

PREPARATION AND CHARACTERIZATION OF PLASMA MEMBRANE-ENRICHED FRACTIONS FROM RAT PANCREATIC ISLETS

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ABSTRACT

Methods have been developed for the isolation on a semi-micro scale of a plasma membrane-enriched fraction from rat islets of Langerhans. An important feature of these experiments is the use of ^{125}I -labeled wheat germ agglutinin as a specific probe for plasma membrane-containing fractions. The partly purified plasma membrane fraction had a density in sucrose of about 1.10 and was enriched in the activities of 5'-nucleotidase, alkaline phosphatase, sodium-potassium, and magnesium-dependent ATPases and adenylate cyclase. It contained only very low levels of acid phosphatase, cytochrome *c* oxidase, insulin, and RNA. Further purification was hampered by the relatively small amounts of fresh plasma membrane material that could be obtained from 16–24 rats in each experiment. When islets were prelabeled with radioactive fucose, the plasma membrane-enriched fraction contained radioactivity at a four- to fivefold higher specific activity than the whole islet homogenate. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of plasma membrane-enriched fractions pooled from several experiments revealed a distinctive pattern of protein bands as compared with other less pure fractions. With respect to rapidity, apparent specificity, and easy reversibility of the labeling of the plasma membrane fraction, ^{125}I -wheat germ agglutinin provides a highly useful tool for the detection of microgram quantities of plasma membrane components which should be applicable to many other systems as well.

Isolated intact pancreatic islets have been successfully used in a wide variety of studies on the regulation of insulin biosynthesis and secretion. But despite an extensive literature on these topics, as well as on the metabolic pathways of islet cells, it is not yet clear how glucose, the main physiological stimulus to the β -cells, generates a signal that leads to increased insulin secretion and biosynthesis. It is still highly controversial whether glucose itself initiates these processes or whether the triggering signal is a metabolic derivative of glu-

cose. Although a direct receptor site has been postulated for glucose in the β -cell plasma membrane (18, 19, 23, 27, 34, 35), the only known glucose recognition site presumably associated with the plasma membrane is the stereospecific glucose transport system which has been observed in both isolated islets and dispersed β -cell suspensions (20, 30). It also has been claimed that glucose-induced insulin secretion involves stimulation of adenylate cyclase (5), but several workers have failed to find any stimulation of adenylate cyclase

activity in homogenates of isolated islets exposed to glucose (22, 25). However, other compounds known to affect the release of insulin are known to modify adenylate cyclase activity, indicating that this enzyme may play an important role in the regulation of secretion. Adenylate cyclase has been localized to rat islet cell membranes by histochemical methods (21). A variety of observations also suggest that the β -cell plasma membrane may be a site of action of other powerful modifiers of insulin secretion such as sulfonylureas, metabolizable and nonmetabolizable amino acids, certain thiol-reagents, and some of the biogenic amines.

So far, there is no clear understanding of the chemical events leading to the fusion of the secretory granule membranes with the inner surface of the plasma membrane, or of how this exocytotic process is regulated. To study these and many related problems concerning the surface components of islet cells requires the isolation of purified plasma membrane fractions. This study describes methods for preparing rat pancreatic islets on a relatively large scale, and methods for partially purifying, identifying, and characterizing the very small amounts of plasma membrane material derived from the few milligrams wet weight of pure islets that are obtained. This paper also describes the use of labeled wheat germ agglutinin (WGA) as a rapid and selective probe for detecting minute amounts of plasma membrane.

MATERIALS AND METHODS

Crude collagenase, type IV, was obtained from Worthington Biochemical Corp., Freehold, N. J. Sigma Chemical Co., St. Louis, Mo. supplied Na and Tris salts of ATP, adenosine 5'-monophosphate (5'-AMP), *N*-acetyl-D-glucosamine, *N*-2-hydroxyethylpiperazine-*N*'-2-ethane sulfonic acid (HEPES), degraded herring DNA, creatine phosphokinase, phosphocreatine (diTris salt), adenosine 3':5'-cyclic monophosphoric acid (cyclic AMP), cytochrome *c*, and fraction V bovine serum albumin (BSA). Wheat germ agglutinin (WGA) and crystallized bovine albumin were purchased from Miles Laboratories Inc., Kankakee, Ill.; methylumbelliferol and 3-isobutyl-1-methyl-xanthine (IBMX) were obtained from Aldrich Chemical Co., Milwaukee, Wis.; Eastman-Kodak, Rochester, N. Y. supplied chloramine-T; Grand Island Biological Co., Grand Island, N. Y. supplied TCM 199, fetal calf serum, horse serum, penicillin, and streptomycin; PEI cellulose-F plates were obtained from Brinkmann Instruments Inc., Westbury, N. Y.; New England Nuclear, Boston, Mass. supplied [^{14}C]adenosine 5'-triphosphate (tetrasodium salt; sp act >400 mCi/mmol), L-[6- ^3H]fucose (12 Ci/mmol), adenosine [G- ^3H]5'-monophosphate (diammonium salt; 11 Ci/

mmol), and Aquasol universal LSC cocktail. ICN Pharmaceuticals Inc., Irvine, Calif. provided [5- ^3H]uridine (21 Ci/mmol), [γ - ^{32}P]adenosine-5'-triphosphate (sodium salt), and methyl umbelliferol phosphate; Na ^{125}I (sp act >300 mCi/ml) was obtained from Industrial Nuclear Co., St. Louis, Mo. and RNase-free sucrose from Schwarz/Mann Div. of Becton, Dickinson & Co., Orangeburg, N. Y. All other reagents were of analytical grade; distilled and deionized water was used throughout the investigation.

Isolation of Islets

This procedure is a modification of the methods of Moskalewski (33) and Lacy and Kostianovsky (26). All glass and plastic wares were pretreated with Siliclad (Clay Adams, Inc., Parsippany, N. J.). Sprague-Dawley or Holtzman strain rats were injected intraperitoneally with 0.25 ml of 4% pilocarpine HCl 2 h before decapitation, which was carried out under sodium pentobarbital anesthesia. The pancreases from 8-16 rats were removed and washed in Hanks' buffer supplemented with 2.8 mM glucose and 0.2 mg/ml BSA (HGA). Fat and lymph nodes were trimmed away, and each two pancreases were quickly minced with scissors for about 2 min in a 20-ml glass scintillation vial containing 10 ml Hanks' buffer with albumin supplemented with 11 mM glucose and 3-4 mg/ml crude collagenase. The vials were gassed for 20 s with a mixture of 95% O₂ and 5% CO₂ before sealing and were shaken either mechanically or by hand at 37°C for 12-18 min.

After incubation the contents of each vial were poured into 30 ml of cold Hanks' in a 50-ml polycarbonate centrifugation tube. The digest was mixed on a vortex mixer for 15-30 s and centrifuged at room temperature for 30 s at 400 g. The supernatant fluid was aspirated and 40 ml of HGA buffer were added, followed by mixing and centrifugation as above. This washing procedure was repeated three times. After the final centrifugation the digest was diluted with HGA and the islets were allowed to sediment at room temperature for 1-2 min, followed by aspiration of the islet-free supernatant liquid. This sedimentation procedure, which was repeated three times, was found to remove the bulk of the digested exocrine pancreas and was followed by centrifugation for 30 s at 800 g, after which the pellets equivalent to two or four pancreases were combined and thoroughly mixed with 8 ml HGA buffer containing 27% (wt/wt) Ficoll (Pharmacia, Uppsala, Sweden). Over this were layered successively 4 ml each of HGA buffer containing 23.5% (wt/wt), 20.8% (wt/wt), and 12.2% (wt/wt) Ficoll. The discontinuous gradient was centrifuged at room temperature for 10 min at 700 g. In general, four to eight gradients representing 8-16 rats were easily handled at one time. After centrifugation, the islets appeared at the interface between the 12.2% and 20.8% Ficoll layers while the residue remained at the bottom. The islets were withdrawn with a Pasteur pipette, added to fresh HGA buffer in a 50-ml centrifuge tube, washed

three times by centrifugation at 200 g for 30 s, and then transferred to a petri dish. Small lymph nodes, fragments of blood vessels, and other debris were removed from the islets under the stereomicroscope. The islets were then transferred to 1.3 × 5 cm cellulose nitrate tubes and washed three times in 5 ml of HGA buffer by centrifugation at 50 g for 10 s. Depending on the number of rats used, this procedure yielded up to 2,000–3,000 islets.

Incubation of Islets

Islets were washed twice by sedimentation in 15 ml of TCM 199 containing 10% horse serum, 5% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (TCM). The islets were pulse labeled for 6 h at 37°C in 300 μl of TCM further supplemented with 20 mM glucose and with 10 μCi of either [5-³H]uridine or [6-³H]fucose. The islets were then washed twice by sedimentation in 15-ml nonradioactive TCM and chased at 37°C for 14–18 h in TCM containing unlabeled uridine or fucose. The labeled islets were finally washed in Mg-borate buffer (10 mM borate buffer, pH 8.0, containing 0.2 mM MgCl₂) along with added freshly prepared islets before being homogenized.

