Biochimica et Biophysica Acta, 311 (1973) 362–380

BBA 76375

PREPARATION OF INVERTED PLASMA MEMBRANE VESICLES FROM ISOLATED ADIPOCYTES

VANN BENNETT and PEDRO CUATRECASAS

Department of Pharmacology and Experimental Therapeutics and Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, Md. 21205 (U.S.A.)

(Received February 20th, 1973)

SUMMARY

Exposure of isolated rat adipocytes at 4 °C to buffers of low ionic strength induces the formation of intracellular vesicles. After gentle lysis of these cells a membrane preparation can be obtained which has properties indicating the presence of inside-out, intact plasma membrane vesicles:

1. ^{125}I-labeled wheat germ agglutinin and ^{125}I-labeled insulin which are bound to the plasma membrane of intact adipocytes (as cell surface markers) prior to endovesiculation exhibit a diminished rate and extent of dissociation from the inverted vesicle preparation

2. A significant proportion of the specific binding sites for ^{125}I-labeled insulin are inaccessible to trypsin in the inverted membrane preparation.

3. The inverted membrane preparation demonstrates decreased binding of insulin, and this can be in part reversed by disruptive procedures such as sonication, digestion by phospholipase C, and treatment with Triton X-100.

4. 80% of the free unbound ^{125}I-labeled wheat germ agglutinin and ^{125}I-labeled insulin which are "trapped" in the intravesicular medium during the process of endovesiculation is not lost under conditions which cause dissociation of the lectin– or hormone–membrane complex.

The retarded dissociation of the ^{125}I-labeled insulin bound to the plasma membranes of these vesicles suggests that at 24 °C "flip-flop" of the insulin–receptor complex to the cytoplasmic surface of the membrane occurs at a very slow or negligible rate. The adenylate cyclase activity of the inverted vesicle preparation exhibits a decreased sensitivity to L-epinephrine and an elevated stimulation by NaF. The relevance of these findings to current concepts of membrane asymmetry and structural organization are considered.

An asymmetrical organization of the plasma membrane of erythrocytes has been established by a variety of techniques which concur on an uneven distribution between the cytoplasmic and external membrane facets of certain proteins, glycoproteins, and saccharides[1–11], as well as particulate enzymes such as the (Mg^{2+}–Na^+–K^+)-ATPase[12], and the avian adenylate cyclase[13]. The work of Steck and his colleagues with inside-out erythrocyte vesicles[11,14–17] suggests that this type of system

may provide a means of examining directly the inner surface of the cytoplasmic membranes.

The present report describes procedures for the preparation from isolated rat adipocytes of membranes which contain a substantial portion of inverted plasma membrane vesicles. Fat cells were chosen because they are metabolically active, they can be obtained by simple procedures, and they contain well characterized membrane-localized hormone receptors. Certain properties of the adipocyte membrane preparations which have been studied using iodinated insulin and wheat germ agglutinin as specific membrane markers indicate the existence of plasma membrane vesicles with an inside-out configuration. The effect of L-epinephrine and NaF on adenylate cyclase activity has also been examined in these adipocyte membrane preparations.

MATERIALS

Trypsin, soybean trypsin inhibitor, phospholipase C from *Clostridium perfringens* and collagenase were purchased from Worthington Biochemicals. Carrier-free $Na^{125}I$ was obtained from Union Carbide. [α-^{32}P]ATP (6.96 Ci/mM) and ^{3}H-labeled cyclic AMP (24 Ci/mM) were bought from Nuclear Chicago. Porcine zinc insulin was obtained from Eli Lilly and Triton X-100 from Beckman. ATP, phosphoenolpyruvate, ovomucoid, *N*-acetyl-D-glucosamine, pyruvate kinase, and neutral alumina were from Sigma. Bovine serum albumin (fraction V) was from Armour. Wheat germ agglutinin was prepared from wheat germ lipase (Worthington Biochemicals) using an ovomucoid affinity column[18]. The vesiculation process was observed with a Zeiss RA-34 microscope equipped with phase contrast and interference contrast optics. Electron microscopy was kindly performed by Dr John Greenawalt of The Johns Hopkins University School of Medicine.

METHODS

Preparation of inverted plasma membrane vesicles

Exposure of isolated rat adipocytes at 4 °C to buffers of low ionic strength results in the formation of large numbers of internalized vesicles with diameters ranging up to several microns. These included vesicles can be released from the parent cells in an intact state by gentle disruption. Following removal of the released lipid deposits the suspension contains two types of membrane vesicles; those which are formed by invagination prior to cell lysis and those which are derived from external membrane fragments. This preparation exhibits properties which strongly suggest that a substantial fraction of the plasma membrane vesicles exist in an inverted configuration. This procedure is monitored by phase and electron microscopy.

Isolated fat cells (about 100 μm in diameter) are prepared from the epididymal fat pads of 180 to 250 g Sprague–Dawley rats, essentially as described by Rodbell[19]. The cells are thoroughly washed with Krebs–Ringer bicarbonate buffer, pH 7.4. The process of invagination is initiated by chilling the packed cells in ice using 15-ml polyethylene conical centrifuge tubes followed by dilution with approx. 2 vol. of a low ionic strength buffer which contains 1 mM $KHCO_3$, 1.5 mM $CaCl_2$, and 2.5 mM $MgCl_2$, pH 7.6. After incubating at 4 °C for 20 to 40 min with occasional

gentle mixing, the cells are disrupted by sealing the tubes with parafilm and inverting and everting the tubes with a sudden wrist motion 25 to 30 times, as described by Rodbell[20] for the preparation of fat cell "ghosts". The cellular components are separated from the lipid deposits which are released into the medium by centifugation for 1 to 2 minutes at $900 \times g$. The infranatant and precipitate, which contain membranes, cytosol, mitochondria and nuclei, are removed with a 15-gauge, 3.5 inch stainless steel hypodermic needle. The procedure is repeated 3–4 times with the remaining fat layer, and the pooled infranatants and precipitates are centrifuged at $35000 \times g$ for 20 min at 0 °C. The pellet consists of 5 to 10 μm vesiculated cell fragments (ghosts), large numbers of small (< 3 μm) free vesicles, and intracellular organelles. It is often necessary to resuspend the pellet in the same buffer and centrifuge once again to remove small amounts of contaminating lipids; this step can be omitted when particularly fresh membranes are required. In order to release the vesicles which are retained within the remaining ghosts, the pellet is resuspended in about 20 vol. of 0.5 mM sodium phosphate buffer, pH 7.6, and vigorously passed two times through a 26-gauge hypodermic needle. This ruptures vesicles larger than 3 to 5 μm but apparently does not significantly damage smaller vesicles. The buffer chosen for this procedure is of even lower ionic strength than that used in the preceding step and consequently facilitates the disruption process. Furthermore, this buffer helps to reduce the concentration of divalent cations, which can cause agglutination of cell fragments into large aggregates which are resistant to rupture.

The resulting suspension of free vesicles is referred to in this report as the whole inverted vesicle fraction. This can be refined further by centrifugation at $3000 \times g$ for 10 min. This step removes nuclei and aggregated material and leaves a supernatant enriched in vesicles. This fraction is termed the inverted vesicle fraction. These suspensions, after adjusting to 1 mM $MgCl_2$ and 1 mM $CaCl_2$ to facilitate sedimentation, are centrifuged for 40 min at 4 °C. The pellets are finally suspended in the original hypotonic buffer at a concentration of 100 to 200 μg of protein per ml and stored at 4 °C.

