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Studies on Isolated Subcellular Components of Cat Pancreas

I. Isolation and Enzymatic Characterization

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Summary. Pancreas of the cat was fractionated into its subcellular components by centrifugation through an exponential ficoll-sucrose density gradient in a zonal rotor. This enables a preparation of four fractions enriched in plasma membranes, endoplasmic reticulum, mitochondria and zymogen granules, respectively. The first fraction, enriched by 9- to 15-fold in the plasma membrane marker enzymes, hormone-stimulated adenylate cyclase, (Na^+K^+)-ATPase, and 5′-nucleotidase, is contaminated by membranes derived from endoplasmic reticulum but is virtually free from mitochondrial and zymogen-granule contamination. The second fraction from the zonal gradient shows only moderate enrichment of the above marker enzymes but contains a considerable quantity of plasma membrane marker enzymes and represents mostly rough endoplasmic reticulum. The third fraction contains the bulk of mitochondria and the fourth mainly zymogen granules as assessed by electron microscopy and marker enzymes for both mitochondria and zymogen granules, namely succinic dehydrogenase, trypsin and amylase. Further purification of the plasma membrane fractions by differential and sucrose step-gradient centrifugation yields plasma membranes enriched 40-fold in basal and hormone-stimulated adenylate cyclase and (Na^+K^+)-ATPase.

A fruitful approach in analyzing the processes underlying some of the mechanisms of pancreatic secretion is to fractionate and isolate pancreatic subcellular components. In this way it is possible to localize different steps involved in secretion and to evaluate the role of each organelle in cellular functions. Compared to other tissues in which successful procedures for isolation of plasma membranes have been described (for a recent review *see* [19], fractionation of pancreatic homogenate is complicated by the presence of digestive proteolytic and lypolytic enzymes and fibrillar material adhering to the fragments of plasma membranes [15–17]. In addition, divalent cations successfully used in other tissues [1] to prevent leakage of nuclear DNA followed by gel formation,

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cannot be applied in pancreatic fractionation, since they cause excessive aggregation of subcellular particles ([15], and our own *unpublished observations*). Accordingly, few studies on purification of pancreatic plasma membranes have been published [15–17], especially concerning activities of enzymes supposed to be located uniquely in cell membranes in other tissues [14, 22].

The choice of proper organelle markers is critical for the identification and assessment of purity of different fractions and may vary for different tissues. Therefore, we used a group of enzymes known to be localized in plasma membranes in different tissues to substantiate our fractionations.

The activity of $(Na^+ + K^+)$-ATPase, 5′-nucleotidase and alkaline phosphatase was monitored during the fractionation procedure so that their enrichment factors and recoveries could be compared. In this way we were able to exclude a possible selective inactivation of a certain enzyme marker during the course of tissue fractionation. In addition to the above-mentioned enzymes, the activity of the peptide hormone-stimulated adenylate cyclase was measured and compared to that of other enzymes. This enzyme is considered to be the most reliable enzyme marker for plasma membranes in this study, since it is generally accepted that peptide hormones exert their effects directly on the basal cell surface (blood side) without crossing the plasma membrane [20] and can therefore be used as a useful tag for basal cell membranes [2].

A possible difficulty in selecting hormone-stimulated adenylate cyclase as a marker in fractionation is due to its lability during the isolation procedure [9, 23]. In the present study this was overcome by proper adjustment of separation conditions including the use of a protease inhibitor and of the Ca^{++} chelator EDTA.

The main subcellular components of pancreatic cells, i.e. plasma membranes, endoplasmic reticulum, mitochondria and zymogen granules, were separated using a combination of zonal density gradient and differential centrifugation. The method applied yielded a substantial amount of membranes highly enriched in plasma membrane markers, such as pancreozymin- and secretin-stimulated adenlyate cyclase $(Na^+ + K^+)$-ATPase and 5′-nucleotidase.