Homogenization of Islets

Islets in siliconized cellulose nitrate tubes were washed three times at 4°C in 5 ml of Mg-borate buffer by centrifugation at 50 g for 10 s. The islet pellet was then suspended by a micropipette in 200 μl of Mg-borate buffer and transferred to a 550-μl polypropylene micro test tube (Milian Instruments, Geneva, Switzerland). The islets were disrupted by mechanical shaking for 60 sec at 4°C in a mixer similar to a Beckman micromixer, model 154 (Beckman Science Essentials, Mountainside, N. J.), and the obtained homogenates centrifuged at 4°C for 60 s at 100 g. After removal of the supernatant fluid, the pellet was shaken and centrifuged again after addition first of 200 μl Mg-borate buffer and, second, of 300 μl Mg-borate, giving a final homogenate volume of 700 μl. When viewed under a stereomicroscope, the remaining pellet contained some pieces of connective tissue and a few intact islets.

Gradient Centrifugation

Sucrose solutions were made in Mg-borate buffer, pH 8.0, containing 1 mM EGTA. Linear sucrose gradients were mixed into 1.3 × 5 cm cellulose nitrate tubes in a final volume of 4 ml and allowed to equilibrate for 3 h at 4°C before use. Two types of gradients were used (Fig. 1): gradient A was composed from top to bottom of 2.5 ml of 3–14% linear sucrose gradient, 750 μl of 31% sucrose, and 750 μl of 45% sucrose, gradient B was a 4-ml 23–45% linear sucrose gradient.

Centrifugations were carried out at 4°C in a SW39L rotor with the Beckman L-2 or LS-65 ultracentrifuge (Beckman Spinco, Palo Alto, Calif.). Gradient A was carefully sampled from the top with a 500-μl siliconized

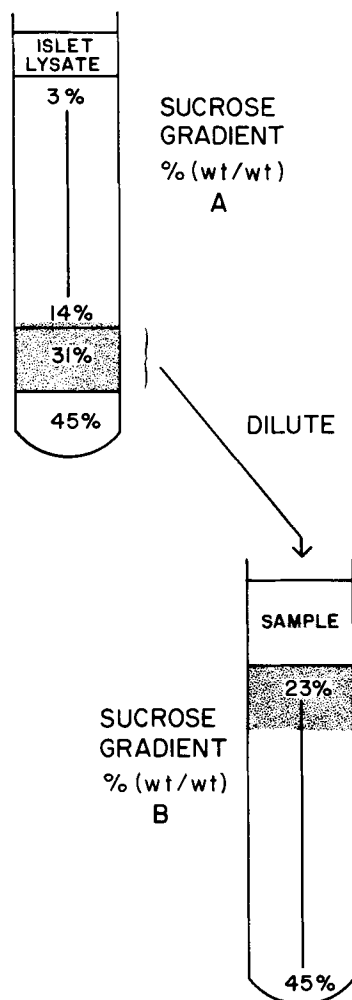


FIGURE 1 Sucrose gradients used for preparation of islet cell plasma membranes. Gradient A was centrifuged at +4°C at 70,000 g for 60 min and was fractionated from above with a micropipette into 10 fractions. Material from gradient A, as indicated, was diluted with 1/2 vol of Mg-borate buffer before being applied to gradient B. Gradient B was centrifuged at 4°C at 70,000g for 90 min and was fractionated from below by piercing the bottom of the tube.

micropipette. Gradient B was pierced at the bottom and about 20 fractions were collected. Sucrose densities were determined in a Bausch & Lomb refractometer (Bausch & Lomb Inc. Scientific Optical Products Div., Rochester, N. Y.). Radioactivity in gradient fractions was determined by adding 5–100-μl aliquots to 700 μl of 0.5 M acetic acid followed by counting in 10 ml of Triton X-100-toluene scintillation mixture (see below). Experimental details are given in the legends to figures and tables.

Analytical Methods

PROTEIN

Samples of homogenates, gradient fractions, and standards (BSA, 0.5–8 μg) were precipitated and washed once with cold 5% TCA in 400- μl polypropylene micro test tubes (Beckman Instruments). Protein was determined on the precipitate in a final volume of 300 μl according to the method of Lowry et al. (31). TCA precipitation was found to be necessary since sucrose influenced the assay in a concentration-dependent manner. The effect of sucrose was potentiated in the presence of 1 mM EGTA. The recovery of protein from the sucrose gradients varied between 85 and 100%.

5'-NUCLEOTIDASE

5'-Nucleotidase activity was assayed in duplicate samples, using a modification of the radioassay described by Avruch and Wallach (2). The assay was carried out in a final volume of 500 μl at 37°C and pH 8.5 in the presence of 40 μM of 5'-AMP and tracer amounts of [^3H -5']AMP. After incubation for 60 min and separation of degraded and nondegraded 5'-AMP by $\text{Zn}(\text{SO}_4)/\text{Ba}(\text{OH})_2$ precipitation, 100- μl aliquots were added to 700 μl of 0.5 M acetic acid, and the radioactivity was counted in 10 ml of Triton X-100/toluene (1:2, vol/vol) scintillation mixture containing 5.5 g/liter PPO and 100 mg/liter POPOP. The activity was expressed as nmol of adenosine liberated in 60 min/ μg protein.

ATPASES

ATPase activities were assayed in duplicate in 310 μl vol of 30 mM histidine buffer, pH 7.4, containing 1.0 mM MgCl_2 , 0.1 mM EDTA, 130 mM NaCl, 20 mM KCl, 1 mM ATP (Na-salt) as well as tracer amounts of γ - ^{32}P -adenosine-5'-triphosphate. Incubations were carried out with or without 1 mM ouabain at 37°C for 60 min. The reaction was stopped by addition of 1 ml of 0.1 M HCl containing 4% (wt/vol) activated charcoal (Norit A), 0.2 mg/ml BSA, 1 mM Na phosphate, and 1 mM Na pyrophosphate. All samples were vortexed and allowed to stand in ice for 30 min to adsorb unreacted ATP before being centrifuged at 4°C for 10 min at 5,000 g. 1 ml of the supernatant fluid was pipetted into 10 ml of distilled H_2O and the Cerenkov radiation of ^{32}P was counted in a Packard liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.). Mg^{2+} -ATPase activity was estimated from the activity obtained in the presence of ouabain. ($\text{Na}^+ + \text{K}^+$)-ATPase activity was estimated from the difference between the activities obtained with and without ouabain. Activity was expressed as nanomoles of Pi liberated in 60 min/microgram protein.

ALKALINE PHOSPHATASE

Alkaline phosphatase activity was determined in duplicates at pH 8.0 in a final volume of 220 μl of 50 mM Tris HCl buffer containing 0.01% BSA according to

Fernley and Walker (13). Methylumbelliferone was used as the standard. Activity was expressed as nanomoles of methylumbelliferone liberated in 3 h/microgram protein.

ACID PHOSPHATASE

Acid phosphatase activity was assayed in the same manner as alkaline phosphatase but at pH 5.5 in a 50 mM citrate buffer containing 0.01% BSA.

CYTOCHROME *c* OXIDASE

Cytochrome *c* oxidase was measured in a final volume of 260 μl as a modification of the assay described by Steiner and Williams (41). Activity was expressed as nanomoles of cytochrome *c* oxidized per minute and microgram protein, by using the millimolar extinction coefficient of cytochrome *c* equal to 19.2.

DNA

DNA was assayed in duplicate samples as described by Lernmark et al. (30). Degraded herring sperm DNA was used as standard.

INSULIN

Insulin was determined by a double antibody radioimmunoassay (32). Human insulin was used as standard.

IODINATION OF WGA

A modification of the chloramine-T method of Freychet et al. (15) was used. To a 2-ml coated Pyrex centrifuge tube was added 10 μg of WGA in 50 μl of 0.3 M sodium phosphate buffer, pH 7.4, as well as 2 mCi of Na ^{125}I (sp act >300 mCi/ml). The reaction was started by the addition of 20 μl of 0.12 mM chloramine-T in sodium phosphate buffer and, after gentle shaking for 3 min, stopped by the addition of 5 μl of 0.05 mM sodium metabisulfite in sodium phosphate buffer and 100 μl of 130 mM borate buffer, pH 7.4, containing 0.5% BSA. The labeled WGA (sp act 20 mCi/mg) was immediately subjected to gel filtration on a 0.8×15 cm superfine Sephadex G-75 column equilibrated with 130 mM borate buffer with 0.5% BSA. The ^{125}I -WGA eluted from the column was completely separated from the free iodide, and migrated as a single peak of radioactivity on SDS polyacrylamide gel electrophoresis. The labeled ^{125}I -WGA was stored at -20°C .

ADENYLATE CYCLASE DETERMINATION

PREPARATION OF SAMPLES: Rat islet homogenates were prepared and, for most experiments, labeled with ^{125}I -WGA as described in Results. Either a sample of the homogenate in Mg-borate was assayed directly for adenylylase activity, or 100 μl of the homogenate was mixed with 5 ml of 25 mM Tris, pH 7.6, containing 1 mM ATP, 1 mM DTT, 1 mM EGTA, 0.05% BSA, and 50 mM *N*-acetyl-D-glucosamine and centrifuged at 100,000 g for 20 min. The protein pellet obtained after centrifugation was frozen on dry ice and stored at -20°C in a solution of 25 mM Tris containing 0.25 M sucrose.

