrifugation. Membranes were solubilized with Triton X-100 (1%) and applied to immunodiffusion plates which also contained Triton X-100 (0.1%). Antiserum from a rabbit which had been injected with rat fat cell intrinsic membrane proteins was then applied to the center well and, after 48 h at 25°C, the plate was washed thoroughly and stained with Coomassie blue.
result indicates that those components of the fat cell intrinsic membrane protein preparation which react with the antiserum to cause immunoprecipitation are not shared by these various other tissues. It should be noted, however, that an obligatory link between biological activity and the formation of a visible immunoprecipitation line in immunodiffusion experiments has not yet been established.

The utilization of glucose by muscle, via glycolysis and glycogenesis, is another insulin-sensitive process which has been studied extensively. The fact that antiserum to fat cell intrinsic membrane proteins did not exhibit a precipitin line in double immunodiffusion analysis when tested against muscle plasma membranes would suggest that these antibodies may not react with muscle cells and, therefore, may not mimic insulin action on muscle glucose utilization. Alternatively, the biologically active antibodies may interact with a component of the muscle membranes present in such small quantities that no immunoprecipitation line could be visualized, but still mimic insulin action on glucose uptake. The data in Table II depict the results of experiments in which paired soleus muscles were removed from rats and glucose utilization studies were performed. \( \text{d}-[5-^3\text{H}]\text{Glucose} \) conversion to glycogen and the production of labeled water by means of glycolysis are both processes which are stimulated by insulin. In order to compare the biological effects of insulin with those of the antisera which we have raised, muscles were incubated in the presence or absence of insulin, normal rabbit serum, or the serum from rabbits injected with intrinsic membrane proteins. The data in Table II confirm that insulin stimulated the utilization of glucose by soleus muscles in that both glycolysis and glycogenesis were increased significantly by 2.4 milliunits/ml of insulin. However, it is apparent that the immunoglobulin fraction from antiserum against fat cell intrinsic membrane proteins did not mimic the stimulatory effect of insulin on glucose utilization by rat soleus muscles. These results demonstrate that the antiserum against the partially purified adipocyte hexose transport system will not react with another cell type that also contains an insulin-sensitive hexose transport system. If the antibodies are indeed producing their biological effect by binding to the fat cell sugar carrier or insulin receptor, then these adipocyte membrane components must differ from their counterparts in the soleus muscle membrane or are inaccessible to the active immunoglobulin molecule.

### Table II

| Additions                      | Conversion of \( \text{d}-[5-^3\text{H}]\text{Glucose} \) to \( ^3\text{H}_2\text{O} \) | \( \text{nmol/mg} \) |
|-------------------------------|---------------------------------|------------------|
|                               | Glycogen                        | H2O              |
| None                          | 0.08 ± 0.01                     | 2.2 ± 0.2        |
| Insulin (2.4 milliunits/ml)    | 0.50 ± 0.02                     | 4.4 ± 0.4        |
| None                          | 0.12 ± 0.01                     | 2.9 ± 0.5        |
| Anti-membrane Ig (1:5 dilution)| 0.16 ± 0.02                     | 2.9 ± 0.3        |
| None                          | 0.07 ± 0.01                     | 2.3 ± 0.3        |
| Normal Ig (1:5 dilution)       | 0.10 ± 0.01                     | 2.7 ± 0.4        |

### Table III

| Adipocyte source | Addition                  | % of control \( \text{d}-[1-^1\text{C}]\text{Glucose} \) to \( ^1\text{CO}_2 \) |
|------------------|---------------------------|----------------------------------|
| Rat              | None                      | 100 ± 10                         |
|                  | Insulin (2.4 milliunits/ml)| 322 ± 3                          |
|                  | Normal Ig*                | 110 ± 5                          |
|                  | Anti-membrane Ig*         | 333 ± 16                         |
| Monkey           | None                      | 100 ± 13                         |
|                  | Insulin                   | 347 ± 1                          |
|                  | Anti-membrane Ig*         | 105 ± 29                         |
| Mouse            | None                      | 100 ± 1                          |
|                  | Insulin                   | 321 ± 24                         |
|                  | Anti-membrane serum*      | 408 ± 21                         |

* Dilution, 1:5.
(Table III). Additional studies with the immunoglobulin fraction of this antiserum (not shown) have confirmed that mouse fat cells respond to these antibodies in the same manner as rat fat cells when glucose oxidation is monitored. These findings demonstrate a correlation between the formation of an immunoprecipitin line and a stimulation of hormone-sensitive hexose transport, at least in those tissues and species studied in this investigation.