Materials and Methods

Materials

All reagents used were of analytical grade. Ficoll (mol wt approx. 400,000), ethyleneglycol-bis(β-aminoethyl ether)N,N′-tetraacetic acid (EGTA) and phenylmethyl sulfonyl-fluo-

ride (PMSF) were obtained from Sigma, St. Louis, Mo. Pyruvate kinase 10 mg/ml, adenosine 5′-triphosphate, adenosine 5′-monophosphate, phospho(enol)pyruvate trisodium salt, protein calibration kit for gel electrophoresis and Na^+-succinate were obtained from Boehringer, Mannheim, Germany. Adenosine 5′-[α-^{32}P]triphosphate (2–5 C/mmole sodium salt) and [8-^{3}H]adenosine 3′,5′-cyclic phosphate (27 C/mmole ammonium salt) were purchased from Radiochemical Centre, Amersham, U.K.; G-strophantin (crystalline), the α-amylase Merckotest and Orcinol (crystalline) were obtained from Merck, Darmstadt, Germany, and enterokinase, grade B, from Calbiochem, Luzern, Switzerland.

Dowex 50 WX 200–400 mesh hydrogen form was purchased from Serva, Heidelberg, Germany, and pancreozymin from Karolinska Institute, Sweden. Vasoactive intestinal polypeptide was a gift from Prof. V. Mutt, Karolinska Institute, Sweden, synthetic secretin was a gift from Prof. E. Wünsch, Max-Planck-Institut, Martinsried/München, Germany, and the octapeptide of pancreozymin was a gift from Dr. M. Ondetti, Squibb-Institute, Princeton, New Jersey.

Methods

I. Preparation of Tissue

Two cats of either sex were anesthetized with Nembutal (60 mg/kg body weight injected intraperitoneally), exsanguinated and the pancreas removed. All subsequent procedures were performed in the cold (0–4 °C). The organ was freed of fat and connective tissue, washed and weighed. The tissue (15 g wet weight) was finely minced with scissors in an ice-cold buffer containing 10% sucrose, 10 mM Tris, pH 7.4, 0.1 mM EGTA, 0.1 mM PMSF, and homogenized in 5 volumes of the same buffer using a hand-operated glass-teflon homogenizer with a clearance of 0.06 mm. Ten strokes were sufficient to homogenize the tissue. The homogenate was filtered twice through two layers of medical gauze and spun in a Sorvall refrigerated centrifuge to remove broken tissue and debris. The rotor was allowed to reach 2,000 rpm (1,400 × *g*) within 8 sec and then the motor was immediately shut off. The supernatant (about 40 ml containing about 500 mg of protein) was used for subsequent fractionation as shwon in Fig. 1. The sample was prepared as the gradient was being loaded in the zonal rotor to reduce the time between homogenization and separation. The time between the homogenization step and loading the zonal rotor with the tissue did not exceed 30 min.

II. Fractionation

A. Fractionation on Zonal Density Gradient (Step I, Fig. 1)

A continuous exponential gradient was formed in a Beckmann Ti 14 zonal rotor (capacity 665 ml) running at 4,000 rpm by means of a piston operated gradient maker (International Equipment Company). The heavier solution (600 ml of ice-cold 45% sucrose w/w) was added at a constant flow rate (25 ml/min) to a mixing chamber containing a lighter solution (ice-cold 10% sucrose w/w plus 5% ficoll w/w). The volume of the mixing chamber was kept constant at 200 ml.

The outlet of the latter chamber was connected to the outer inlet of the head of the zonal rotor. After 450 ml had been pumped into the rotor, its remaining space was filled with 60% ice-cold sucrose w/w using a peristaltic pump (Cole-Parmer, Chicago). Then 20 ml of ice-cold solution containing 10% sucrose + 5% ficoll was layered at a rate of 10 ml/min on the top of the gradient followed by 40–50 ml of the pancreatic homogenate and then by an overlay of 50 ml of ice-cold homogenization buffer. All solutions containing sucrose were checked for density by an Abbé refractometer. Unloading was performed at 4,000 rpm by displacement with 60% sucrose w/w, and 15 ml fractions were collected. Each fraction was checked for density by refractometry and for protein content by measuring