A 500- μ l aliquot of partially purified material from gradient B (see above) was pelleted and stored in the same manner. The peak fractions of 125 I-WGA from gradient B representing the purified plasma membrane fraction, and the gradient fractions surrounding the plasma membrane fraction were combined (usually in groups of three) and also stored and pelleted as above.

Assay Procedure

The system used was similar to that described by Howell and Montague (22). A 25- μ l aliquot of the sample to be assayed was added to 50 μ l of a reaction mixture containing 25 mM Tris, 1.5 mM EDTA, 7.5 mM MgCl_2 , 0.075% albumin, 0.75 mM IBMX, ATP- ^{14}C at a concentration of either 1.5 mM or 0.15 mM (sp act \sim 100,000 cpm/nmol) and an ATP regenerating system of 30 mM phosphocreatine and approximately 1.15 mg/ml of creatine phosphokinase. The final pH of the reaction mixture was adjusted to 7.6. Reaction blanks consisting of 25 μ l of buffer plus 50 μ l of the above reaction mixture were included in each experiment.

The effect of the duration of incubation on cyclic AMP formation was determined by incubating 25 μ l of the total homogenate, prepared in Mg-borate and not pelleted, with 50 μ l of the above reaction mixture in 37.5 mM Tris, so that the final Tris concentration equaled 25 mM. At a final ATP concentration of either 1.0 mM or approximately 0.1 mM, cyclic AMP formation was linear for at least 30 min. Accordingly, this incubation period was used for all assays.

The effect of protein concentration on cyclic AMP formation was determined using pelleted samples of the whole homogenate or the partially purified membrane fraction obtained from the first gradient, which were stored frozen and then diluted in 25 mM Tris, pH 7.6, containing 0.25 M sucrose. Cyclic AMP formation was proportional to added protein up to a concentration of 8 $\mu\text{g}/\text{tube}$. Samples were appropriately diluted so as to fall within this concentration range. The protein concentration used in assays of purified plasma membrane fractions ranged from 0.62 μg protein/tube to 5.91 μg protein/tube.

At the end of the 30 min incubation period, the reaction was terminated by the addition with rapid mixing of 25 μ l of 20% perchloric acid (PCA) containing 40 mM unlabeled cyclic AMP. To separate cyclic AMP from the other nucleotides present, a 50- μ l aliquot of the supernatant fluid, obtained after centrifugation in the Beckman 152 microfuge for 5 min at 4°C, was chromatographed on PE1 cellulose F thin-layer plates (3) along with authentic cyclic AMP in the system—95% ethanol-1.15 M ammonium acetate (74:26 vol/vol) (42). The carrier cyclic AMP band was identified under UV light, marked, and then scraped directly into a scintillation vial to be counted in 15 ml of Aquasol. Results are expressed as picomoles of cAMP formed \times 30 $\text{min}^{-1} \times \mu\text{g}$ protein $^{-1}$.

Electron Microscopy

Homogenates or gradient fractions (100–600 μ l) in cellulose nitrate tubes (1.3 \times 5 cm) were fixed by the addition of 4 ml of cold 2% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.4. After mixing, the fixed material was pelleted by centrifugation for 20 min at 100,000 g. The pellet was then resuspended in 600 μ l of glutaraldehyde and kept in 5 \times 42-mm cellulose nitrate tubes at 4°C overnight before being pelleted by centrifugation as above. By transferring to a micro tube, a visible pellet could be obtained which was postfixed in 1% phosphate-buffered osmium tetroxide, pH 7.3, rinsed in distilled water, dehydrated in ethanol, and finally, via propylene oxide, embedded in Epon 812. Sections, cut on an LKB ultratome (LKB Instruments, Inc., Rockville, Md.), were stained with lead citrate and uranyl acetate and examined in a Philips EM300 electron microscope.

RESULTS

Homogenization of Islets

Previous studies on isolated mouse islets suggested that they can be dispersed into cells by rapid mechanical shaking. Brief shaking for 10–15 s yielded mostly free cells while prolonged shaking markedly diminished the recovery of intact cells (29). Since such a procedure seemed to be more gentle than ordinary homogenization, it was adopted for the preparation of islet plasma membranes. It was found that shaking in the hypotonic Mg-borate buffer for at least 60 s completely disrupted the islets and the islet cells. When viewed in the phase contrast microscope, blood vessels and connective tissue seemed to remain intact. However, the nuclei appeared markedly swollen in hypotonic buffer, and this could be prevented by the addition of MgCl_2 (0.2 mM) but less so by CaCl_2 . Aggregation of particles occurred if the MgCl_2 or CaCl_2 concentration was increased to 0.5 or 1 mM.

Fractionation Procedure

In a homogenate prepared in Mg-borate buffer, around 70% of the 5'-nucleotidase activity of the homogenate remained in the supernatant fluid after centrifugation for 5 min at 800 g while 50% was pelleted after centrifugation for 10 min at 10,000 g. Because some 5'-nucleotidase was lost in the initial centrifugation to remove nuclei, freshly prepared homogenates were layered on the sucrose gradients shown in Fig. 1. Rate zonal centrifugation of fresh islet homogenates on 3–14% (wt/wt) linear sucrose gradients for 60 min at

10,000 g resulted in a broad peak of 5'-nucleotidase and Mg^{2+} ATPase activities near the top of the gradient. However, the activity of 5'-nucleotidase in this peak accounted for only about 16% of the total activity added to the gradient, the rest of the activity was recovered at the bottom of the tube. When the slowly sedimenting peak was collected and applied to gradient B (Fig. 1) and centrifuged for 90 min at 70,000 g, the 5'-nucleotidase activity was fully recovered in the density region corresponding to about 1.10.

After centrifugation of homogenates on linear 3–14% sucrose gradients for 60 min at 70,000 g, very little 5'-nucleotidase, Mg^{2+} -ATPase, or ouabain-sensitive ($Na^+ + K^+$)-ATPase remained on the gradient, in contrast to the amount of protein added which was pelleted to the extent of only about 50%. However, further purification of pelleted material was disadvantageous because the pellet was difficult to suspend, giving rise to an altered and variable distribution of 5'-nucleotidase activity on gradient B.

By adding two discontinuous zones of higher density below the linear 3–14% gradient of sucrose, gradient A, shown in Fig. 1, was designed. The behavior of various marker activities, including 5'-nucleotidase and Mg^{2+} -ATPase, on this gradient is summarized in Fig. 2. If not otherwise stated, the results in this and the following figures are given as percent of total activity applied to the gradient. Major amounts of 5'-nucleotidase and Mg^{2+} -ATPase activity were recovered in fractions 8 and 9. Before further purification on gradient B, these fractions were combined and diluted as indicated in Fig. 1. The characterization of this partially purified material is included in Table I under "PM fraction after Gradient A." As shown in Table I, roughly 23% of the added protein remained in this fraction resulting in a three- to fivefold increase in specific activity of membrane markers such as 5'-nucleotidase, Mg^{2+} -ATPase, and alkaline phosphatase. Around 15% of the added DNA, 40% of the insulin, and 26% of the acid phosphatase activity also remained in the partially purified material. More than 100% of the activities of ouabain-sensitive ($Na^+ + K^+$)-ATPase and cytochrome *c* oxidase were found in the partially purified material, indicating that these enzyme activities might have been partially inhibited in the islet whole homogenate.

On gradient A, with loads of 0.3–1.4 mg total homogenate protein, a faint zone of particles

could be seen in the 31% sucrose. Some aggregates of particles could be seen at the interface between 31% and 45% sucrose, especially with higher starting protein concentrations. When the gradient was sampled from above with a micropipette, this material was not included in the partially purified fraction. This sampling procedure was adopted since some material was pelleted at the bottom of the tube, making it impractical to collect these gradients from below.

For further purification of the plasma membrane-containing fraction, the partially purified sample from gradient A was added to gradient B (Fig. 1), centrifuged for 90 min at 70,000 g, and collected from below in 18–20 fractions. In no instance could any bands be seen in the region of the gradient that contained the major peaks of plasma membrane markers. Gradient B was analyzed for various markers as shown in Figs. 3 and 4; the results for the plasma membrane peak are summarized in terms of percentage distribution and specific activities in Table I. The recovery of protein in the plasma membrane-containing fraction amounted to 0.5–1.5% of the islet homogenate. A severalfold purification over the islet homogenate of plasma membrane markers such as 5'-nucleotidase, alkaline phosphatase and Mg^{2+} -ATPase was found. Alkaline phosphatase showed a small distinct peak at higher densities. The specific activities of DNA and insulin were decreased while that for cytochrome *c* oxidase remained unchanged relative to the partially purified membrane fraction. The plasma membrane fraction retained 1–3% of the total homogenate acid phosphatase activity, and there was a slight increase in its specific activity relative to the homogenate.