Control membrane preparation

Suspensions containing normally oriented membrane vesicles are obtained by homogenizing intact adipocytes in Krebs–Ringer bicarbonate buffer, pH 7.4, for 30 s with a Polytron PT-10 (Brinkmann) at 3000 rev./min; this procedure effectively ruptures cells but spares nuclei. The resulting dispersion of fat and cell fragments is centrifuged at $35000 \times g$ for 40 min at 0 °C and the pellet is resuspended in hypotonic buffer and centrifuged again. The final pellet is suspended in hypotonic buffer at a concentration of 100 to 200 μg of protein per ml and stored at 4 °C. Both the control and the inverted vesicles appear to be stable for up to three days at this temperature.

Iodination of insulin[21] and wheat germ agglutinin[22]

Iodination was performed with carrier-free $Na^{125}I$ stored in 0.1 M NaOH, using chloramine-T as an oxidant[23]. The specific activity of the ^{125}I-labeled insulin varied from 1.4 to 2.0 Ci per μmole, and the preparations were 95 to 99% precipitable by 10% (w/v) tichloroacetic acid using 0.5% albumin as a carrier protein[21]. The specific activity of the ^{125}I-labeled wheat germ agglutinin derivatives was 2 to 6 μCi per μg, and they were 80 to 90% precipitable by trichloroacetic acid[22].

Assays

All assays except those for adenylate cyclase activity are performed in 12.5 mm×150 mm plastic tubes. The specific binding of ^{125}I-labeled insulin to membranes is determined using the polyethylene glycol precipitation method described previously in detail[24,25]. The concentration of labeled insulin which was used in the binding studies varied between 0.6 to 1.2 nM. The specific conditions for the incubations are described in the legends of the figures and tables.

The rate of dissociation of the ^{125}I-insulin–receptor complex is determined by measuring the rate of disappearance of radioactivity from the ^{125}I-labeled insulin-labeled membranes. The dissociation reaction is initiated by adding native insulin (10^{-6} M) and incubating the samples at 24 °C. The amount of ^{125}I-labeled insulin bound is assayed by rapidly adding 4 ml of ice-cold Krebs–Ringer bicarbonate buffer, pH 7.4, containing 0.1% (w/v) albumin to the samples and immediately filtring the suspension under vacuum on Millipore filters (EG, cellulose acetate, 0.25 μm pore size). The filter well is washed with 10 ml of the same buffer. The filtration and washing procedures consume about 10 to 15 s per tube. The radioactivity remaining on the filter is measured in a well-type gamma counter operating at 50% efficiency.

The dissociation of ^{125}I-labeled wheat germ agglutinin from the lectin–membrane complex is determined in a manner similar to that described above for insulin except that the membranes are incubated in an ice bath and the dissociation reaction is initiated by adding *N*-acetyl-D-glucosamine and ovomucoid. The details ot the assays are provided in the legends to Figs 1 and 2.

Adenylate cyclase activity is determined as described in Table III using a modification of the method of Pohl *et al.*[26]. The membrane preparations are maintained at 4 °C and assayed within 3 to 4 h of cell disruption.

Protein is measured after hydrolysis in 4 M NaOH at 90 to 100 °C using the method of Lowry *et al.*[27], with bovine serum albumin as a standard. The protein content was considered to be proportional to the amount of plasma membrane for purposes of comparison between different preparations, provided that these had a similar organelle composition. Thus, the amount of insulin binding per mg of protein by the whole vesicle preparation can be compared directly to that of the control membranes since both contain nearly all of the particulate cellular components and consequently have the same proportion of plasma membrane per mg of protein. It has been established independently that insulin-binding structures are restricted to the cytoplasmic membranes of fat cells[25].

RESULTS

Attempts to purify inverted membrane vesicles

Since it was apparent (to be described shortly) that the vesicle preparations contained a mixture of inverted and normally oriented plasma membranes, serious attempts were made to purify these two classes of membrane particles. Steck and coworkers have achieved substantial separation of inside-out erythrocyte vesicles from evented membranes by centrifugation on "Dextran" gradients[11,14–17]. This method, and various modifications of it, proved unsatisfactory in the present studies because of the long time periods required and because of incomplete separation

of the two plasma membrane populations. When the inverted vesicle fraction was layered on a linear "Dextran 70" gradient, a discrete band of plasma membrane material formed at a density of 1.02 after a 20-h centrifugation at $100000 \times g$. However, on the basis of the fall in the specific binding of insulin and of the retardation of release of bound ^{125}I-labeled wheat germ agglutinin, as will be described below, these preparations contained at most a 10 to 15% increase in the proportion of inverted plasma membranes. Very recent studies[16] also indicate that such gradients may not always effectively separate the two classes of membrane vesicles even in erythrocyte preparations.

Studies of the binding of concanavalin A–ferritin conjugates to red cell membranes by Nicolson and Singer[9] indicate that the membrane oligosaccharides responsible for the formation of cell–lectin complexes occur primarily on the external surface of the erythrocyte plasma membrane. This finding suggested the possibility of purifying inverted membranes by specific bioadsorption of the everted vesicles on lectin–agarose affinity columns. It was reasoned that the normally oriented vesicles might adsorb to these columns while the inverted vesicles would not due to a lack of available binding sites. Chromatography of inverted vesicle preparations on Sepharose-4B columns containing covalently bound concanavalin A (2 to 6 mg per ml of agarose) or wheat germ agglutinin (0.5 to 1 mg/ml) resulted in a modest increase in the fraction of inverted membranes (up to 25 per cent on the basis of the retarded release of ^{125}I-labeled wheat germ agglutinin bound to the membranes). These procedures were not adopted for the present studies since substantial purification required continuous chromatography of the membrane suspensions over periods as long as 10 to 15 h; this resulted in a destruction of adenylate cyclase activity and the yields obtained were not high.

The study by Eylar *et al.*[10] indicates that membrane sialic acid, the moiety which is apparently responsible for most of the cellular zeta potential, is localized on the external surface of the erythrocyte plasma membrane. This suggests that a separation of inside-out from normal vesicles may be accomplished on the basis of this externally-oriented charge density. Accordingly, vesicle preparations were chromatographed on ion-exchange resins such as diamino dipropylamino Sepharose at a pH of 7.2. These procedures were not effective in enriching the proportion of inverted plasma membranes in the vesicle suspensions.

Since repeated attempts by various means to separate the two types of membranes by practical procedures were unsatisfactory, the principal studies were continued using the mixed vesicle preparation.

Inclusion of ^{125}I-labeled wheat germ agglutinin within inverted vesicles

^{125}I-labeled wheat germ agglutinin binds very tightly to saccharide determinants of the plasma membrane of adipocytes[22] and is therefore a useful marker for the external surface of these cells. However, since wheat germ agglutinin can also bind to membranes of intracellular organelles[22], it is necessary that the membrane–lectin complex be formed initially in the intact cell, before any disruption procedures are instituted. Accordingly, the cells are washed to remove all the free iodoprotein in the medium before exposing them to disruptive conditions. The lectin will adhere very tightly to the membrane during the washing and subsequent procedures provided these are performed at 4 °C. Dissociation of the complex can

then be achieved when desired by the addition of *N*-acetyl-D-glucosamine or ovomucoid to the medium[22].