It was also of interest to investigate the role of immunoglobulin divalency in the biological activity of the anti-membrane antibodies used in these studies. Kahn et al. (25) have recently suggested that the antibodies which they have isolated from patients suffering with Type B syndrome insulin resistance and acanthosis nigricans, which are thought to bind to sites on the insulin receptor, may stimulate fat cell glucose transport by agglutinating membrane components. They have shown that monovalent Fab' fragments of the immunoglobulin were ineffective in stimulating hexose transport while anti-Fab added to fat cells treated with monovalent Fab' fragments induced a stimulation of sugar transport. In the present study, Fab fragments were prepared by papain digestion of the immunoglobulin fraction from normal rabbit serum and antiserum against intrinsic membrane proteins. As shown in Fig. 9, goat anti-rabbit Fab reacted on immunodiffusion plates with all of the antisera fractions tested, including the Fab fractions obtained by papain digestion. Goat anti-rabbit Ig also reacted with all of the immunoglobulin fractions tested, although reactions of partial identity can be observed. The Fab fragments lack the Fc region of the IgG molecule and this resulted in the appearance of spurs when tested adjacent to a sample containing intact immunoglobulin molecules. The immunodiffusion pattern in Fig. 9 was consistent with the patterns expected of Fab fragments obtained by papain digestion of rabbit sera. In addition, immunoprecipitin lines did form between solubilized intrinsic membrane proteins and either antiserum or the immunoglobulin fraction from antiserum against intrinsic membrane proteins. However, no reaction was seen between the membranes and Fab fragments of the immunoglobulin fraction from antiserum against intrinsic membrane proteins. As expected, no precipitin lines were observed with any of the fractions of normal rabbit serum tested against solubilized membranes. These results confirm that the Fab fragments prepared in these studies did react with anti-Fab serum and they reacted with anti-Ig serum in a reaction of partial identity with a known immunoglobulin fraction.

The data presented in Table IV depict the results of experiments in which rat fat cells were incubated in the presence or absence of insulin (2.4 milliunits/ml) or the immunoglobulin or Fab fractions from either normal rabbit serum or antiserum against rat adipocyte intrinsic membrane proteins. It can be seen that glucose oxidation by fat cells was significantly stimulated by insulin (2.4 milliunits/ml) and by the immunoglobulin fraction from antiserum against intrinsic membrane proteins. The addition of the Fab fractions to fat cells had no significant effect on glucose utilization nor did the addition of commercial goat antiserum against rabbit Fab fragments. When fat cells were incubated with Fab fragments from antiserum against intrinsic membrane serum prior to addition of antiserum to rabbit Fab fragments, a significant stimulation of glucose oxidation was observed. Control samples which received Fab fragments from normal rabbit serum followed by the antiserum against rabbit Fab fragments were unaffected. Another condition tested involved the combination of Fab fragments with antisera against rabbit Fab fragments for 10 min prior to addition to rat fat cells. It can be seen in Table IV that this combination of anti-membrane Fab plus anti-Fab caused a significant increase in glucose oxidation, while control Fab plus anti-Fab had no effect. These results are consistent with the hypothesis that the anti-membrane antibodies stimulate glucose oxidation subsequent to their binding to antigenic sites on the fat cell surface and an event or events which require multivalgacy of the immunoglobulin.

Table IV

| Additions to adipocytes | Glucose oxidation (%)* |
|------------------------|------------------------|
| None                   | 100±2                  |
| Insulin (2.4 milliunits/ml) | 569±27                |
| Ig from normal rabbit serum | 131±25                |
| Ig from antiserum against intrinsic membrane proteins | 420±53 |
| Control Fab* | 93±11 |
| Anti-membrane Fab* | 120±12 |
| Anti-Fab* | 84±9 |
| Control Fab, followed by anti-Fab | 121±15 |
| Anti-membrane Fab, followed by anti-Fab | 263±13 |
| Control Fab plus anti-Fab* | 107±6 |
| Anti-membrane Fab plus anti-Fab* | 257±55 |

*Fab fragments from the immunoglobulin fraction of normal rabbit serum.

†Fab fragments from the immunoglobulin fraction of rabbit antiserum against rat adipocyte intrinsic membrane proteins.

‡Commercial goat antiserum to rabbit Fab fragments.
DISCUSSION

The data presented in this communication demonstrate that antibodies against rat adipocyte intrinsic membrane proteins are capable of binding to the outer surface of the rat fat cell and producing a stimulation of glucose transport as well as an inhibition of norepinephrine-stimulated lipolysis. The inhibition of lipolysis occurs in the absence of exogenous n-glucose and this fact argues against the hypothesis that the antibodies are acting solely through a direct interaction with the glucose transport system of the adipocyte membrane. It is of course possible that different membrane antigens mediate the effects of antibodies on glucose transport and lipolysis and that the transport system is one such antigen. Previous data (11) have shown that the antibodies do not inhibit $^{125}$I-insulin binding to the fat cells, indicating that the antibodies against intrinsic membrane proteins do not bind to the insulin binding site of the insulin receptor. This conclusion is further verified by the studies involving trypsin digestion of adipocytes; enzymatic degradation of insulin binding sites on the fat cell membrane did not eliminate the response of the cells to antibodies (Fig. 3), suggesting that the antibodies do not potentiate hexose transport by a direct interaction with the insulin binding site. The results of experiments involving tissue specificity and species specificity also indicate that the antibodies are not binding to a site on the insulin receptor close to the site of insulin attachment, since the insulin binding site is believed to be very similar in a wide variety of species and tissues (26).