Ouabain-sensitive ($Na^+ + K^+$)-ATPase activity could hardly be detected in the islet homogenate, but the activity of this enzyme markedly increased both after partial purification (Table I) and after final centrifugation on gradient B (Fig. 3). There was an apparent 25-fold increase in the specific activity of this enzyme over the homogenate in the purified plasma membrane fraction.

Figs. 5 and 6 show the distribution of various markers after a freshly prepared homogenate was layered on gradient B (Fig. 1) and centrifuged for 90 min at 70,000 g. Fig. 5 shows that 5'-nucleotidase was recovered as a major peak, at a density of about 1.10, which represented 23% of the total activity recovered from the gradient. About 35% of the protein applied to the gradient remained at

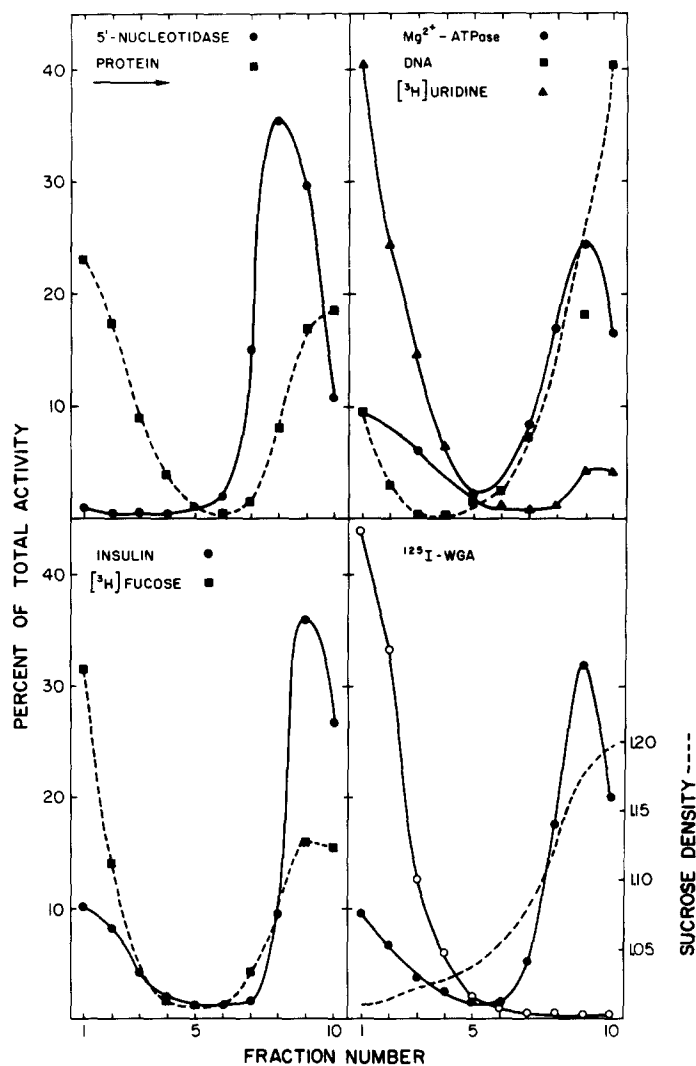


FIGURE 2 Fractionation of islet homogenates on gradient A. The relative distributions of 5'-nucleotidase, protein, Mg²⁺-ATPase, DNA, insulin, and uridine and fucose radioactivities from prelabeled islets are shown. The lower right panel shows the distribution of ¹²⁵I-WGA either added directly to the gradient (without islet homogenates) (○—○) or representing radioactivity in fractionated islet homogenates (●—●). The islet homogenates were obtained from intact islets incubated with ¹²⁵I-WGA in 130 mM borate buffer, pH 7.4, containing 0.5% BSA for 30 min at room temperature. The incubation was followed by extensive washing at 4°C in nonradioactive borate buffer before the islets were washed and homogenized. Each curve represents the mean of two to four separate experiments. Direction of sedimentation is from left to right.

the top, and no significant peaks of protein were observed throughout the gradient.

Although a high percentage of the insulin and acid phosphatase activity was found at the top of the gradient, peaks of both also appeared in the density region around 1.15. The specific activity of insulin in the peak fraction amounted to around

58 ng insulin/μg protein, representing a threefold purification over the whole homogenate (Fig. 6). Centrifugation of the gradients for 4 h at 70,000 g did not appreciably change the distribution of 5'-nucleotidase activity although the peak of insulin shifted to a somewhat higher density (not shown).

The peak of 5'-nucleotidase activity also con-

TABLE I
Distribution of protein and various markers

	Homogenate	PM fraction after gradient A	PM fraction after gradients A and B
Protein			
μg	919	166	9
% recovery	100	18	1
DNA			
ng · μg protein ⁻¹	21	35	6
RA	1	1.6	0.2
% recovery	100	13	0.2
Insulin			
ng · μg protein ⁻¹	15.1	25.1	8.3
RA	1	1.7	0.6
% recovery	100	41	0.2
5'-Nucleotidase			
nmol · μg protein ⁻¹ · 60 min ⁻¹	1.4	5.1	21.4
RA	1	3.5	17.3
% recovery	100	63	12
(Na⁺-K⁺)-Mg²⁺ ATPase			
nmol · μg protein ⁻¹ · 60 min ⁻¹	0.3	2.3	7.1
RA	1	8.8	27.4
% recovery	100	377	54
Mg²⁺ ATPase			
nmol · μg protein ⁻¹ · 60 min ⁻¹	19.9	23.7	55.5
RA	1	1.2	2.8
% recovery	100	51	6
Alkaline phosphatase			
nmol · μg protein ⁻¹ · 3 h ⁻¹	0.2	1.0	1.4
RA	1	5.6	8.3
% recovery	100	64	6
Acid phosphatase			
nmol · μg protein ⁻¹ · 60 min ⁻¹	0.5	0.4	0.6
RA	1	0.9	1.4
% recovery	100	30	2
Cytochrome c oxidase			
nmol · μg protein ⁻¹ · min ⁻¹	0.05	0.19	0.19
RA	1	4.1	4.6
% recovery	100	119	5
[³H]Uridine distribution			
cpm · μg protein ⁻¹	341	119	86
RA	1	0.4	0.3
% recovery	100	11	0.1
[³H]Fucose distribution			
cpm · μg protein ⁻¹	50	81	237
RA	1	1.6	4.7
% recovery	100	7	2

Distribution of protein, DNA, insulin, and various markers in the islet homogenate, plasma membrane (PM) fraction from gradient A and the plasma membrane (PM) fraction obtained after centrifugation on both gradients A and B. RA denotes relative specific activity. Each value represents the mean of 2-10 different experiments. The experiments with [³H]uridine and [³H]fucose utilized islets prelabeled with these isotopes as described in the text.

tained major activities of Mg²⁺-ATPase, ouabain-sensitive (Na⁺ + K⁺)-ATPase (Fig. 6) as well as of adenylate cyclase (not shown). In each case, about a fourfold increase in specific activity over the starting homogenate was obtained. It seemed that the fractions containing plasma membrane markers were contaminated with some of the soluble proteins which sedimented only slightly or not at all. The high concentrations of insulin and acid

phosphatase at the top of the gradients also suggested that some disruption of lysosomes and secretion granules had occurred. Centrifugation of islet homogenates on gradient A effectively removed these contaminants.

Experiments with Uridine-Labeled Radioactive Islets

Initially, NADH-dependent cytochrome *c* reductase activity was chosen as a marker for the endoplasmic reticulum. Although available methods were scaled down to a final reaction volume of 200 μl, the assay was not sensitive enough to allow determinations on the individual fractions from the sucrose gradients. Islets were therefore

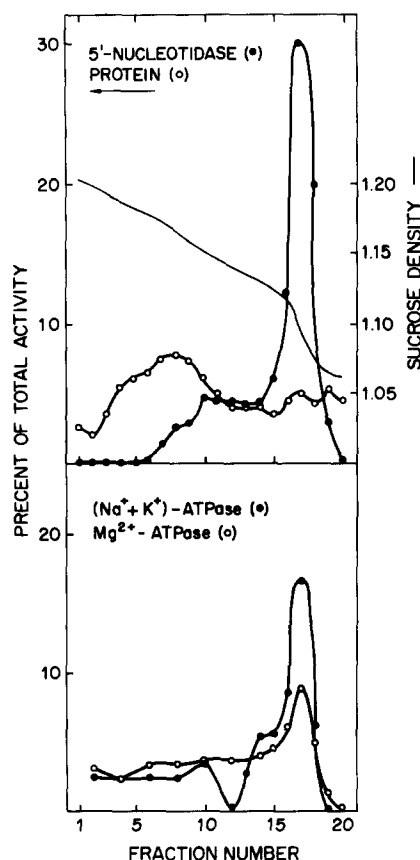


FIGURE 3 Fractionation of partially purified islet homogenates on gradient B centrifuged for 90 min at 70,000 g. The relative distributions of total activity recovered from the gradient for protein, 5'-nucleotidase, (Na⁺ + K⁺)-ATPase, and Mg²⁺-ATPase are shown. There was an 85-100% recovery of activities added to the gradients. Each curve represents the mean of two to four separate experiments.