The rate and extent of dissociation of a lectin molecule which is bound to the inner aspect of a membrane vesicle should in principle be quite retarded since the dissociating molecules (especially ovomucoid) cannot have free access to the lectin–membrane complex, and since the lectin molecule which is released from the complex cannot readily traverse the vesicular membrane and gain access to the medium. A population of mixed vesicles (inverted and everted) which is prepared from adipocytes previously exposed to ^{125}I-labeled wheat germ agglutinin should, therefore, exhibit an apparently diminished dissociation of the bound lectin compared to that of a preparation which is composed principally of everted plasma membrane vesicles. Accordingly, the rates and extents of dissociation of ^{125}I-labeled lectin–membrane complexes were compared in control and inverted vesicles prepared from fat cells previously labeled on their external surface with ^{125}I-labeled wheat

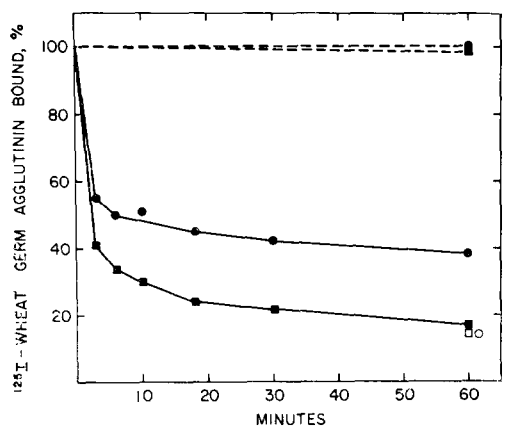

Fig. 1. Rate of release of ^{125}I-labeled wheat germ agglutinin from inverted (●, ○) and control (■, □) membranes prepared from intact fat cells which had previously been complexed with the iodinated lectin. ^{125}I-labeled wheat germ agglutinin-labeled adipocytes were prepared by incubating a suspension of 4 ml of packed fat cells in 8 ml of Krebs–Ringer bicarbonate buffer, pH 7.4, with 10 ng per ml of ^{125}I-labeled wheat germ agglutinin and 10 μg per ml of native lectin (specific activity, 0.2 μCi per μg) for 15 min at 24 °C. The cells were washed 3 times with Krebs–Ringer bicarbonate buffer and divided into two portions, one for preparation of control vesicles and the other for inverted vesicles, as described in the text. The control and inverted vesicle preparations were finally suspended in a buffer containing 50 mM NaCl, 1.0 mM $KHCO_3$, 1.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, pH 7.6, at concentrations of 102 μg of protein per ml (598 cpm per μg) and 72 μg of protein per ml (701 cpm per μg), respectively. The release of membrane-bound lectin at various times was measured at 4 °C with samples (0.1 ml) of the membrane suspensions in the presence (———) and absence (– – –) of 1.7 mg per ml of ovomucoid and 78 mM *N*-acetyl-D-glucosamine. The amount of radioactivity at each time was determined by rapidly adding 4 ml of ice-cold Krebs–Ringer bicarbonate buffer containing 0.1% (w/v) bovine serum albumin to the samples and filtering through EG cellulose acetate Millipore filters (pore size 0.25 μm). The filters were washed with 10 ml of the same buffer, and assayed for ^{125}I in a well-type gamma counter. The values are expressed as a per cent of the radioactivity present at the beginning of the experiment in the absence of dissociating agents, and were determined in duplicate. The effect of incubation (60 min, 4 °C) with Triton X-100 (0.05%) in addition to *N*-acetyl-D-glucosamine and ovomucoid was also determined for control (□) and inverted (○) vesicles; these values are expressed as the per cent of radioactivity which is retained compared to samples exposed only to Triton X-100.

germ agglutinin (Fig. 1). In the absence of *N*-acetyl-D-glucosamine or ovomucoid virtually no dissociation occurs during 60 min at 4 °C. However, upon addition of these dissociating agents rapid dissociation of the lectin–membrane complex occurs. Although the rate of dissociation is somewhat slower in the inverted vesicle preparation, the most striking differences are found in the extent to which dissociation occurs. In the experiment described in Fig. 1 about 82% of complex dissociates (60 min, 4 °C) in the control preparation compared to 60% in the inverted vesicle fraction. Since the inverted vesicle fraction consists of a mixture of normally-oriented and inside-out vesicles, it can be expected that the observed rate of dissociation in this case would be contributed principally by the normally-oriented vesicle components and may thus not differ very significantly from the rates observed in the control preparations. However, the proportion of bound molecules which are capable of dissociating under these conditions should differ by an amount which should be roughly proportional to the relative content of both types of vesicles in the preparations. From the data in Fig. 1 it may therefore be inferred that in this experiment at least 25% of the vesicles may be of the inverted type. It is notable that in the presence of 0.05% of the detergent Triton X-100, which as will be described shortly probably destroys the integrity of the vesicle orientation, the extent of dissociation is identical in both membrane preparations (Fig. 1).

The dissociation of wheat germ agglutinin was also studied under conditions where the concentration of ovomucoid was kept constant (1.7 mg per ml) and the concentration of *N*-acetyl-D-glucosamine was varied (Fig. 2). The differences between the two membrane preparations are readily detectable at all concentrations of *N*-acetyl-D-glucosamine. Since at 4 °C the rate of dissociation of wheat germ

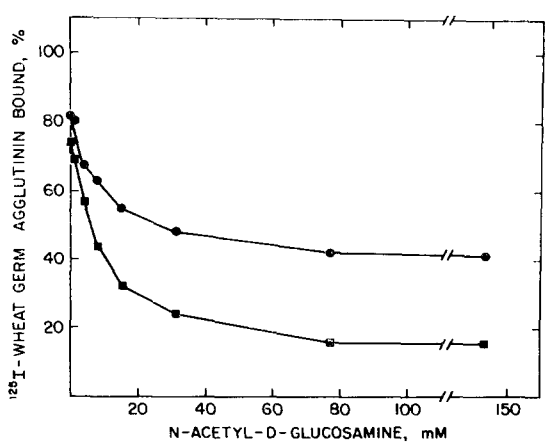

Fig. 2. The effect of increasing the concentration of *N*-acetyl-D-glucosamine on the release of membrane-bound ^{125}I-labeled wheat germ agglutinin from control (■) and inverted (●) vesicles prepared from intact fat cells previously incubated with the iodinated lectin. The control and inverted membranes were prepared and suspended as described in Fig. 1. The dissociation of the bound lectin was initiated by adding to the membrane samples (0.1 ml) 20 μl of solutions containing ovomucoid (10 mg per ml) and *N*-acetyl-glucosamine (at the indicated concentration). The suspensions were incubated at 4 °C for 100 min, and the amount of radioactivity retained at each concentration of *N*-acetyl-D-glucosamine was determined as described in Fig. 1. The values are expressed as the per cent of radioactivity compared to the samples which were not exposed to ovomucoid or *N*-acetyl-D-glucosamine.

agglutinin is not increased by raising the concentration of ovomucoid, the amount of the glycoprotein was not varied.