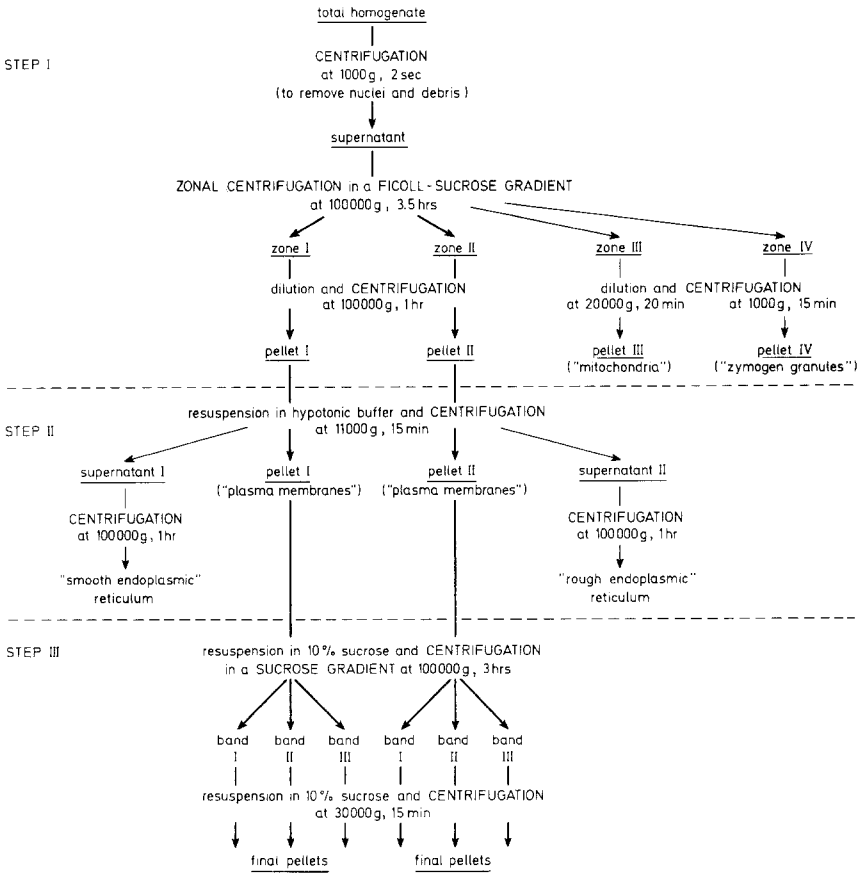

Fig. 1. Procedure to fractionate cat pancreatic homogenate. The terms: "plasma membranes", "endoplasmic reticulum", "mitochondria", and "zymogen granules" refer to fractions enriched in the corresponding cell components

optical density at 280 nm after 11-fold dilution with water. Four fractions were recovered including two 15-ml fractions of the highest protein peaks in Fractions I, III and IV and four 15-ml fractions of highest protein peaks in Fraction II. The rest was discarded. The highest densities of the fractions collected corresponded to sucrose densities of 1.07, 1.10, 1.17, and 1.20 at the interface of the gradient and the 60% sucrose (Fig. 2). They are referred to hereafter as Fractions I, II, III, and IV (zones I, II, III, IV, in Fig. 1). These fractions were pelleted as follows: Fraction I, containing higher ficoll concentration than other fractions, was diluted 8 times by homogenization buffer and spun down for 1 hr at $100{,}000 \times g$. Fractions II and III were diluted down to 10% w/w sucrose and centrifuged for 1 hr at $100{,}000 \times g$ and for 20 min at $20{,}000 \times g$, respectively. Fraction IV was diluted with double-distilled water to a sucrose concentration of 10% and spun down at $1{,}000 \times g$ for 15 min.