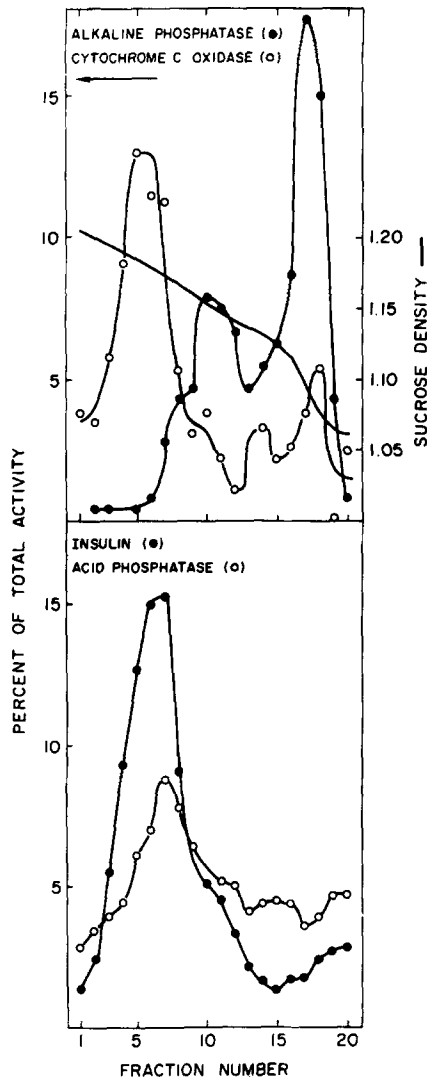


FIGURE 4 Fractionation of partially purified islet homogenates on gradient B centrifuged for 90 min at 70,000 *g*. The relative distributions of total activity recovered from the gradients are shown for alkaline phosphatase, cytochrome *c* oxidase, insulin, and acid phosphatase. There was an 85–100% recovery of activities added to the gradient. Each curve represents the mean of two to four separate experiments.

labeled with [³H]uridine for a 6-h pulse period followed by a chase period of more than 10 h to allow incorporation into a stable cytoplasmic RNA pool, presumably localized in the rough endoplasmic reticulum and free ribosomes or polysomes. Some of the labeled islets were lysed in 1% SDS and centrifuged on 5–20% sucrose/Na acetate gradients. Uridine radioactivity was re-

covered in fractions sedimenting as 28S, 18S, and slower sedimenting RNA species. Almost 80% of the uridine radioactivity was recovered in the three uppermost fractions on gradient A (Fig. 2), and roughly 9% was recovered in the partially purified membrane material. On the second gradient a major portion of the uridine radioactivity was recovered in a density region of about 1.15–1.17, and only roughly 0.1% of the homogenate radioactivity was found in the plasma membrane fraction.

Experiments with Fucose-Labeled Islets

The distribution of radioactive fucose from homogenates of prelabeled islets on gradients A and B is shown in Figs. 2 and 7, respectively. Approximately 50% of the fucose radioactivity added to gradient A was recovered in the three uppermost

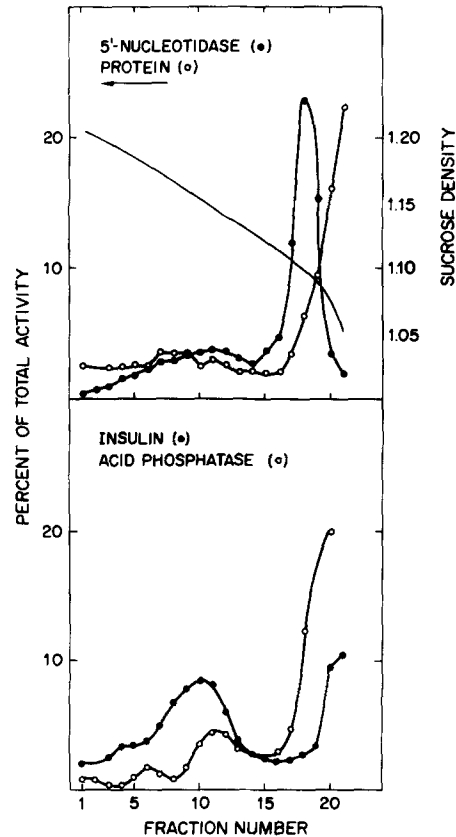


FIGURE 5 Fractionation of islet homogenates on gradient B showing distribution of protein, insulin as well as activities of 5'-nucleotidase and acid phosphatase. Each curve represents the mean values of two separate experiments.

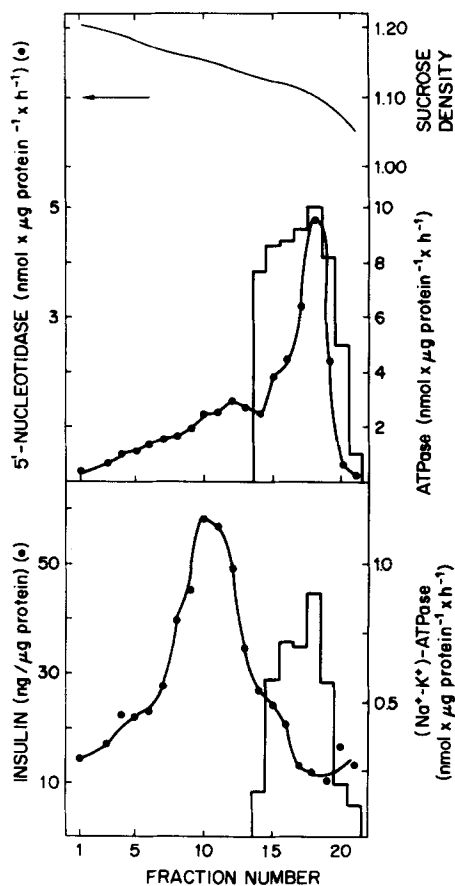


FIGURE 6 Fractionation of islet homogenates on gradient B. The distribution of specific activities of 5'-nucleotidase, Mg^{2+} -ATPase, $(Na^+ + K^+)$ -ATPase, and insulin is shown. Each curve represents the mean values of two separate experiments.

fractions. The fucose radioactivity did not show a marked peak on gradient A in the high sucrose density fractions. Partially purified membrane material (fractions 8 and 9), centrifuged on gradient B, revealed a peak of radioactivity in the plasma membrane fraction (Fig. 7), with a four- to five-fold increase in specific activity over the islet homogenate (Table I).

Experiments with ^{125}I -Wheat Germ Agglutinin (WGA)-Labeled Islets

A simple method for following the fractionation of the islet cell plasma membrane would be to use a radioactive marker which binds firmly to the membrane surface without redistributing during homogenization and fractionation. For further analytical studies on the membrane fraction (e.g.,

enzyme assays or electron microscopy), it is also desirable that the labeled marker be able to be dissociated. For this purpose, isolated islets or islet homogenates were labeled with ^{125}I -WGA in an approach similar to that suggested by Chang et al. (6).

The distributions of radioactivity on gradients A and B of islet homogenates prepared from intact isolated islets incubated with 10^{-9} M ^{125}I -WGA are shown in Figs. 2 and 8. As can be seen in the lower right panel of Fig. 2, free ^{125}I -WGA did not sediment on gradient A. When intact islets were prelabeled with ^{125}I -WGA before homogenization, a peak of radioactivity appeared at fractions 8 and 9 (Fig. 2). Similar results were obtained

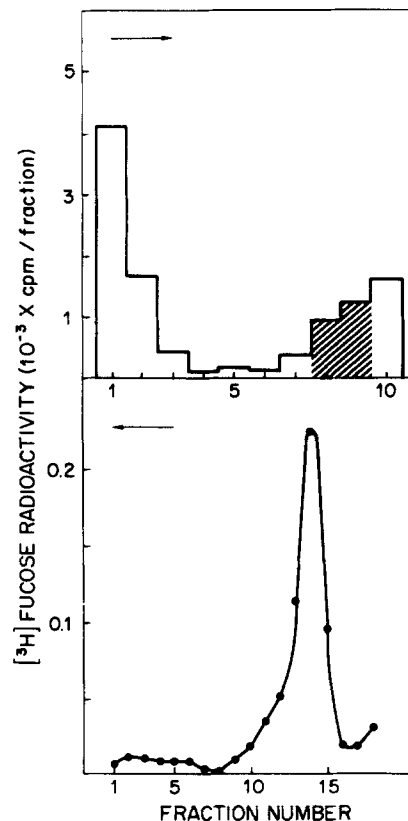


FIGURE 7 Distribution of fucose radioactivity on gradients A and B of homogenates from islets prelabeled with $[6-^3H]$ L-fucose as described in Materials and Methods. After fractionation on gradient A (upper panel), the material in fractions 8 and 9 (shaded) was added to gradient B (lower panel) and centrifuged for 90 min at 70,000 g. The distribution of radioactivity in 18 fractions collected is shown. The peak of radioactivity coincided with that of 5'-nucleotidase activity.