Inclusion of ^{125}I-labeled insulin within inverted vesicles

Recent studies with ^{125}I-labeled insulin of very high specific activity [21,25,29] suggest that the high-affinity insulin-binding site (dissociation constant about 10^{-10} M) may serve as a very sensitive and specific indicator of certain cytoplasmic plasma membranes. Therefore, inclusion of experiments analogous to those with wheat germ agglutinin were also performed with labeled insulin as a plasma membrane marker.

The rate of dissociation of insulin from its receptor at 4 °C is less than 0.1% of the rate at 24 °C (ref. 29). Thus, insulin can be complexed very tightly with available receptors simply by incubating cells with the labeled hormone at room temperature followed by chilling in an ice-bath. The cells can be washed to remove the free iodoinsulin in the medium without significant losses of bound insulin. The control and inverted vesicle preparations can then be prepared by the usual procedures. Receptor-bound insulin presumably would be sequestered on the inner surface of the inside-out portion of the plasma membrane vesicles. Dissociation experiments can be conducted when desired simply by raising the temperature to 24 °C.

It must be recognized that the number of binding sites per fat cell for insulin is about 10^4 times lower than for the lectin, and that a significant amount of binding may occur by nonspecific adsorption of the hormone to the cell. It is therefore necessary to include appropriate control samples to ascertain that the observed binding of ^{125}I-labeled insulin involves only the biologically important insulin receptor. In the past[21,24,25], the specific binding of insulin has been determined by subtracting from the total amount of bound radioactivity that amount which can be displaced by incubating the cells first with relatively high concentrations of the native hormone. In the present experiments the nonspecific adsorption of insulin was determined and its contribution was corrected for in a similar manner. Membranes were prepared from two sets of cells; one was incubated with $5 \cdot 10^{-10}$ M ^{125}I-labeled insulin alone, and the other was exposed to 10^{-6} M native insulin before addition of the labeled hormone ($5 \cdot 10^{-10}$ M). The specifically bound insulin was determined by subtracting the radioactivity per mg protein of the membranes prepared from the cells which had been exposed to native insulin from the value obtained with the membranes obtained from the cells which had been incubated with the labeled hormone alone. Less than 10% of the insulin-binding activity was nonspecific by these criteria in either the control or the inverted vesicle preparations.

The dissociation of specifically bound ^{125}I-labeled insulin at 24 °C was determined with the different membrane preparations (Fig. 3). The inverted vesicles contain a significantly greater fraction of nondissociable insulin–receptor complexes than the control membranes, again indicating the presence of a sequestered portion of the plasma membrane in the inverted vesicle fraction. Incubation with 0.05% Triton X-100 again destroys this segregated microenvironment and equalizes the amount of ^{125}I-labeled insulin lost from control and inverted vesicles. This effect of the detergent is almost certainly not due to a direct alteration of the insulin–receptor interaction itself since no diminution in insulin-binding is detected with two-times higher concentrations of the detergent.

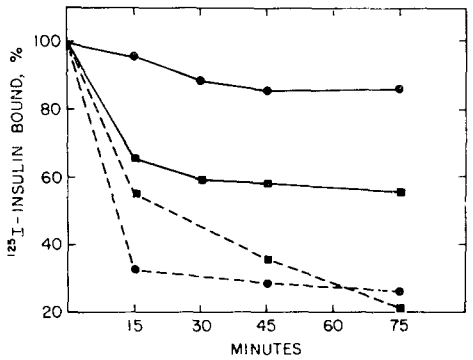

Fig. 3. Rate of dissociation of specifically bound ^{125}I-labeled insulin in the absence (———) and presence (– – –) of 0.05% Triton X-100 from control (■) and inverted (●) vesicles prepared from isolated fat cells which have previously been incubated with iodinated insulin. ^{125}I-labeled insulin-labeled adipocytes were prepared by incubating a suspension of 5 ml of packed fat cells in 10 ml of Krebs–Ringer bicarbonate buffer, pH 7.4, 0.1% (w/v), bovine serum albumin, with $5 \cdot 10^{-10}$ M ^{125}I-labeled insulin (1.5 Ci per μmole) for 40 min at 24 °C. The cells are washed 2 times with ice-cold Krebs–Ringer bicarbonate buffer and kept at 4 °C. The cells were then divided into two portions, one for the preparation of control vesicles, and the other for inverted vesicles. The final control and inverted membranes are suspended in a buffer containing 1 mM $KHCO_3$, 1.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 0.5% bovine serum albumin, pH 7.6, at a concentration of 0.22 mg of protein per ml (6600 cpm per mg of specifically bound ^{125}I-labeled insulin) and 0.29 mg of protein per ml (10300 cpm per mg of specifically bound ^{125}I-labeled insulin), respectively. Dissociation of specifically bound insulin was initiated by adding native insulin (10^{-6} M) and by warming the samples (0.2 ml) to 24 °C. The amount of particle-bound radioactivity was determined as described in Fig. 1 and the text. The nonspecific component of binding (not shown in figure) was determined by preparing control and inverted membranes from cell samples which were treated in identical fashion except that 10^{-6} M native insulin was added before exposure to the labeled hormone. The radioactivity (per mg protein) for these membranes was less than 10% of the value obtained with membranes prepared from cells exposed only to ^{125}I-labeled insulin; these values did not change significantly after incubation at 24 °C. The values in the figure have been corrected for this nonspecific fraction. 10 μl of Triton X-100 (1.0%) was added to some (– – –) samples 10 min before initiating the dissociation reaction. Values are expressed as the per cent of radioactivity compared to that of the samples assayed before initiation of the dissociation experiment.

Protection of insulin binding from tryptic digestion in inverted vesicles

Tryptic digestion of adipocytes, liver and fat cell membranes, and the solubilized insulin receptor severely ablates the ability of these preparations to specifically bind insulin[25,30]. If a population of intact, inverted plasma membrane vesicles exists, the internally localized insulin receptors may be inaccessible to trypsin and may thus retain their insulin-binding capacity after digestion with the enzyme. It was of interest, therefore, to determine if insulin-binding activity could be recovered in the inverted vesicle fraction following exposure of the inverted vesicles to trypsin.

In these experiments the fat cells were not exposed to ^{125}I-labeled insulin before preparation of the membrane fractions. The amount of specific insulin binding to each membrane preparation was determined directly before and after disrupting the vesicles by sonication. Protection of the insulin-binding sites from tryptic digestion was determined by comparing the binding of samples sonicated before tryptic digestion to the value of those disrupted after enzymic digestion. Proteolysis

was halted when desired by the addition of soybean trypsin inhibitor. The insulin-binding assay was conducted in the presence of the same concentrations of trypsin and the inhibitor for the three types of samples, although the order of addition was different in these.

A significant amount of specific insulin binding was measured in the inverted vesicles which were sonicated after tryptic digestion, while those vesicles which were disrupted before exposure to trypsin exhibited a nearly total loss of insulin binding (Table I). The emergence of insulin receptors following sonication of the undigested or digested inverted vesicle preparation is not an artifact of the sonication procedure since sonication of control membranes prepared from trypsinized adipocytes does not increase specific insulin binding (Table I). Also, results qualitatively similar to those of Table I are obtained when the membranes are disrupted by exposure to Triton X-100 instead of by sonication. The findings are in harmony with the earlier inclusion experiments which indicate that a substantial amount of inside-out plasma membranes exist in the inverted vesicle preparation.