B. Separation of Zonal Fractions

1. Differential centrifugation (step II, Fig. 1). The pellets obtained after centrifugation of the material recovered in Fractions I and II were rendered hypotonic by suspending

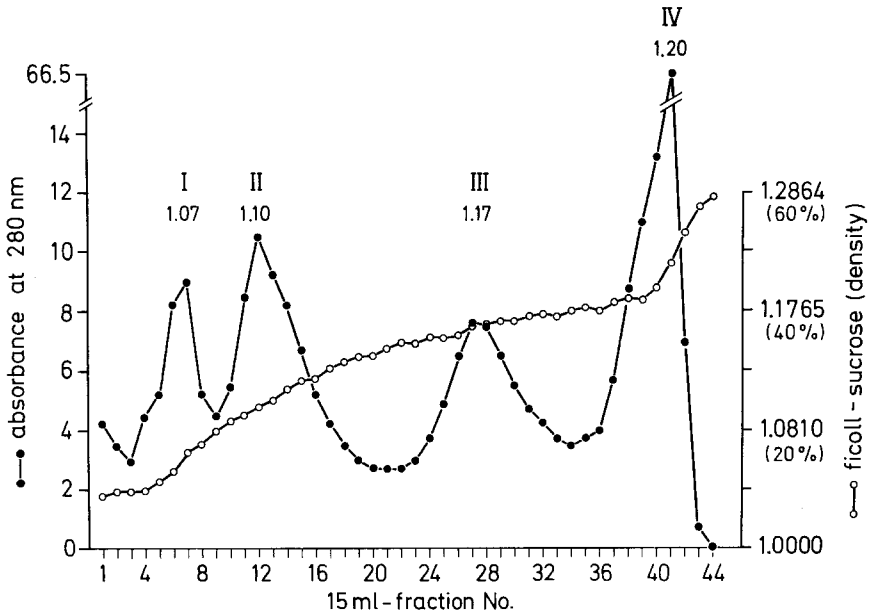

Fig. 2. Fractionation on a zonal ficoll-sucrose density gradient. ●—● absorbance at 280 nm; ○—○ density of ficoll-sucrose

them in 40 ml of a buffer containing 10 mM Tris, pH 7.4, 0.1 mM EGTA, and 0.1 mM PMSF to give a protein concentration of about 0.3 mg/ml and spun down at $11{,}000 \times g$ for 15 min. About one-third of total protein was pelleted by this step. The pellet was resuspended and saved for enzyme determination or for further fractionation. The supernatant was spun down at $100{,}000 \times g$ for 1 hr.

2. Fractionation by sucrose step gradient (step III). The $11{,}000 \times g$ pellet from Fractions I and II, respectively, was resuspended in 10% (w/w) sucrose (4–6 mg of protein per 3 ml) and layered over a discontinuous gradient prepared in 16 ml cellulose acetate tubes (Beckmann) using equal volumes of 15, 27, 32 and 40% (w/w) sucrose. Six tubes were used in parallel so that the amount of protein per tube did not exceed 2 mg. The tubes were placed in a Beckmann SW 27 swing-out rotor and centrifuged for 3 hr at $135{,}000 \times g$ (4 °C). Three protein bands were recovered at the 15/27, 27/32 and 32/40% sucrose interfaces of the step gradient. Longer centrifugation times did not improve separation. Bands were collected, diluted with the same buffer as above, and spun down at $30{,}000 \times g$ for 15 min. Final pellets were resuspended in buffer as above and saved for further analysis.

C. Duration of Procedures

All three steps of the procedure were usually performed en bloc. However, it is possible to interrupt the procedure after each step, storing the material as a dense suspension in a buffer containing 10 mM Tris-Cl, pH 7.4, 0.1 mM EGTA, 0.1 mM PMSF (6–8 mg of protein/ml) at −30 °C. All enzyme activities including the hormone-stimulated adenylate cyclase were preserved under these conditions for at least three weeks.

To assess the influence of the time needed for the preparation of tissue and manipulation on the sensitivity of adenylate cyclase to different hormones, a quicker procedure for tissue preparation was performed for comparison as previously described [18]. Briefly,

it consists of low speed centrifugation of the homogenate at $4{,}000 \times g$ and washing of the pellet by hypotonic buffer. This crude fractionation allowed determination of adenylate cyclase activity within 1.5 hr after homogenization.