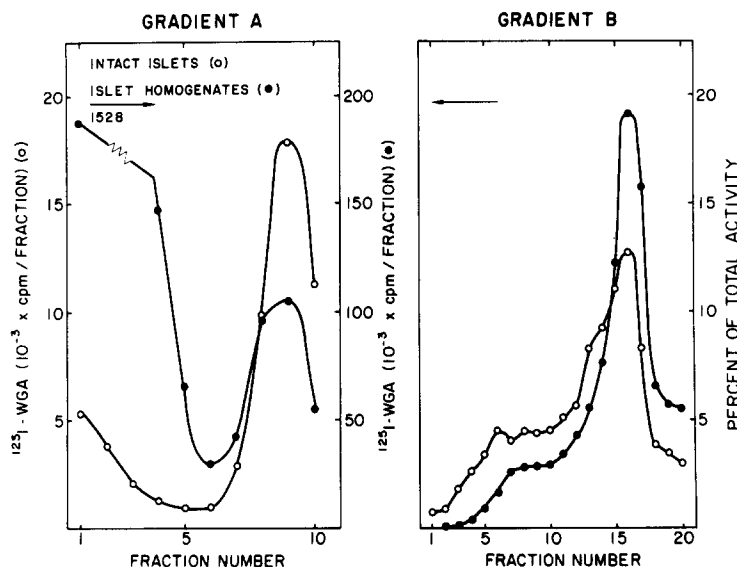


FIGURE 8 Distribution of ^{125}I -WGA radioactivity on gradients A and B. Intact freshly isolated islets were incubated at room temperature for 30 min with 10^{-9} M ^{125}I -WGA ($4\text{--}5.5 \times 10^6$ cpm) in $500 \mu\text{l}$ of 130 mM borate buffer containing 0.5% BSA. The islets were then washed three times at 4°C by low-speed centrifugations in 5-ml volumes of the above nonradioactive borate BSA buffer before being washed and homogenized in the Mg-borate buffer. The homogenate of intact islets (○—○) was then centrifuged on gradient A, fractions 8 and 9 were brought together, diluted with Mg-borate, and this sample was finally centrifuged on gradient B. Gradient B was centrifuged for 90 min at $70,000 g$ and fractionated, the result of which is shown in the right panel. When counting the ^{125}I -WGA radioactivity, the whole fractions obtained from gradients A and B were counted in a Packard gamma scintillation spectrometer.

In experiments with broken islets, freshly prepared islet homogenates ($700 \mu\text{l}$) were incubated with an aliquot ($10\text{--}100 \mu\text{l}$) of ^{125}I -WGA ($4\text{--}5.5 \times 10^6$ cpm) for 30 min at 4°C . The result of centrifugating the labeled homogenate on gradient A is shown in the left panel (●—●). The material in fractions 8 and 9 was then centrifuged on gradient B for 90 min at $70,000 g$, the fractionation of which is shown in the right panel (○—○). The curves represent mean values from two to three experiments.

upon incubation of islet homogenates with ^{125}I -WGA (Fig. 8). When the material in the peak fractions of gradient A (Fig. 8) was centrifuged on gradient B, WGA radioactivity was recovered as a sharp peak of radioactivity in the density region around 1.10. The labeled profile corresponded closely to that obtained for 5'-nucleotidase as was confirmed by direct measurements of the enzyme in the same experiments (data not shown).

The binding of ^{125}I -WGA in relation to protein is summarized in Table II. A severalfold enrichment in specific activity was achieved in the plasma membrane fraction when either intact islets or islet homogenates were labeled with the lectin. A higher specific activity was obtained in the plasma membrane fraction when islet homogenates were labeled (Table II).

Fig. 9 shows that the binding of ^{125}I -WGA is reversible. Incubation of partially purified material from gradient A with 50 mM *N*-acetyl-D-glucosamine displaced a major part of the radioactivity from the plasma membrane fraction to the top of the gradient. D-glucose did not alter the binding of the lectin.

The patterns of ^{125}I -WGA radioactivity on gradient B after labeling intact islets or islet homogenates were strikingly similar (Fig. 8). There was no indication of major binding to particles in a higher density region. In fact, the shoulder of ^{125}I -WGA radioactivity seen on gradient B after labeling intact islets was less pronounced after labeling the islet homogenates. However, binding studies with purified subcellular fractions should be carried out to further validate the specificity of WGA-binding to islet cell plasma membranes.

Adenylate Cyclase Measurements

The distribution of unstimulated adenylate cyclase activity in combined fractions from the 23% to 45% (wt/wt) continuous sucrose gradient (B),

TABLE II
Distribution of [¹²⁵I]Wheat Germ Agglutinin

	Homogenate	PM fraction gradient A only	PM fraction gradients A and B
Intact islets labeled			
cpm μg protein ⁻¹	66	203	367
RA	1	3.2	5.6
% recovery	100	31	4
Islet homogenate labeled			
cpm μg protein ⁻¹	—	1,731	8,321
RA	—	1	5.4
% recovery	—	100	24

Distribution of [¹²⁵I]WGA radioactivity in islet homogenates, plasma membrane (PM) fraction from Gradient A, and the plasma membrane (PM) fraction obtained after centrifugation on both Gradients A and B. RA denotes relative specific activity. Each value represents the mean of three (intact islets) and five (islet homogenates) separate experiments. Intact islets or islet homogenates were labeled with [¹²⁵I]WGA and fractionated on gradients A and B as described in the legend to Fig. 8.

along with the binding of ¹²⁵I-WGA to each gradient fraction, is depicted in Fig. 10. Maximum adenylate cyclase activity is present in the combined gradient fraction containing the peak of ¹²⁵I-WGA and surrounding fractions, demonstrating the enrichment of adenylate cyclase in the plasma membrane fraction.

The increase in the specific activity of adenylate cyclase as compared to the activity in pellets of the initial islet homogenate and the partially purified fraction is shown in Table III. After partial purification (pelleted), adenylate cyclase activity increased from 1.50 pmol of cAMP per 30 min/μg protein in the pelleted homogenate to 2.89 and increased further to 3.68 in the pelleted plasma membrane fraction. Thus, a 2.5-fold purification of basal adenylate cyclase activity was achieved in the plasma membrane fraction.

Electrophoretic Pattern of Membrane Fraction Proteins on SDS Gels

The patterns of proteins in the islet cell homogenate and the plasma membrane fractions after electrophoresis on polyacrylamide gels containing SDS are shown in Fig. 11. The amount of plasma membrane protein applied (about 30 μg) was insufficient to give distinct bands for either clear photographic reproduction or scanning. Thus, in the figure the banding pattern has been drawn adjacent to the photographs of the gels for clarity.

The plasma membrane fraction showed a distinct band pattern from the homogenate which contained many additional bands. Further experiments will be necessary to describe fully the pro-

tein pattern typical for islet cell plasma membranes.

Electron Microscopy

Representative electron micrographs of the final plasma membrane containing fraction are shown in Fig. 12. This fraction consisted almost entirely of smooth membrane vesicles and sheets, very rarely with associated ribosomal particles. Occasionally, small, dense, membrane-surrounded particles, resembling vesicles from the Golgi apparatus or contracted cristae from disrupted mitochondria, as well as rare secretion granules, also could be seen in some areas.

Electron micrographs of whole islet homogenates showed the presence of swollen mitochondria, intact secretion granules, lysosome-like particles, and an abundance of membrane vesicles, many with attached ribosomes (not shown). Frac-

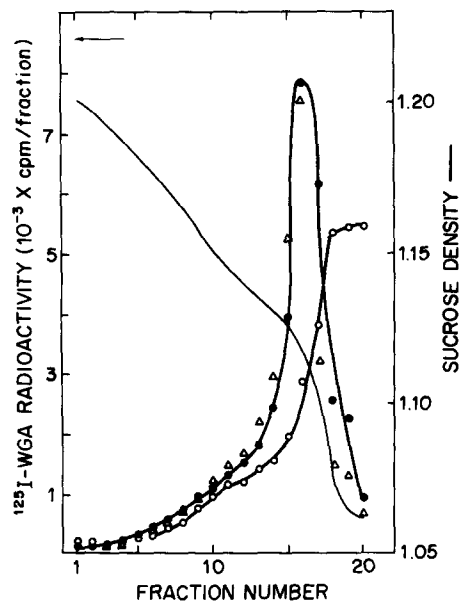


FIGURE 9 Effect of *N*-acetyl-D-glucosamine (O—O) and D-glucose (Δ—Δ) on the distribution of ¹²⁵I-WGA radioactivity on gradient B. Islet homogenates were incubated with ¹²⁵I-WGA and centrifuged on gradient A as described in Fig. 9. Fractions 8 and 9 were combined from gradient A and divided into three samples. These were diluted to a final volume of 1 ml with Mg-borate buffer only (●—●), or buffer containing either *N*-acetyl-D-glucosamine (O—O) or D-glucose (Δ—Δ) to a final concentration of 50 mM. All samples were allowed to stand at room temperature for 15 min before being centrifuged on gradient B as described in Fig. 1.