Absorption studies have demonstrated that all biologically active antibodies prepared in our hands can be absorbed from antisera against either adipocyte plasma membranes or against adipocyte intrinsic membrane proteins. Intact rat fat cells, plasma membranes, or intrinsic membrane proteins, as well as mouse plasma membranes, can all be used for the absorption of the biologically active antibodies. Most importantly, intrinsic membrane proteins can bind all of the biologically active antibodies from antisera against plasma membranes (Table I). This result is a clear indication that antibodies bind to a specific component(s) of the intrinsic membrane protein preparation, which we have employed to immunize rabbits and to partially purify the hexose transport system (6, 7, 10–13).

The nature of that antigenic site which elicits biological activity remains to be determined, but it seems likely that the carbohydrate portion of a glycoprotein would possess the correct location as well as a high degree of antigenicity. Antibodies against human erythrocyte Band 3, however, have been characterized recently (27) and these antibodies are directed against regions of the protein exposed on the internal cytoplasmic surface of the red blood cell plasma membrane. These antibodies have no effect on anion transport in erythrocyte ghosts, despite the fact that Band 3 is thought to be involved in anion transport. It is not clear why the sites on the externally oriented regions of adipocyte membrane proteins were effective antigenic determinants, while those on the red cell surface were not. Perhaps the crucial red cell protein or carbohydrate residues were not recognized as foreign by the rabbit, or perhaps the rat adipocyte membrane proteins were altered by treatment with dimethylmaleic anhydride in such a way as to make them more antigenic than native membrane proteins. This latter possibility seems less likely, in view of experiments in which intact adipocyte plasma membranes were used to immunize rabbits and antisera was raised which was still capable of accelerating glucose transport and reacting with intrinsic membrane proteins.

One possible explanation for the effects of the antisera is that antibodies bind to membrane proteins and cause an aggregation of membrane components; this type of mechanism also has been suggested for the stimulation of adipocyte hexose transport by concanavalin A (28). Recent experiments by Kahn, Flier, and their co-workers (29–34) have shown that patients suffering from Type B syndrome insulin resistance and acanthosis nigricans have antibodies in their sera which block insulin binding to a wide variety of human and rat cells and mimic the action of insulin to stimulate glucose uptake into rat adipocytes. In addition, it has recently been shown that monovalent fragments of the immunoglobulin fraction from these sera are ineffective in stimulating glucose transport (25), a finding which is consistent with the hypothesis that antibodies may activate the adipocyte hexose transport system by aggregating membrane components. A recent report by Jacobs et al. (35) shows that antibodies against a preparation of solubilized rat liver insulin receptors will also cause an activation of rat adipocyte hexose transport activity. It is intriguing to speculate that the mechanisms whereby antibodies against rat liver insulin receptors (35), against rat adipocyte intrinsic membrane proteins (11–13), and against human insulin receptors (29–34) stimulate sugar transport into rat fat cells are similar. The experiments involving Fab fragments (Fig. 9, Table IV) have provided a strong indication that divalent aggregation of membrane components must be involved in the biological activity of the antibodies used in these present studies. With this model in mind, it is possible that the antisera which mimic insulin action do so by interacting with the same membrane components with which insulin interacts. The antisera raised by others presumably bind to the insulin receptor itself (29–35), while our antisera bind to an intrinsic membrane protein, which may or may not be the glucose transport system or the insulin receptor.

A number of agents have been shown to mimic the action of insulin on fat cell glucose transport (36) but, unfortunately, the list of these oxidizing and polyvalent compounds did not appear to possess a common characteristic which might clarify a mechanism of action for all reagents that activate hexose transport. The current observation, that divalent clustering of intrinsic membrane components is requisite for the effects of these antibodies on fat cell glucose metabolism, raises the possibility that aggregation of membrane proteins by substances as diverse as H$_2$O$_2$ and concanavalin A might, in fact, explain the ability of these compounds to mimic insulin action. It is intriguing to speculate that concanavalin A and the antibodies which we have characterized in this report bind to the carbohydrate moieties of a key intrinsic membrane glycoprotein which is exposed to the exofacial surface of the adipocyte and cause cross-linking of adjacent monomers. Oxidants could cross-link this same membrane component by catalyzing disulfide formation between sensitive sulfhydryl residues on adjacent monomers, as previously suggested (36). In this fashion, antibodies, lectins, and oxidizing agents may all stimulate glucose transport activity through a common mechanism which depends on an aggregation phenomenon.

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