III. Preparation of Zymogen Granule Membranes

Following the zonal ficoll-sucrose-gradient centrifugation (step I) and pelleting the material recovered in Fraction IV for 15 min at $1{,}000 \times g$, zymogen granule membranes were prepared according to the method of Meldolesi *et al.* [15] with some modification. This involves lysis of zymogen granule content by 150 mM Tris-HCl (pH 7.8) in the presence of 0.1 mM PMSF. Particulate material was separated from the lysed granule contents by differential centrifugation ($100{,}000 \times g$ for 1 hr) followed by separation of membranes from intact granules and other dense contaminants on a sucrose step gradient (15 and 31% sucrose w/w at $100{,}000 \times g$ for 1 hr). After centrifugation, membranes of zymogen granules were recovered at the 15/31% interface.

IV. Enzyme Assays

Adenylate cyclase was measured according to the method of Krishna *et al.* [11] with slight modification [18]. Separation of cyclic adenosine 5′-monophosphate (cAMP) was by chromatography on Dowex 50 WX columns (0.5×3 cm) followed by precipitation by $ZnSO_4/Ba(OH)_2$. Incubations were performed at 30 °C for 20 min in 0.1 ml of medium containing 0.8 mM ATP $\alpha^{32}P$, 5 mM $MgCl_2$, 40 mM Tris-Cl, pH 7.4, 10 mM theophylline, 1 mM cAMP, 50–150 µg of membrane protein and an ATP regenerating system consisting of 5 mM phospho(enol)pyruvate and 20 µg pyruvate kinase. Under these conditions linear reaction rates are obtained in the presence and absence of hormones for at least 35 min.

$(Na^+ + K^+)$-stimulated-Mg^{++}-dependent ATPase, HCO_3^--stimulated-Mg^{++}-dependent ATPase, Ca^{++}-stimulated ATPase and Mg^{++}-stimulated ATPase activities were measured in the presence of appropriate ions [32]. The phosphate liberated by hydrolysis of ATP was estimated by a modification of the method of Fiske and Subbarow [7]. 5′-nucleotidase and alkaline phosphatase were determined as described previously [32]. Succinic dehydrogenase was determined according to King and Howard [10] and ADPase as described by Emmelot *et al.* [4]. Rotenone-insensitive nicotinamide-adenine-dinucleotide (NADH) cytochrome-*c*-reductase was measured at 22 °C according to Sottocasa *et al.* [29]. Trypsin was measured by the Boehringer Biochemical Test Combination No. 15950 using benzoyl-arginine-*p*-nitranilid as substrate after preincubation with entero-kinase B (1 mg/mg protein) for 30 min at 37 °C to activate the enzyme. The activity of amylase was determined with the α-amylase Merckotest based on the method of Street and Close [31].

Protein was determined according to Lowry *et al.* [12] after precipitation of the protein by 10% TCA in the cold and dissolution of the precipitate in 1 N NaOH [32]. Ribonucleic acid (RNA) was determined by the orcinol method of Mejbaum [13] after precipitation with 1 M $CdCl_2$ as described by Hatcher and Goldstein [8].

V. Polyacrylamide Gel Electrophoresis

Electrophoresis was carried out essentially as previously described [26]. Twenty to 50 µg of protein were dissolved in 1% SDS-5% mercaptoethanol at 37 °C for 10 min and solubilized proteins were fractionated on polyacrylamide columns 5×60 mm. Stacking gel (3.5% acrylamide) was prepared in 0.1 M Tris-Cl, pH 6.8, and separation gels were 10% acrylamide prepared in 0.4 M Tris-Cl, pH 8.6. In each gel 2.5% of the acrylamide was

N,N′-methylene-bis-acrylamide. Polymerization was performed at 15 °C for 40 min using tetramethylethylenediamine (TEMED) and ammonium persulfate. Reservoir buffer was 0.19 M glycine-Tris base at pH 8.6. All solutions contained 0.1% SDS. Electrophoresis was performed at 2 mA per tube for 3.5 hr. Gels were stained for protein with Coomassie blue and destained as described by Fairbanks *et al.* [5].

Polyacrylamide columns were calibrated for molecular weight using trypsin inhibitor (mol wt 21,500), bovine serum albumin (mol wt 68,500) and RNA polymerase (α, β and β' form, mol wt 39,000, 155,000 and 165,000, respectively) available as a protein standard kit from Boehringer, Mannheim.

