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Purification of Plasma Membranes from Rat Pancreas: A Rapid Method[1] (39799)

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Introduction. The role of adenylate cyclase and cyclic nucleotides in the secretory processes of the exocrine pancreas is not well defined. It has been reported that adenylate cyclase activity can be increased by secretin and, to a lesser degree, by pancreozymin (1, 2). Kempen *et al.* (3) found that phospholipid addition increased the sensitivity of adenylate cyclase to pancreozymin. Moreover, some authors have reported that dibutyryl cAMP induces enzyme secretion (4-6), while others found no effect (7, 8).

In spite of these controversial results, it is accepted that cyclic AMP could be involved as an intracellular mediator for hydralatic secretion (9, 10), while cyclic GMP might be an intracellular messenger for enzyme secretion (11, 12).

Purification of plasma membranes is a prerequisite for the study of molecular mechanisms underlying the hormonal and cholinergic responses of the exocrine pancreas. We describe here a rapid and simple method for the isolation of plasma membranes from the rat pancreas.

Materials and methods. ATP, GTP, AMP, yeast RNA, β-mercaptoethanol, Tris-ATP, creatine kinase, cAMP, phosphocreatine, and albumin fraction V were obtained from Sigma. [α-^{32}P]ATP (150 Ci/mmole) was purchased from New England Nuclear (Montreal); Metol was a product of Kodak. Synthetic terminal octapeptide of porcine cholecystokinin was a gift of Dr. M. A. Ondetti, Squibb Institute for Medical Research, Princeton, N. J. Natural porcine secretin was obtained from the GIH Research Unit, Karolinska Institutet, Stockholm, Sweden.

Adenylate cyclase assay. Adenylate cyclase activity was measured according to a modification (13) of the technique of Drummond and Duncan (14). The incubation was carried out for 15 min at 37° in a medium containing 40 μg/ml of soya bean trypsin inhibitor (SBTI), 0.02% albumin, 0.8 m*M* [α-^{32}P]ATP (4 μCi/assay), 10 m*M* theophylline, 5.5 m*M* $MgCl_2$, 40 m*M* Tris-HCl (pH 7.4), 50 μg of enzyme fraction protein, 100 μg of creatine kinase, and 20 m*M* phosphocreatine in a final volume of 100 μl.

Electron microscopy. Pellets obtained from the different fractions of membranes were fixed for 60 min in 2.5% ice-cold glutaraldehyde adjusted to pH 7.2 with 0.1 *M* cacodylate buffer. After a 45-min washing in the same buffer, they were postfixed in 2% osmium tetroxide in 0.1 *M* cadodylate buffer, pH 7.2. They were dehydrated in ethanol, embedded in Epon 812, sectioned, and stained with uranyl acetate and lead citrate. Electron micrographs were taken with a Philips EM 201 electron microscope.

RNA, DNA, and protein determinations. RNA contents were estimated by the method of Schneider (15) using yeast RNA as the standard. DNA contents were estimated by the method of Volkin and Cohn (16) using calf thymus DNA as the standard. Protein concentrations in the different fractions were measured according to Lowry *et al.* (17) using bovine serum albumin as a standard.

Enzymatic markers. For the ATPase assay, samples of each fraction were incubated in the presence of 5m*M* Tris-ATP, 10 m*M* $MgCl_2$, 40 m*M* Tris, pH 8.0, for 15 min at 37°. Inorganic phosphate release was measured according to a modification of the method of Sims (18). The 5'-nucleotidase activity was measured according to Bodan-

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sky and Schwartz (19). Succinate dehydrogenase was assayed according to Maeno *et al.* (20) and α-amylase activity was determined according to Bernfeld (21).