TABLE I

RECOVERY OF SPECIFIC [^{125}I]-INSULIN BINDING FOLLOWING DIGESTION OF INVERTED MEMBRANE BY TRYPSIN

The whole vesicle fraction was prepared as described in Methods and suspended in the usual hypotonic buffer with 5 mg per ml of bovine serum albumin. Digestion was performed by the addition of 20 μl of trypsin (0.2 mg per ml of 1 mM HCl) to membrane samples (0.2 ml) followed by an incubation of 12 min at 37 °C. Proteolysis was halted by the addition of 20 μl of soybean trypsin inhibitor (1 mg per ml). When no digestion was desired, the inhibitor was added first, followed by the enzyme. Sonication (10 s) was performed while the samples were immersed in ice. Trypsinized adipocytes were prepared in a separate experiment by incubating a cell suspension (5 ml of packed cells in 5 ml of Krebs–Ringer bicarbonate buffer, 1% (w/v) bovine serum albumin, pH 7.4) with trypsin (25 μg per ml) for 15 min at 37 °C. Membranes were then isolated from these adipocytes by the method described in the text for the preparation of control vesicles, and suspended in a final volume of 2.0 ml (120 μg of protein per ml). The binding assay was conducted with 0.2 ml aliquots, as described above. Specific insulin-binding was determined using polyethylene glycol precipitation methods[24, 25] after incubating the samples for 40 min at 24 °C. These results were qualitatively reproduced in three similar experiments.

Membrane treatment	*Specific ^{125}I-labeled insulin binding* cpm × 10^{-3}*
Sonicated, no digestion	6.5 ± 0.8
Sonicated prior to digestion	0 ± 0.1
Sonicated after digestion	0.9 ± 0.1
Sonicated membranes prepared from trypsinized cells	0.1 ± 0.1
Untreated membranes prepared from trypsinized cells	0.1 ± 0.2

* Mean value ± half range of duplicate determinations.

Decreased binding of ^{125}I-labeled insulin in the inverted vesicles

The previous experiment suggests that in the inverted vesicle preparation a significant number of insulin receptors are inaccessible to macromolecules in the

medium. Since insulin present in the incubation medium would presumably also be excluded from the vesicle interior, it should in principle be possible to demonstrate that the inverted vesicle preparation binds less ^{125}I-labeled insulin than the control membranes. Specific insulin binding per mg of protein is indeed diminished in the whole inverted vesicle preparation relative to control vesicles (Table II). This apparent "loss" of hormone receptors is at least in part reversible by procedures which disrupt membranes such as incubation with Triton X-100, phospholipase C digestion, and vigorous sonication (Table II). These results are in accord with the occurrence of a reversed plasma membrane configuration in the inverted vesicle preparation. The results also suggest that the ability to specifically bind insulin is limited to the external surface of the cytoplasmic membrane.

TABLE II

REVERSIBLE DECREASE IN THE SPECIFIC BINDING OF ^{125}I-LABELED INSULIN FOLLOWING MEMBRANE INVAGINATION

Control and whole vesicle membrane fractions were prepared as described in Methods and suspended at a concentration of 0.3 mg and 0.5 mg of protein per ml, respectively, in the hypotonic buffer, pH 7.6. The binding assay was initiated by the addition of 20 μl of ^{125}I-labeled insulin (1.5 Ci per μmole) to samples (0.2 ml) of the membrane suspensions, to give a final concentration of $6 \cdot 10^{-10}$ M insulin. Nonspecific insulin-binding was determined by the addition of 20 μl of 10^{-4} M native insulin three minutes prior to the labeled hormone, and these values were subtracted from the total amount of insulin bound. The samples were incubated for 100 min at 2 °C, and then assayed for insulin binding using the polyethelene glycol precipitation method described previously[24, 25].

Membrane treatment	*Specific ^{125}I-labeled insulin binding*† *(cpm/mg protein × 10^{-4})*		*Ratio of the binding of inverted and control vesicles*
	Control vesicles	*Inverted vesicles*	
No addition	9.1 ± 0.3	5.9 ± 0.2	0.65
Phospholipase C digested*	10.6 ± 0.3	7.9 ± 0.3	0.75
Sonicated**	6.9 ± 0.4	7.9 ± 0.4	1.14
Triton X-100 preincubation***	9.8 ± 0.3	8.4 ± 0.3	0.86

* Membranes were digested for 60 min at 37 °C with 75 μg per ml of phospholipase C in the presence of 5 mg per ml of bovine serum albumin.

** Membranes were sonicated twice for 20-s intervals while immersed in ice.

*** Membranes were incubated for 30 min with 0.1% Triton X-100 at 20 °C.

† Mean value ± standard error of the mean (four observations).

Adenylate cyclase activity

The adenylate cyclase of the inverted vesicle preparation exhibits a considerable increase in NaF-stimulated activity and a decreased response to L-epinephrine, compared to the control membranes (Table III). The results suggested that the inverted vesicles may possess fewer solvent-exposed L-epinephrine receptors and, in addition, that the NaF-stimulated portion of the adenylate cyclase system might exist in a different state in this preparation. To examine the latter possibility, the adenylate cyclase activity of control and inverted vesicles was measured as a function

TABLE III

RESPONSE OF ADENYLATE CYCLASE ACTIVITY OF CONTROL AND INVERTED VESICLE PREPARATIONS TO L-EPINEPHRINE AND NaF

Control and vesicle fraction membranes were suspended in 50 mM Tris·HCl, pH 7.6, and assayed within 4 h of cell disruption, using a modification of the method described by Pohl *et al.*[26]. The incubation mixture consisted of 0.1 ml containing 50 mM Tris·HCl, 7 mM $MgCl_2$, 1 mM EDTA, 2.5 mM aminophylline, 1 mg per ml bovine serum albumin, 3 mM [α-^{32}P]ATP (2.0 μCi), 5 mM phosphoenolpyruvate, 60 μg of pyruvate kinase, and 80 to 90 μg of protein. After incubating at 30 °C for 12 min, the reaction was terminated by placing the tubes in boiling water for 3 min, and 0.5 ml of a recovery mixture containing cyclic [^{3}H]AMP was added to each sample. Cyclic AMP was then isolated by chromatography on a column containing 1 g of neutral alumina, which was eluted with 2 ml of 50 mM Tris·HCl, pH 7.6, directly into glass counting vials.