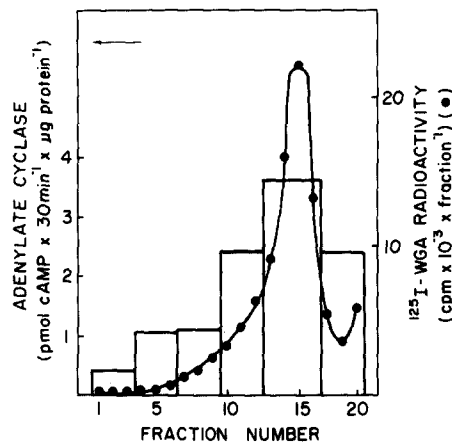


FIGURE 10 Distribution of specific adenylate cyclase activity and total ^{125}I -WGA radioactivity on gradient B. Islet homogenates were incubated with ^{125}I -WGA and centrifuged on gradient B as described in Fig. 9. Partially purified material was diluted with Mg-borate buffer and centrifuged on gradient B at 70,000 g for 90 min. Twenty fractions from 5.3-ml gradients were collected, and the whole fractions were counted in a Packard gamma scintillation spectrometer. The pattern of ^{125}I -WGA radioactivity was found to coincide with that of 5'-nucleotidase. In order to obtain enough material for the adenylate cyclase assay, 3-4 fractions were combined, diluted in Tris buffer containing 50 mM *N*-acetyl-D-glucosamine, and particles were pelleted by centrifugation at 100,000 g for 20 min (see Materials and Methods).

tions 4-6 from gradient B (density approximately 1.17) which contained the highest activities of cytochrome *c* oxidase, acid phosphatase, and insulin (see Fig. 4) contained fragments of rough endoplasmic reticulum, microsomal particles, intact secretion granules, swollen mitochondria, and mitochondrial fragments (not shown).

DISCUSSION

This study describes methods for the isolation of a fraction enriched in plasma membranes from rat islets of Langerhans. By means of sensitive fluorescence and radiometric enzyme assays, as well as by binding of a ^{125}I -labeled lectin, it was possible to identify and partially characterize the membrane fraction obtained from only a few milligrams of islet protein. To obtain even this limited amount of starting material from rat pancreas required the modification to a larger scale of existing collagenase digestion methods for islet isolation (26, 33) and the use of Ficoll density gradients to separate the islets from the pancreatic digests.

This modified method provided roughly 2,000-4,000 islets from 8-16 rats within 2-3 h. We also noted that pretreatment of the animals with pilocarpine seemed to give an improved yield of islets, as was recently reported by Kuo et al. (25). In separate experiments, we found that these islets were able to increase both the synthesis and the release of insulin severalfold in response to glucose stimulation. It has recently been reported, however, that glucose-induced insulin release is impaired in isolated islets from mice pretreated with pilocarpine (1).

Indirect evidence suggests that the plasma membrane may be involved in the initiation of insulin release by glucose (19, 23, 34), certain amino acids (7, 12), hormones (17), and sulfonyleureas (17), as well as certain chemical reagents (4). These are only indirect observations, and few attempts have been made to investigate the plasma membrane directly. However, adenylate cyclase activity has been localized to the β -cell plasma membrane by electron microscopic histochemistry (21), and cyclic AMP-dependent protein kinase activity has been detected in a membrane fraction prepared from fish and mouse islets (9).

In order to detect and characterize the islet plasma membrane fraction, it was necessary to utilize a number of appropriate markers. For this purpose, we have studied the distribution of adenylate cyclase, Mg^{2+} -ATPase, ouabain-sensitive

TABLE III
Distribution of Adenylate Cyclase Activity

Fraction	Adenylate cyclase (pmol cAMP \cdot μg protein $^{-1}$ \cdot 30 min $^{-1}$)	Relative activity
Total particulate	1.50	1
Gradient A only	2.89	1.9
Gradients A and B	3.68	2.5

Activities of adenylate cyclase in particulate fractions of isolated rat islets. Islet homogenates were labeled with ^{125}I -WGA and fractionated on gradients A and B. A sample of the homogenate, and of the peak fractions of gradient A as well as the total peak fractions of ^{125}I -WGA radioactivity from gradient B were diluted in Tris-buffer containing *N*-acetyl-D-glucosamine to displace ^{125}I -WGA. All samples were pelleted by centrifugation for 20 min at 100,000 g. In the presence of *N*-acetyl-D-glucosamine, 88-96% of the ^{125}I -WGA radioactivity was found in the supernate. The pelleted material was finally assayed for adenylate cyclase activity and protein as described in Materials and Methods. The values represent the mean of three different experiments.

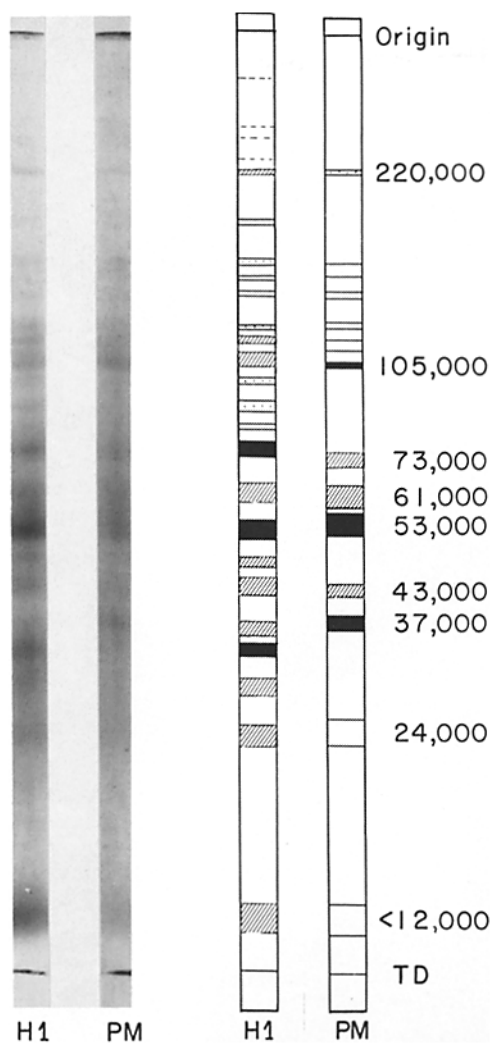
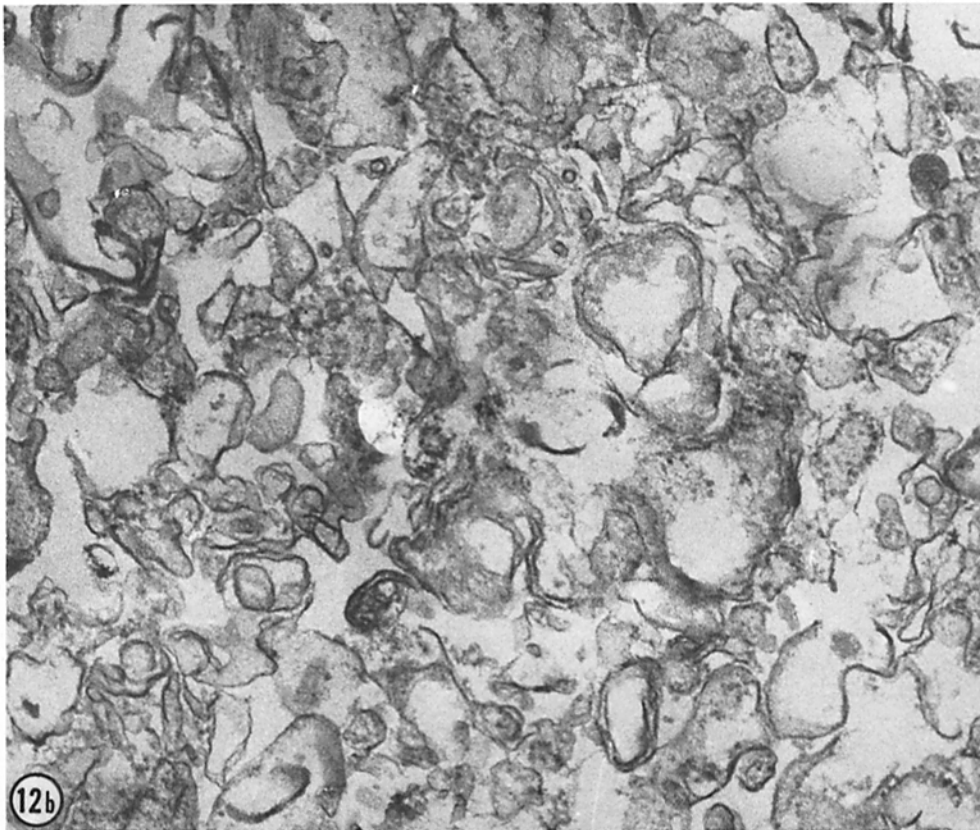
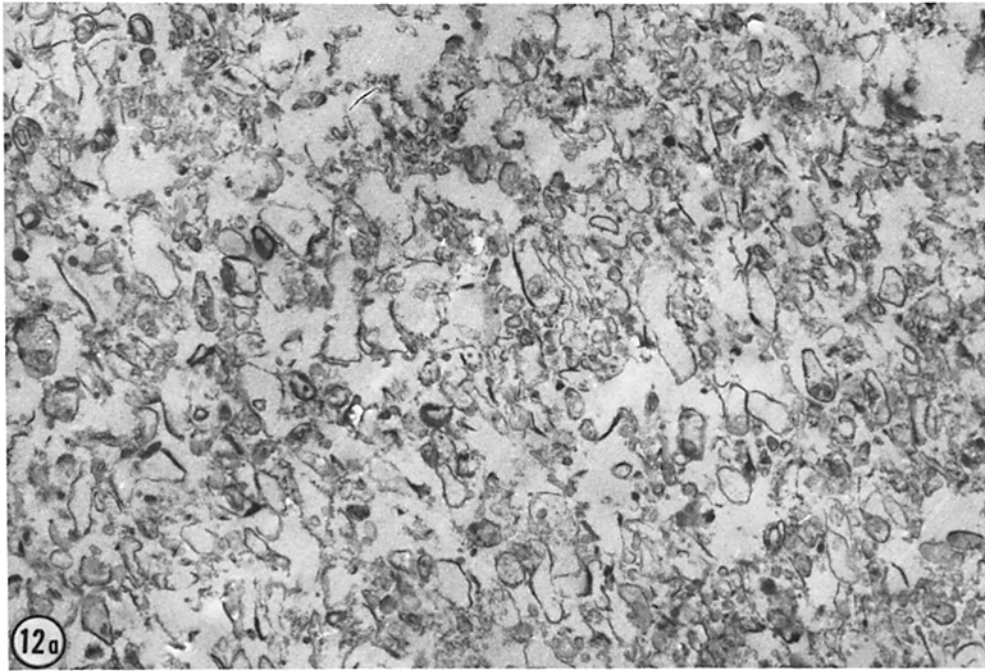