Isolation of the membrane fractions. Male Sprague–Dawley rats weighing between 300 and 350 g were used for these experiments. Pancreases were quickly excised and, after removal of the adipose tissue, were rinsed in the homogenization buffer A containing 40 m*M* Tris–HCl, pH 7.0, 0.25 *M* sucrose, 5 m*M* β-mercaptoethanol, 3 m*M* $MgCl_2$, 1 m*M* EDTA, 200 μg/ml of soya bean trypsin inhibitor (SBTI), and 1 mg/ml of albumin. The pancreases were homogenized in 10 vol of buffer A with 10 strokes of a tight-fitting Teflon–glass homogenizer. The homogenate was filtered through four layers of cheesecloth and centrifuged at 50*g* for 5 min in order to remove red blood cells, unbroken cells, and connective tissue. The 50*g* supernatant was centrifuged at 5100*g* for 20 min; a pellet with a brownish top layer was thus obtained. The top layer of this 5100*g* pellet was carefully removed for further purification on a sucrose gradient. In this second step, the separated brownish top layer was mixed with 75% (w/v) sucrose to a final concentration of 40% (w/v) and inserted in a stepwise sucrose gradient of 25, 30, 35, 40% (w/v) (containing the 5100*g* pellet). The above gradient was centrifuged for 1 hr at 115,000*g* in a SW.27 Beckman rotor. The different fractions obtained on the gradient were carefully removed with a syringe and diluted with 2 vol of buffer B containing 40 m*M* Tris–HCl, pH 7.4, 0.25 *M* sucrose, 1 mg/ml of albumin, and 5 m*M* β-mercaptoethanol. They were then centrifuged for 10 min at 30,000*g*. The characterization of the different fractions was based on morphological and enzymatic criteria.

Results. Morphological criteria. Figure 1 shows a low-magnification (15,000 ×) electron micrograph of the material found at the interface 30–35%. This fraction consists mainly of smooth vesicular membranes, although some contamination by rough endoplasmic reticulum can still be observed. The electron micrograph of Fig. 2 represents a higher magnification (55,000 ×) of the same preparation, and it shows smooth ve-

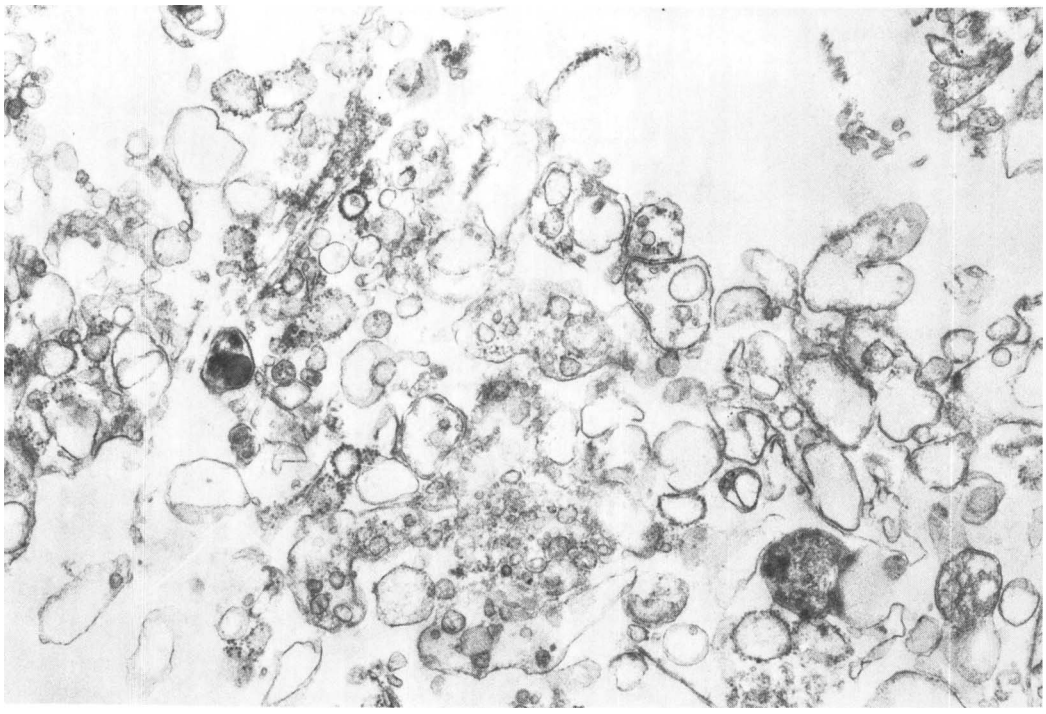

FIG. 1. Electron micrograph of the material recovered at the interface of sucrose 30–35%. It consists mainly of smooth vesicular membranes with a small contamination by rough endoplasmic reticulum. 15,000 ×.