VI. Electron Microscopy

The material recovered in the four protein peaks (I–IV, step I of fractionation scheme, Fig. 1) from the zonal gradient after pelleting was fixed at 4 °C with 2.5% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.3). After washing with the same buffer, specimens were postfixed with osmium tetroxide (1% OsO_4; 0.1 M cacodylate buffer, pH 7.3) for 1 hr and stained with an aqueous solution of 2% uranyl acetate for 2 hr. Subsequently, they were dehydrated in a series of alcohols and embedded in a low-viscosity resin [30]. Sections were stained with lead citrate (double-staining) and examined in a Philips 300 electron microscope.

Results

I. Fractionation on Zonal Density Gradient

Fig. 2 shows separation of the pancreatic homogenate obtained following centrifugation for 3.5 hr at 43,000 rpm in the continuous ficoll-sucrose gradient. Four distinct protein peaks as estimated by 280 nm absorbance appeared at refractometric densities of 1.07, 1.10, 1.17, and 1.20.

About 30% of the total protein added in this step was recovered from all fractions as particulate material, whereas the remainder could not be pelleted by $140{,}000 \times g$ for 3 hr. The distribution of marker enzymes in the particulate material recovered from four protein peaks of zonal separation, plotted according to the method of de Duve [3], is shown in Figs. 3*A* and 3*B*. The height of the blocks gives the relative enrichment of the marker, in a given fraction and the area the per cent recovery in a given fraction.

A. Fraction I. It can be seen in Fig. 3*A* that the occurrence of all plasma marker enzymes tested decrease with increasing density of the gradient. So Fraction I shows the highest enrichment of various typical plasma membrane marker enzyme activities, such as basal adenylate cyclase, secretin-stimulated and pancreozymin-stimulated adenylate cy-