FIGURE 11 SDS-polyacrylamide gel electrophoretograms of the plasma membrane-containing fraction (PM) and sedimentable components of the islet homogenate (H1). Both samples were centrifuged (100,000g, 40 min) and the pellets were solubilized and applied to 6.5-cm gels as described by Fairbanks et al. (11). Gels were stained with Coomassie Blue G and photographed. The schematic representations on the right were prepared by direct visual inspection of the stained gels. Molecular weight reference proteins included: cytochrome c, ribonuclease, IgG, alcohol dehydrogenase, ovalbumin, bovine serum albumin, phosphorylase a, β -galactosidase and myosin.

(Na^+ - K^+) Mg^{2+} ATPase, 5'-nucleotidase, and alkaline phosphatase in islet homogenates subjected to rate-zonal and isopycnic centrifugation. All these enzymes are thought to be localized in the

plasma membrane in various cells, although this may not constitute a unique localization for all of them (39, 40). However, the labeling of the cell surface with ^{125}I -WGA and the coseparation of this probe along with the above enzyme activities in a density region of about 1.10 in sucrose (90 min centrifugation at 70,000 g) suggest that the plasma membrane in pancreatic islet cells is also an important locus of these enzymes. The binding sites for ^{125}I -WGA appear to be cell surface carbohydrates (8, 38), and the label remains firmly bound even during conditions of homogenization, as shown recently in attempts to isolate plasma membranes from liver cells, lymphocytes, fat cells, and yeast cells (6, 36). Labeling the cell surface with a sensitive probe that can be measured rapidly and easily, and that can also be readily removed, provides a highly useful technique for the small-scale isolation and characterization of plasma membranes from islet cells, and it would presumably be applicable to other tissues where the availability of starting material is limited.

In fractions from islets labeled with ^{125}I -WGA and subsequently washed with *N*-acetyl-D-glucosamine after the final gradient centrifugation, the specific activity of adenylate cyclase was increased threefold in the plasma membrane-containing fraction as compared to the total particulates of the homogenate. It was estimated that about half the protein in the homogenate remains in the supernatant fluid after the 100,000 g centrifugation. However, even after correction for this loss of protein, the enrichment of adenylate cyclase activity in the membrane fractions obtained after centrifugation on both gradients A and B seemed to be lower than that obtained for, for example, 5'-nucleotidase. This may be due to the fact that, in contrast to the other marker enzymes, several fractions had to be pooled and pelleted by centrifugation to amass enough material for the adenylate cyclase assay. In addition, there may be losses of activity during purification, as observed in other cell systems (39), as well as losses of activity during treatment with ^{125}I -WGA and *N*-acetyl-D-glucosamine. Further experiments are required to rule out the possibility that certain parts of the plasma membrane have been selectively purified. The parallel separation of adenylate cyclase activity with other plasma membrane markers indicates that the present preparation will be useful for detailed studies of islet cell adenylate cyclase activity, including its sensitivity to fluoride stimulation and various insulin secretagogues. So far, such



studies have been carried out only in islet whole homogenates (22, 25).

Notwithstanding its general importance as a marker for and constituent of plasma membranes, the assay for adenylate cyclase is too difficult and cumbersome to be useful for rapid screening of gradient fractions. For this purpose, we found the 5'-nucleotidase assay to be useful and convenient. However, since 5'-nucleotidase activity may not be uniquely associated with the plasma membrane (39), activity was assayed at an alkaline pH and in the presence of Mg^{2+} as required for optimal measurement of 5'-nucleotidase activity in plasma membranes of liver and fat cells (14). We did not encounter any major discrepancies in the recovery of 5'-nucleotidase activity when monitoring gradients A and B, indicating that this enzyme might be suitable for calculating the degree of purification. The specific activity of 5'-nucleotidase in the plasma membrane fraction obtained after centrifugation on both gradients A and B is similar to that reported for purified mouse liver cell plasma membranes (10).

In contrast to the results with 5'-nucleotidase activity, there were marked changes in the recovery of the Mg^{2+} ATPase activities during the fractionation procedures. After centrifugation on gradient A, the recovery of, for example, ouabain-sensitive ($Na^+ + K^+$) ATPase activity was increased more than 300% as compared to the total homogenate. This indicates a marked activation of the enzyme after the gradient centrifugation, perhaps due to a removal of an inhibitor present in the whole homogenate. The present preparation will thus allow a more meaningful characterization of islet Mg^{2+} ATPases. Such enzymes are thought to be important for the insulin secretory process (24, 37).

Similar to the activities of Mg^{2+} dependent ATPases, there was also an increased recovery of cytochrome *c* oxidase activity after islet homogenates were centrifuged over gradient A. The major peak of cytochrome *c* oxidase was well separated from the plasma membrane-containing fraction and occurred in the density region around 1.18 (Fig. 7). The specific activity of this enzyme was found to be the same in the membrane fraction as in the starting material added to gradient

B, but the increases in total recovery of this and some other markers after gradient centrifugation preclude the calculation of relative purification based on observed specific activities. The assay conditions used for the markers were provisional, based on reported properties of these enzymes in other systems; the optimal conditions for assaying the various marker enzymes in their respective locations have not been determined. For a more complete description of the behavior of the mitochondria in our isolation procedure, experiments will be undertaken to follow the distribution of monoamine oxidase and glutamate dehydrogenase, which are believed to be localized in regions of the mitochondria other than the inner membrane, the site of cytochrome *c* oxidase.

The SDS-gel band pattern in Fig. 12 shows the protein composition of the plasma membrane-containing fraction. The band pattern of the starting material is shown for comparison. This technique will, of course, be of importance for further characterization of the islet cell plasma membrane proteins. Glossman and Neville (16) reported that no subunit accounting for more than 1% of the total membrane proteins is common to the plasma membrane fractions from rat liver, kidney, and erythrocytes. Similarly, there was no resemblance between the electrophoretic patterns obtained with membranes isolated from the anterior, intermediate, or posterior pituitary lobes (28). Thus, the major protein species appearing in the plasma membranes of various differentiated cells may be largely organ or tissue specific. The implication of these findings for studies of differentiation and development, as well as of viral and autoimmune susceptibility, is immediately apparent. Further studies of the detailed structure of islet cell membrane components should contribute to our understanding of both the secretory mechanism for insulin and its regulation, as well as of the pathogenesis of diabetes.

The authors are indebted to Professor Hewson Swift and Mr. Chris Chow in Chicago and Professor Gunnar Bloom and Miss Mariane Borg in Umeå, Sweden, for their kind assistance in the preparation of electron micrographs of various isolated fractions, and to Ms. Christine Kuo, Ms. Carol Lipsey, Ms. Lee Anne Szyska and Mr.

FIGURE 12 Electron micrographs of the purified plasma membrane-containing fraction (a) $\times 16,500$; (b) $\times 38,340$.

Raymond Carroll who provided technical assistance. We also thank Ms. Sandra Stein for valuable assistance in the preparation of this manuscript. Portions of this work were supported by grants from the USPHS (AM-17046 and AM-13914) and from the Kroc Foundation, the Lolly Coustan Memorial Fund, and the Swedish Research Council (12x-3923).

Received for publication 19 April 1976, and in revised form 6 July 1976.

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