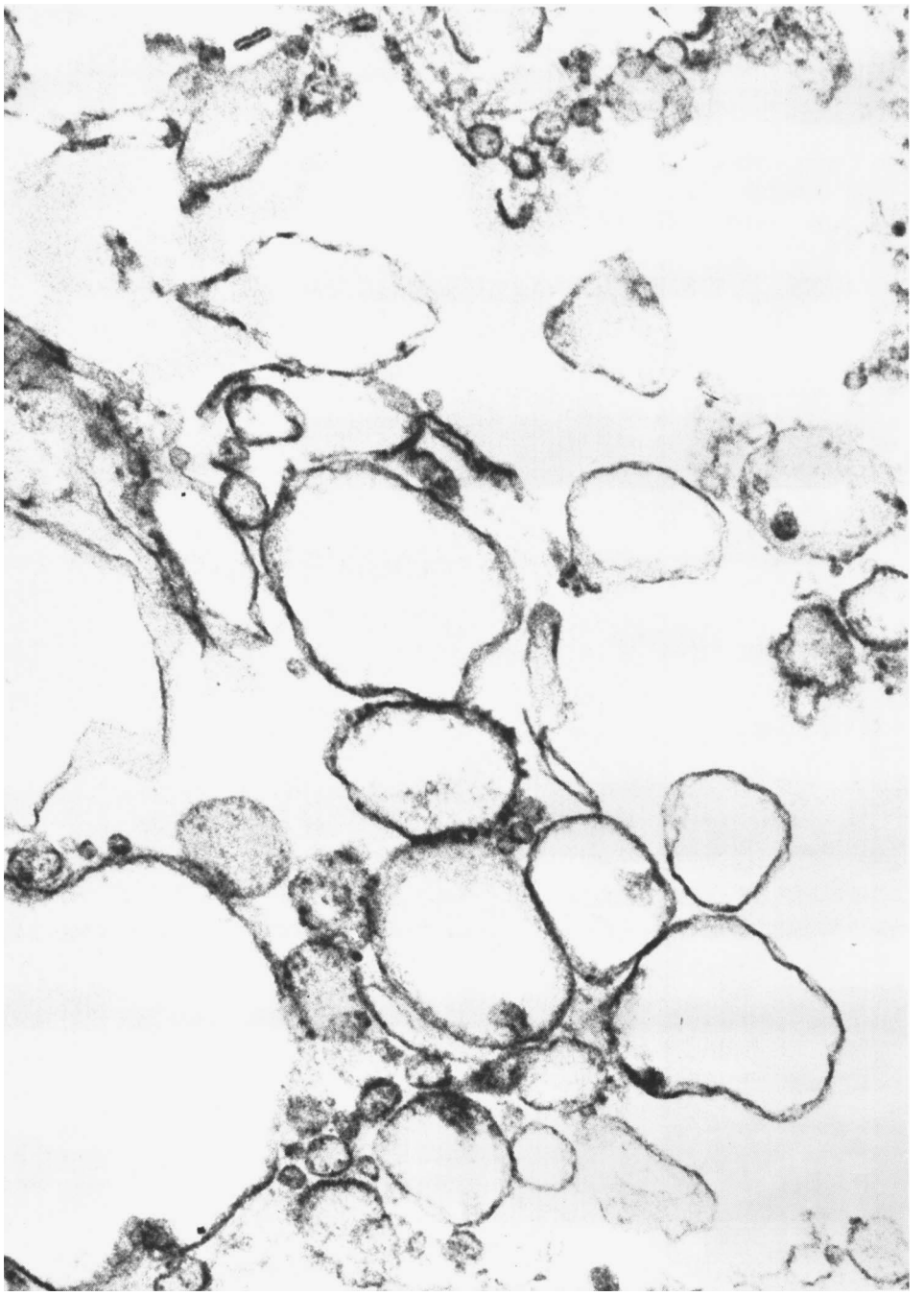

FIG. 2. Electron micrograph of the material shown in Fig. 1 showing a different field at a greater magnification. 55,000 ×.

sicular membranes and a small proportion of rough endoplasmic reticulum.