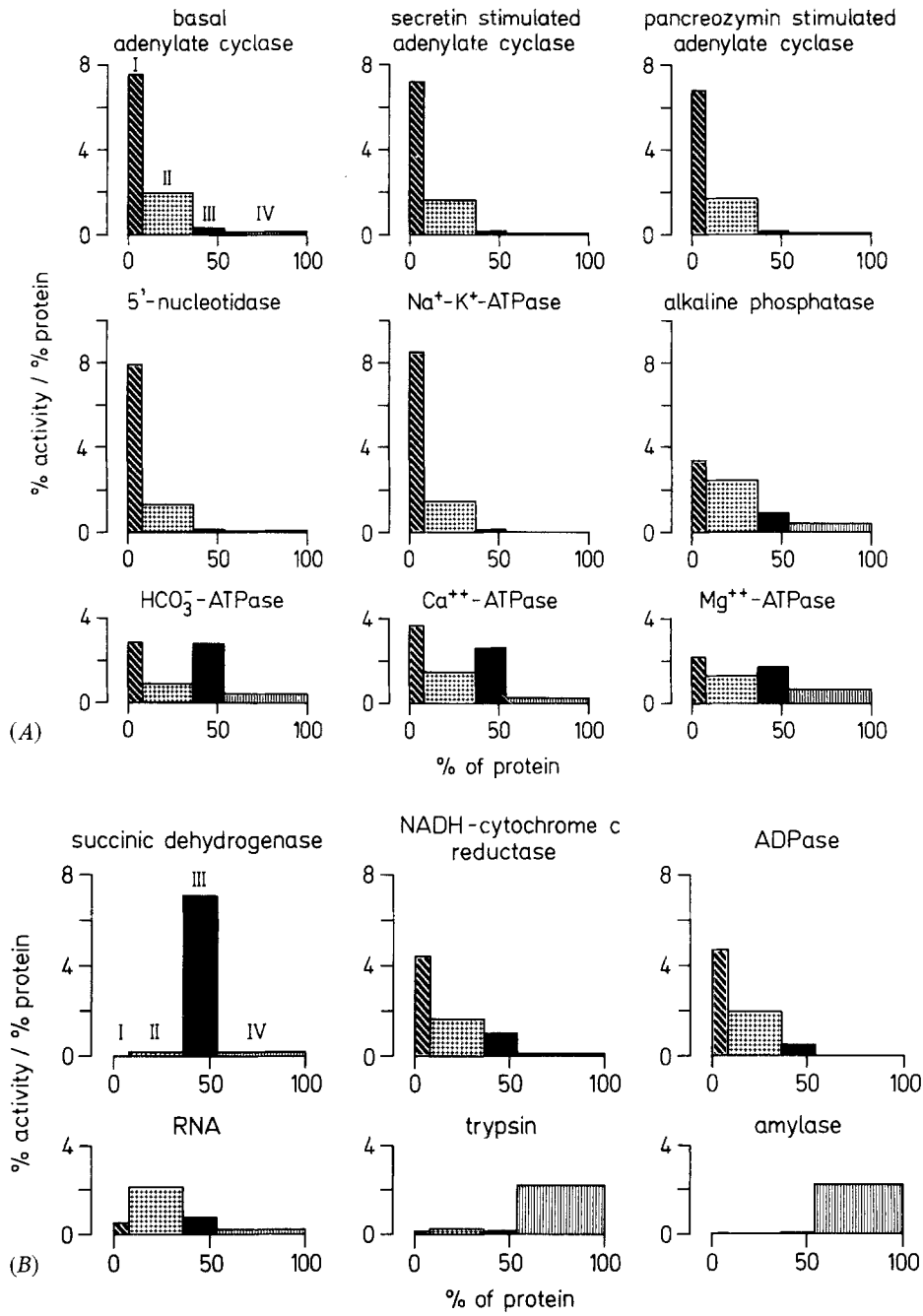

Fig. 3. Enzyme distribution patterns in four fractions (I–IV) recovered from the ficoll-sucrose gradient. The data are plotted according to de Duve [3]. The height of each column represents the relative enrichment (recovery of the enzyme activities divided by protein recovery), whereas the width shows the protein recovery. Thus, the surface area is the total enzyme recovery in each fraction. The average values of enzyme activities from all experiments were used for the plots. Only the protein recovered from the four fractions indicated was used for calculation. (*A*) Distribution of plasma membrane markers; (*B*) Distribution of markers for mitochondria, endoplasmic reticulum and zymogen granules

clase, $(Na^+ + K^+)$-stimulated-Mg^{++}-dependent-ATPase, and 5′-nucleotidase.

All these different enzymes have similar enrichment factors (height of the columns) and recoveries (surface area) in Fraction I suggesting that they move together as a group of enzymes. In addition, this fraction is enriched in Ca^{++}-stimulated ATPase, Mg^{++}-stimulated ATPase and HCO_3^--stimulated-Mg^{++}-dependent ATPase; however, the latter three enzymes are more broadly distributed among the first three fractions of the zonal gradient. Fig. 3*B* shows the distribution of marker enzymes for mitochondria, endoplasmic reticulum and zymogen granules. ADPase, considered to be a smooth endoplasmic reticulum marker [4], and rotenone-insensitive NADH-cytochrome-*c*-reductase, a marker for endoplasmic reticulum [29], although present in substantial amounts in Fraction I, are comparably less enriched than plasma membrane markers in this fraction. As the amount of ribonucleic acid (RNA) present in this fraction is small in comparison with Fraction II, the contribution of the rough endoplasmic reticulum (ER) in Fraction I is estimated to be relatively low. The mitochondrial marker succinic dehydrogenase is virtually absent from this fraction, whereas the zymogen granule markers trypsin and amylase are substantially lower than in other fractions. It would appear therefore that the first fraction represents enriched plasma membranes contaminated by the membranes derived from smooth endoplasmic reticulum. It is possible to further separate both types of membranes from each other as will be described below. An electron micrograph of Fraction I shows smooth membrane vesicles of different size with moderate ribosomal contamination. No other organelles are seen (Fig. 4*A*).