Additions	*Adenylate cyclase activity (moles cyclic AMP/mg protein per min × 10^{11})**	
	Control vesicles	*Inverted vesicles*
None	1.1 ± 0.3	0.3 ± 0.1
L-Epinephrine, 10^{-5} M	3.5 ± 0.2	1.8 ± 0.2
L-Epinephrine, 10^{-4} M	4.5 ± 0.1	2.6 ± 0.2
NaF, 10^{-2} M	6.5 ± 0.4	8.4 ± 0.2

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

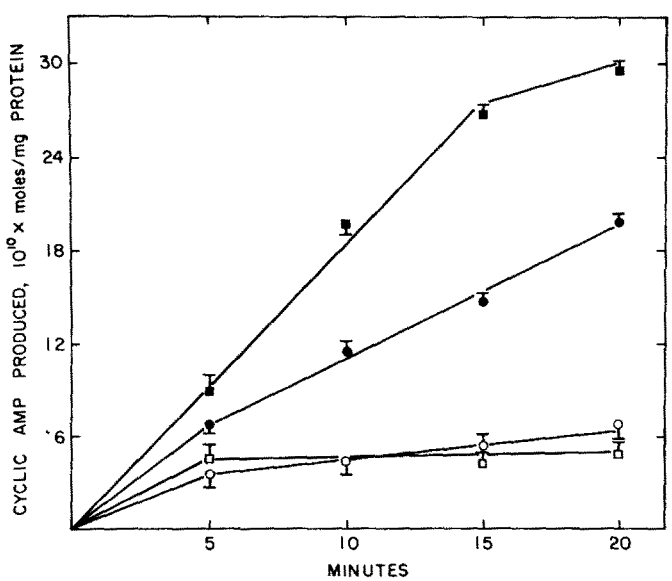

Fig. 4. Base-line (□, ○) and NaF-stimulated (■, ●) adenylate cyclase activity of control (●, ○) and inverted vesicles (■, □) as a function of time. The adenylate cyclase assay was performed as described in Table III using control and whole vesicle membrane preparations suspended in 50 mM Tris·HCl, pH 7.6. Some of the samples were incubated with 0.01 M NaF, while the others received no additions. Error bars are equal to one standard error of the mean (three determinations).

of time in the presence and absence of 10 mM NaF (Fig. 4). The baseline (unstimulated) enzyme activities of the two preparations did not differ significantly. However, the F^--activated enzyme of the inverted vesicles sustains a clearly greater rate of cyclic AMP production during the first 15 min of incubation. The adenylate cyclase activity of these vesicles appears to be less stable, however, since a marked decline in activity occurs between fifteen and twenty minutes. Whole vesicle preparations were used in these experiments in order to validate comparisons of activity on the basis of protein content and to conserve time in the preparation of the membranes.

Release of intravesicular, free ^{125}I-labeled wheat germ agglutinin and ^{125}I-labeled insulin

The previous experiments indicate that a significant population of inside-out plasma membrane vesicles are present in the inverted membrane preparation. Since such vesicles presumably form by a process of membrane invagination, it is possible that a portion of the original medium may be included within the vesicles in the process of vesicle formation. Macromolecules in the medium may also be trapped within the vesicular lumen. Subsequently such intravesicular molecules may not be released into the medium as long as the vesicles remain intact and as long as they retain permeability barriers to macromolecules. Thus, the loss of trapped molecules may provide a means of examining directly the intact nature of the inverted vesicles. The possible processes involved in the translocation of small proteins across the membrane can in principle also be evaluated by comparing the rate of their appearance in the medium relative to that of other large, non-diffusible molecules. It was of interest, therefore, to examine the loss of trapped ^{125}I-labeled wheat germ agglutinin and ^{125}I-labeled insulin from the inverted vesicles.

In these experiments it was essential that neither ^{125}I-labeled wheat germ agglutinin nor ^{125}I-labeled insulin be bound to the membrane. Specific binding of insulin to receptors was minimized by first exposing the intact fat cells to 10^{-6} M native hormone for 30 min at 24 °C, and then allowing invagination to occur in the presence of $5 \cdot 10^{-9}$ M ^{125}I-labeled insulin and 10^{-6} M native insulin at 4 °C for 20 min. The attachment of ^{125}I-labeled wheat germ agglutinin to membrane sites was similarly prevented by preincubating the lectin with ovomucoid, a glycoprotein to which it binds readily. The cells were exposed to wheat germ agglutinin in the presence of 10^4-fold excess of ovomucoid by weight. The membrane preparation containing ^{125}I-labeled wheat germ agglutinin was washed two times with a solution containing 20 mM *N*-acetyl-D-glucosamine, which promotes the rapid dissociation of the lectin.

The amount of free, intravesicular iodoprotein was determined by measuring the amount of radioactivity immediately lost upon sonication of the preparation at 4 °C. About half of the total radioactive material was released in this manner from vesicles containing either insulin or wheat germ agglutinin. Digestion of the vesicles with phospholipase C and incubation with Triton X-100 (0.05%) resulted in a similar loss of radioactivity. It is possible that the disruption procedures merely alter the amount of membrane retained on the Millipore filters. However, this seems unlikely since membranes prepared from ^{125}I-labeled wheat germ agglutinin-adipocytes can tolerate similar treatment with less than an 8% change in radioactivity adsorbed to filters.

The inverted vesicles retained about 80% of the trapped insulin and wheat

TABLE IV

RELEASE OF TRAPPED, UNBOUND ^{125}I-LABELED WHEAT GERM AGGLUTININ AND ^{125}I-LABELED INSULIN FROM INVERTED VESICLES

6 ml of packed, isolated adipocytes were divided into 2 equal portions, and one of them was incubated with $2 \cdot 10^{-6}$ M native insulin for 30 min at 24 °C. Each aliquot of cells was suspended in 3 ml of the usual hypotonic buffer and chilled to 2 °C. A solution (0.3 ml) containing $3.2 \cdot 10^7$ cpm of ^{125}I-labeled insulin and 50 μg of native insulin were added to the cells pretreated with the hormone, while 0.3 ml of a solution containing $2.9 \cdot 10^7$ cpm of ^{125}I-labeled wheat germ agglutinin (5 Ci per g) and 3 mg of ovomucoid were added to the other cells. After a 30-min period of incubation at 2 °C with occasional gentle mixing, the medium was removed and the cells were subjected to the whole vesicle preparation procedure. The membranes from the cells exposed to ^{125}I-labeled wheat germ agglutinin were washed two additional times with a buffer containing 40 mM NaCl, 20 mM *N*-acetyl-D-glucosamine, 1 mM $KHCO_3$, 1.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, pH 7.6, and the final suspension was allowed to incubate for 3 h at 2 °C prior to the assay. The total amount of trapped, unbound material was determined in each case by filtering samples through Millipore filters (EG), as described in Fig. 1 and the text, before and immediately after sonication for 10 s. Vesicle disruption in this manner resulted in a 65% loss of radioactivity from the ^{125}I-labeled wheat germ agglutinin membranes and a 60% decrease from the insulin containing fraction. The amount of labeled material retained by these vesicle preparations was measured at 24 °C by removing 0.2 ml aliquots at the designated times, filtering through Millipore filters (EG). These values, which are corrected for the adsorbed material not released by sonication, are expressed as the per cent of radioactivity compared to samples assayed at the beginning of the experiment and were determined in duplicate.

Time (min)	*Trapped material remaining (%)*	
	^{125}I-labeled insulin	*^{125}I-labeled wheat germ agglutinin*
10	91	95
15	88	92
25	77	81
40	77	80

germ agglutinin after incubation for 40 min at 24 °C (Table IV). It has been shown previously that wheat germ agglutinin does not appreciably penetrate intact fat cells during a 30-min period of incubation at 24 °C[22]. Since the membrane orientation is presumably reversed in the inverted vesicles which trap the medium lectin, the loss of free wheat germ agglutinin from these vesicles should be comparable to the process of entry of the lectin into normally oriented membranes and should consequently occur at a negligible rate. It is therefore likely that the observed 20% fall in retained lectin reflects a loss of the sealed nature of a portion of the inverted vesicles. This could result from a gross disruption of the integrity of the vesicles, or from some less drastic alteration which results in a relaxation of the normal permeability barriers. The similarity in the behavior of trapped insulin and wheat germ agglutinin suggests that insulin also does not significantly penetrate the plasma membrane under the conditions of these studies.