Distribution of enzymatic activities. Table I displays the subcellular distribution of different enzyme activities. The specific activity of adenylate cyclase in the material sedimenting at the interface 30–35% and identified as smooth vesicular membranes by elec-

tron microscopy demonstrates a 44-fold increase in adenylate cyclase activity as compared to the homogenate. The 5′-nucleotidase, a plasma membrane marker enzyme (22, 23), and the Mg^2+-dependent ATPase activity which has been found to be associated with thyroid plasma membranes (24) show, respectively, four- and fivefold increases in this purified fraction. Succinate dehydrogenase activity, a mitochondrial marker (20), was low in the adenylate-cyclase-rich fraction and α-amylase was practically absent from the purified fraction.

RNA and DNA contents. The fraction found at the interface 30–35% of the sucrose gradient shows a sevenfold decrease in RNA content as compared to the total homogenate. The small proportion of RNA found in that fraction can be accounted for by a small contamination by rough endoplasmic reticulum. As expected, this fraction had a very low DNA content (data not included).

Yield of plasma membranes. The yield of plasma membranes was low: 0.34 to 0.45 mg of protein/g of tissue wet weight. This is, however, typical of yields encountered in such studies and reported by Wolff and Jones for thyroid plasma membranes (24), by Pohl *et al.* for liver membranes (25), by Abou-Issa and Reichert (26) for rat testes tubule membranes, and by Gospodarowicz for bovine corpus luteum (27).

Stimulation of adenylate cyclase activity by secretin and pancreozymin-octapeptide. Using this purified fraction, the response of adenylate cyclase to secretin and PZ-octapeptide was measured. Figure 3 shows that the optimal concentration of secretin necessary to obtain maximal stimulation of adenylate cyclase activity was 2 clinical units (CU)/ml. Table II shows that secretin, at a concentration of 2 CU/ml, brings about a sixfold increase in adenylate cyclase activity

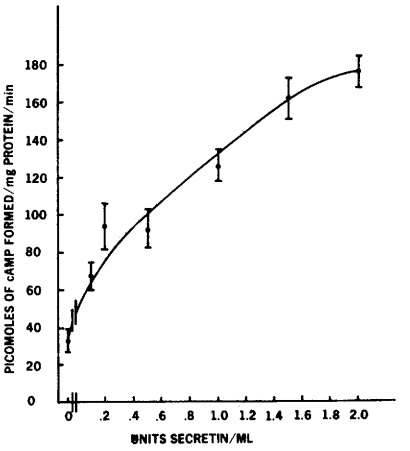

FIG. 3. The response of adenylate cyclase in purified (30–35% fraction) exocrine pancreas membrane preparation to secretin.

TABLE II. HORMONE-SENSITIVE ADENYLATE CYCLASE IN PANCREATIC PLASMA MEMBRANES.[a]

Experiment	Picomoles of cAMP formed per milligram of protein per minute
Control	33.3 ± 6.1 (4)
1 m*M* GTP	20.0 ± 3.0 (4)
10 m*M* NaF	668.4 ± 38.1 (4)
Secretin, 2 CU/ml	181.1 ± 35.7 (4)
Secretin, 2 CU/ml, + 1 m*M* GTP	307.8 ± 7.1 (4)
PZ-Octapeptide 10^{-6} *M*	72.1 ± 13.7 (4)

[a] Number of experiments is shown in parentheses. Each experiment was done in quadruplicate.

TABLE I. SPECIFIC ACTIVITIES OF ADENYLATE CYCLASE, 5′-NUCLEOTIDASE, Mg^{2+} ATPase, SUCCINATE DEHYDROGENASE, α-AMYLASE, AND RNA CONTENTS IN THE DIFFERENT SUBCELLULAR FRACTIONS.[a]

Fraction	Adenylate cyclase[b]	5′-Nucleotidase[c]	Mg^{2+} ATPase[c]	Succinate dehydrogenase[d]	Amylase[e]	Micrograms of RNA per milligram of protein
Homogenate	15.0 ± 5.4 (2)	0.71 ± 0.04 (2)	4.28 ± 0.105 (2)	5.59 ± 0.6 (2)	157.9 ± 7.8 (4)	255 ± 33 (5)
5100*g* Supernatant	36.4 ± 1.7 (2)	0.694 ± 0.012 (2)	2.99 ± 0.036 (2)	3.0 ± 0.5 (2)	115.1 ± 14.1 (4)	311 ± 28 (5)
5100*g* Pellet	91.5 ± 17.0 (2)	0.599 ± 0.002 (2)	5.99 ± 0.12 (2)	6.9 ± 0.6 (2)	155.2 ± 22.2 (4)	220 ± 14 (5)
Fraction 25–30% sucrose	177.0 ± 30.0 (2)	0.860 ± 0.008 (2)	5.97 ± 0.11 (2)	3.2 ± 0.5 (3)	15.5 ± 7.1 (3)	61 ± 22 (4)
Fraction 30–35% sucrose	668.0 ± 38.0 (5)	2.83 ± 0.07 (2)	19.8 ± 0.3 (2)	0.93 ± 0.1 (3)	6.0 ± 1.5 (3)	34 ± 9 (4)
Fraction 35–40% sucrose	182.6 ± 23.7 (2)	1.09 ± 0.05 (2)	8.88 ± 0.08 (2)	2.79 ± 0.18 (3)	14.3 ± 3.7 (3)	83 ± 30 (4)

[a] Results of experiments are expressed as mean ± SEM. Number of experiments is shown in parentheses.
[b] Adenylate cyclase activity was measured in the presence of 10 m*M* NAF; picomoles of cyclic AMP formed per milligram of protein per minute at 37°.
[c] Micromoles of Pi released at 37° per 30 min per milligram of protein.
[d] Micromoles of succinate oxidized at 25° per minute per milligram of protein.
[e] Micromoles of maltose produced at 37° per milligram of protein per minute.

which is further increased by the addition of 1 m*M* GTP. NaF at a concentration of 10 m*M* increased the adenylate cyclase activity by 20-fold, while pancreozymin-octapeptide at a concentration of 10^{-6} *M* is associated with a 2.2-fold increase.

Discussion. The isolation and purification of plasma membranes from the exocrine pancreas is complicated by the fact that the pancreatic tissue is made of different cell types. Attempts have already been made to purify these membranes from rat (28) and guinea pig pancreas (29, 30). The present method must be considered as an improvement over previous ones for the following reasons. In the first study on guinea pig pancreas (29) the isolated plasma membrane fraction showed no decrease in cytochrome oxidase activity as compared to the homogenate, while we find in our preparation a significant decrease in succinate dehydrogenase specific activity. Furthermore, no marker has been used for rough endoplasmic reticulum (30), while we have monitered the contamination by this cell fraction by measuring the RNA content.

It is generally agreed that adenylate cyclase is associated with particulate fractions in different tissues (13, 24, 25, 31). The plasma membrane fraction shows a very satisfactory increase in adenylate cyclase specific activity; similar results have been obtained during the purification of thyroid plasma membranes (24). Also, the membranes show an increase in 5′-nucleotidase specific activity, a plasma membrane marker (22, 32), and an increase in Mg^{2+}ATPase activity which has been found to be associated with thyroid plasma membranes (24). The purified fraction exhibits a low succinate dehydrogenase activity which shows little contamination by mitochondrial material as confirmed by electron microscopy. The small contamination by rough endoplasmic reticulum in the plasmalemmal fraction, as observed by electron microscopy, is shown by the low RNA content of this fraction as compared to the homogenate.

The low levels of α-amylase and DNA indicate the absence of intact granules and nuclear material from the purified fraction. The fact that adenylate cyclase does not copurify with 5′-nucleotidase reflects, in our opinion, the diversification of plasma membrane composition; a similar observation has been made during the purification of renal plasma membranes (33).

The membranes (30–35% sucrose) respond to secretin and pancreozymin; this, therefore, shows that some of their receptors are still associated with the adenylate cyclase complex.

This method provides an excellent tool for study of the interaction of secretin, pancreozymin, and other hormones with the membrane; the lipid requirement of the adenylate cyclase complex has been recently studied using this purified fraction (34). Studies are in progress to characterize its associated muscarinic receptor (35).

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