DISCUSSION

The geometry of the gross membrane invaginations observed initially in the intact cells suggested that these internalized vesicles may have undergone a reversal

of the usual membrane configuration. Morphological studies of pinocytic vacuoles of fat cells[31] indicate that an inversion of the plasma membrane does, in fact, accompany invagination. The work of Steck and colleagues[11,14–17] with inverted erythrocyte vesicles suggests that such systems may permit direct examination of the heretofore hidden cytoplasmic facet of the plasma membrane. It was therefore of interest to establish if the "inverted" membrane preparation of fat cells described above did indeed contain a population of inside-out vesicles.

In approaching this problem, it was assumed that the fat cell plasma membrane is relatively impermeable to most macromolecules and consequently that the interior of membrane vesicles constitutes a separate phase, distinct from the surrounding medium, with respect to such molecules. Therefore, provided that the vesicles remain intact, external molecules presumably cannot readily penetrate their interior and internal material cannot readily escape. Two general types of experiments are possible with this system: those based on the premise that molecules trapped within the vesicles are retained, and those devised on the assumption that the vesicle interior is inaccessible to macromolecules in the medium.

Specific means of identifying the outer surface of the cytoplasmic membrane are required in such proposed studies. Recent studies of the interaction of ^{125}I-labeled insulin of high specific activity with adipocytes have demonstrated the exclusive localization of a high affinity insulin-binding moiety in the plasma membranes of these cells[21,25,29]. Furthermore, the total loss of insulin binding in cells or homogenates of cells previously digested with trypsin-agarose[25] also suggests that the receptor structures for this hormone are exclusively oriented externally, at least averaged over the period of digestion. It was not known at the outset of this study whether the insulin receptor would rapidly exchange between an external and internal position in the membrane. However, the present experiments with ^{125}I-labeled insulin indicate that such a process is very unlikely.

^{125}I-labeled wheat germ agglutinin provided another probe for the outer membrane surface. This lectin was chosen because of a fortuitous combination of favorable properties. Native and iodinated wheat germ agglutinin can be purified "functionally" in a single step with ovomucoid affinity columns[18]. A large number (about 10^8) of binding sites are present on the surface of fat cells, and the innate (spontaneous) rate of dissociation of the lectin-membrane complex is extremely slow, especially at 4 °C[22]. Adipocytes do not internalize detectable quantities of wheat germ agglutinin during the time necessary to achieve equilibrium binding of the lectin[22]. Moreover, the membrane-bound wheat germ agglutinin can be dissociated at will by the addition of specific ligands, such as *N*-acetyl-D-glucosamine and ovomucoid, which decrease the half-life of the lectin-complex from more than 40 h to 5 min or less. Thus, if intact cells are briefly exposed to labeled wheat germ agglutinin, the lectin binds in stable form to the external side of the cytoplasmic membrane and the binding is readily reversible by simple procedures.

The inclusion experiments were performed by specifically attaching iodinated insulin and wheat germ agglutinin to the outer membrane surface prior to instituting endovesiculation. The bound molecules are thus adsorbed on the inside phase of any inverted vesicles which are formed from the labeled cell membrane. In these experiments it was necessary to control the dissociation of the membrane–marker complexes. Dissociation of the bound insulin is easily achieved by manipulating

the temperature since little dissociation occurs at 4 °C while at 24 °C the half-life of the hormone–receptor complex is about 30 min[29]. The membrane–wheat germ agglutinin combination is quite sensitive to the presence of *N*-acetyl-D-glucosamine and ovomucoid and can be quickly terminated by the addition of these molecules[22]. The existence of inside-out vesicles was determined by comparing the rate and extent of release of the markers in the control and invaginated vesicles. The preparation (inverted vesicle fraction) containing a portion of previously internalized vesicles retains a significant portion of both markers. Membrane disruption by Triton X-100 in both cases normalizes the loss of marker to that of the control vesicles.

Experiments were also designed on the premise that the interior of membrane vesicles may be inaccessible to externally localized macromolecules. The ability to specifically bind insulin was used as a probe for the inner surface of inside-out vesicles. In some experiments, all of the exposed insulin binding sites were destroyed by tryptic digestion. However, a significant portion of the total specific binding was recovered following sonication. This indicated the existence of a sequestered region of the external surface of the plasma membrane in the inverted preparation, and suggested that a fraction of the insulin receptors should not be available for binding. A 35% decrease in specific ^{125}I-labeled insulin binding of the inverted membranes relative to a control preparation was observed. The apparent loss of plasma membrane components was reversed completely by vigorous sonication and partially by phospholipase C digestion and incubation with Triton X-100. These results indicate that the inverted vesicles contain a fraction of the external plasma membrane surface within a separate microenvironment which can be reintegrated by disruptive procedures.

The present experiments permit examination of the possibility that insulin-binding sites might undergo significant reorientation from the external surface to the inner aspect of the plasma membrane. In the ^{125}I-labeled insulin inclusion experiments, the receptor-bound hormone was presumably internalized within inside-out vesicles. Externalization of these sequestered receptors would be equivalent, in terms of the membrane configuration, to internalization of normally directed receptors on the cell surface. Provided that the inverted vesicles remain intact, internally-bound insulin can escape only by migrating through the membrane to an external position while attached to the receptor, followed by dissociation of the hormone into the medium. The number of ^{125}I-labeled insulin receptors available for such externalization will depend on the relative rates of the two competing processes: internal dissociation of bound insulin, and insulin–receptor reorientation. In such experiments (Fig. 3) only 2% of the radioactivity is lost from the inverted vesicles during the interval from 30 to 75 min. It therefore follows that reorientation of the insulin receptor complex from the internal to the external surface of these vesicles occurs at a negligible rate compared to the process of dissociation of the complex.

One possible mechanism for such an interchange between the two aspects of the membrane would be rotary diffusion of the receptor about an axis parallel to the plane of the membrane. Singer and Nicolson[32] have predicted that this type of motion does not occur with membrane glycoproteins because of a high energy required for the penetration of hydrophilic residues through the membrane lipid phase. The retention of internally-bound insulin observed in the present studies

suggests that if there is significant diffusion of the receptor to the opposite side of the membrane, either the binding site does not penetrate completely or the reoriented insulin–receptor complex has a reduced rate of dissociation. The data can exclude only the rotation during incubation at 24 °C of the hormone–receptor moiety from the external surface of the membrane to an equivalent position on the opposite side in a conformation permitting unaltered dissociation of the bound insulin. It would be of interest to determine if a similar restriction of motion occurs above the liquid crystal–liquid transition temperature for some membrane lipids. It was not possible to perform such studies because at these temperatures (38 to 42 °C) the vesicles became unstable.

The study by Oye and Sutherland[13] with turkey erythrocytes suggested that the NaF-sensitive component of the adenylate cyclase enzyme was localized on the inner surface of the plasma membrane, while the structures responsible for the epinephrine-regulated activity were situated externally. They concluded that the particulate adenylate cyclase contained two types of subunits: an external, hormone-binding portion and an internal catalytic subunit which can be activated directly by NaF. It is significant that the inverted vesicles exhibit a decreased response to L-epinephrine and a concomitantly elevated activity with NaF. These results suggest that the preparation may contain diminished numbers of binding sites for epinephrine, and are consistent with an asymmetric arrangement of the enzyme in the membrane.

The NaF-stimulated adenylate cyclase activity of the inverted vesicles has an apparently greater turnover number than that of control preparations. This suggests that measurement of adenylate cyclase activity in normally oriented vesicular membrane preparations may not necessarily reflect the true intrinsic kinetic properties of the enzyme. For example, it is possible that in ordinary measurements of enzymic activity a rate-limiting step may be the translocation of the substrate, ATP, to the catalytic component of the enzyme on the inner aspect of the membrane. A more detailed study of this system is obviously required, and may provide some insight into certain puzzling aspects of the adenylate cyclase system, such as the site of fluoride action.

A consequence of the formation of vesicles by membrane invagination may be the trapping of external medium within the vesicle interior. This was detected by adding iodinated insulin and wheat germ agglutinin as medium markers during the process of endovesiculation. The normal binding of these molecules to membrane sites was blocked before vesiculation and controls were included to insure that the internalized iodoprotein markers were free. About 80% of the trapped insulin and wheat germ agglutinin were retained after a 40-min period of incubation at 24 °C. Thus, the assumption in the previous experiments that the vesicles remain intact and grossly impermeable to large molecules is approximately correct. These findings emphasize an important problem of studies which use the present vesicle system. It is impossible with the statistical approach employed in this study to determine whether the external emergence of a supposedly internalized structure is due to alterations at discrete points on a single vesicle or simply to generalized vesicle disintegration throughout the preparation. Direct morphologic examination of single vesicles by special electron microscopic techniques will be necessary to obtain an unambiguous interpretation.

The approximately equivalent release of insulin and the non-diffusible lectin, wheat germ agglutinin, suggests also that insulin does not readily penetrate the intact plasma membrane. This finding reaffirms the concept of a strictly external site of action of this hormone, and is in accord with studies indicating that insulin covalently attached to agarose beads is biologically active[33–37].

The process of membrane invagination observed here has not been systematically examined but it may be analogous to pinocytosis. While the amount of internalized membrane may appear to be high (35% on the basis of the decrease in specific insulin-binding), similar and even higher values have been reported in studies of phagocytosis by HeLa cells[38] and amoebae[39]. Further studies of the cytoplasmic facet of the plasma membrane may require a pure preparation of inside-out vesicles available within 3 to 4 h of cell disruption. In the present studies purification has been attempted using Dextran gradients, affinity chromatography with wheat germ agglutinin and concanavalin A attached covalently to agarose beads, and ion exchange chromatography. None of these methods have been completely satisfactory.

ACKNOWLEDGEMENT

Supported by grants from N.I.H. (AM14956), National Science Foundation (GB34300), American Cancer Society, and American Diabetes Association. V. Bennett is a recipient of a Medical Scientist Scholarship funded by the Home Life Insurance Company. P. Cuatrecasas is a recipient of P.H.S. Career Development Award AM31464.

REFERENCES

1 Phillips, D. R. and Morrison, M. (1971) *Biochemistry* 10, 1766–1771
2 Nicholson, G. L., Marchesi, V. T. and Singer, S. J. (1971) *J. Cell Biol.* 51, 265–272
3 Bretscher, M. S. (1971) *Nat. New Biol.* 231, 229–232
4 Bretscher, M. S. (1971) *J. Mol. Biol.* 58, 775–781
5 Bender, W. W., Garan, H. and Berg, H. C. (1971) *J. Mol. Biol.* 58, 783–797
6 Cook, G. M. W. and Eylar, E. H. (1965) *Biochim. Biophys. Acta* 101, 57–66
7 Berg, H. C. (1969) *Biochim. Biophys. Acta* 183, 65–78
8 Lenard, J. (1970) *Biochemistry* 9, 1129–1133
9 Nicolson, G. L. and Singer, S. J. (1971) *Proc. Natl. Acad. Sci. U.S.* 68, 942–945
10 Eylar, E. H., Madoff, M. A., Brody, O. V. and Oncley, J. L. (1962) *J. Biol. Chem.* 237, 1992–2000
11 Steck, T. L., Fairbanks, G. and Wallach, D. F. H. (1971) *Biochemistry* 10, 2617–2624
12 Marchesi, V. T. and Palade, G. E. (1967) *J. Cell Biol.* 35, 385–404
13 Oye, I. and Sutherland, E. W. (1966) *Biochem. Biophys. Acta* 127, 347–354
14 Steck, T. L., Weinstein, R. S., Straus, J. H. and Wallach, D. F. H. (1970) *Science* 168, 255–257
15 Steck, T. L., Straus, J. H. and Wallach, D. F. H. (1970) *Biochim. Biophys. Acta* 203, 385–393
16 Kant, J. A. and Steck, T. L. (1972) *Nat. New Biol.* 240, 26–28
17 Steck, T. L. and Kant, J. A., *Methods in Enzymology*, in the press
18 Marchesi, V. T. (1972) in *Methods in Enzymology* (Ginsburg, V., ed.), pp. 354–356, Academic Press, New York
19 Rodbell, M. J. (1964) *J. Biol. Chem.* 239, 375–380
20 Rodbell, M. J. (1967) *J. Biol. Chem.* 242, 5744–5750
21 Cuatrecasas, P. (1971) *Proc. Natl. Acad. Sci. U.S.* 68, 1264–1268
22 Cuatrecasas, P. (1973) *Biochemistry* 12, 1312–1322
23 Hunter, W. M. and Greenwood, F. G. (1962) *Nature* 194, 495–496
24 Cuatrecasas, P. (1972) *Proc. Natl. Acad. Sci. U.S.* 69, 318–322

25 Cuatrecasas, P. (1972) *J. Biol. Chem.* 247, 1980–1991
26 Pohl, S. L., Birnbaumer, L. and Rodbell, M. (1971) *J. Biol. Chem.* 246, 1849–1856
27 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
28 Burger, M. M. and Goldberg, A. R. (1967) *Proc. Natl. Acad. Sci. U.S.* 57, 359–366
29 Cuatrecasas, P. (1971) *J. Biol. Chem.* 246, 7265–7274
30 Cuatrecasas, P. (1971) *J. Biol. Chem.* 246, 6522–6531
31 Cushman, S. W. (1970) *J. Cell Biol.* 46, 326–341
32 Singer, S. J. and Nicolson, G. L. (1972) *Science* 175, 720–731
33 Cuatrecasas, P. (1969) *Proc. Natl. Acad. Sci. U.S.* 63, 450–457
34 Turkington, R. W. (1970) *Biochem. Biophys. Res. Comm.* 41, 1362–1367
35 Blatt, L. M. and Kim, K. H. (1971) *J. Biol. Chem.* 246, 4895–4898
36 Oka, T. and Topper, Y. J. (1971) *Proc. Natl. Acad. Sci. U.S.* 68, 2066–2068
37 Oka, T. and Topper, Y. J. (1972) *Nat. New Biol.* 239, 216–217
38 Wetzel, M. G. and Korn, E. D. (1969) *J. Cell Biol.* 43, 90–104
39 Heine, J. W. and Schnaitmain, C. A. (1971) *J. Cell Biol.* 48, 703–707