B. Fraction II. As seen in Fig. 3*A* the second fraction recovered from the zonal gradient banding at a refractometric density of 1.10, shows only a moderate enrichment, but contains a considerable quantity of plasma membrane marker enzymes. This fraction contains also Ca^{++}-ATPase, Mg^{++}-ATPase and HCO_3^- stimulated-Mg^{++}-dependent ATPase activities as well as rotenone-insensitive NADH-cytochrome-*c*-reductase and ADPase (Fig. 3*B*). All these enzymes are less enriched in Fraction II as compared to Fraction I. However, recovery of all these enzymes in both fractions is comparable. The most prominent feature of this fraction, in contrast to Fraction I, is its high content and enrichment of RNA. Furthermore, this fraction is characterized by low activity of trypsin and amylase and is almost free of SDH. Electron microscopy

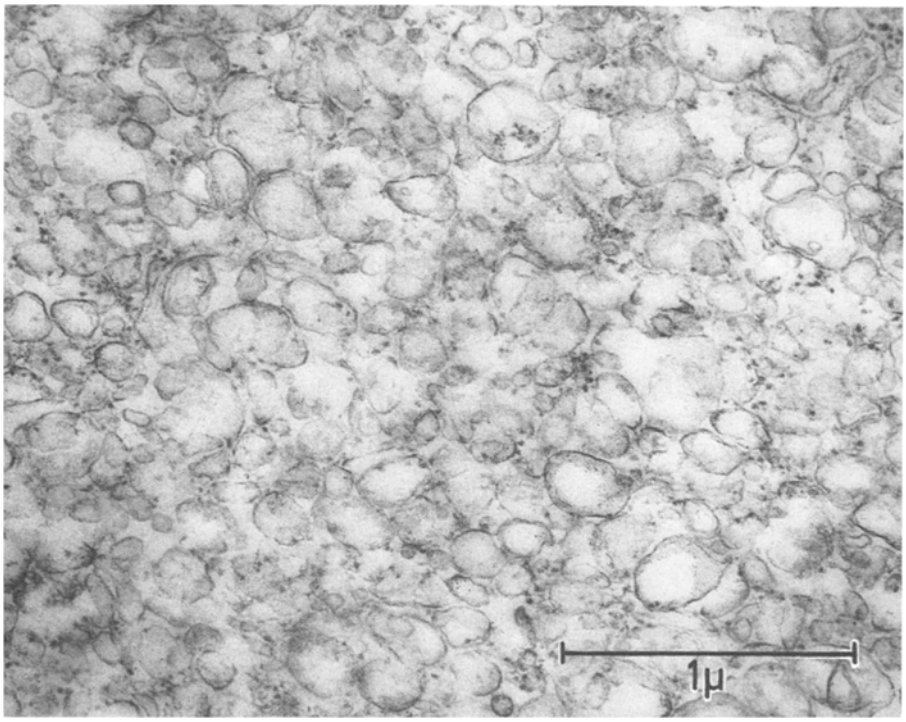

Fig. 4*A*

Fig. 4. (*A*) Section through a pellet of smooth membranes derived from Fraction I of the zonal ficoll-sucrose density gradient. The fraction consists mainly of smooth membrane vesicles of different size with some ribosomal contamination (39,000×). (*B*) Section through a pellet of Fraction II from zonal density gradient. Small vesicles with attached ribosomes derived most likely from rough endoplasmic reticulum can be seen. Smooth vesicles probably originating from cytoplasmic membranes contaminate this fraction (39,000×). (*C*) Section through a mitochondrial fraction (zonal density gradient, zone III). Most mitochondria have an electron opaque matrix and are of irregular shape. Some mitochondria with a matrix of lower density are round. Besides small whorls of smooth membranes probably originating from broken mitochondria, the picture demonstrates some contamination with rough endoplasmic reticulum (8,300×). (*D*) Section through the pellet of zone IV from the zonal density gradient. Beside zymogen granules of markedly different size and staining intensity, some vesicles derived from rough endoplasmic reticulum are also to be seen (6,300×)

shows mainly small vesicles with attached ribosomes derived from rough endoplasmic reticulum with some contamination by smooth vesicles of bigger size (Fig. 4*B*).

C. Fraction III. The third peak in Fig. 2 banding at a density of 1.17 contains the bulk (80%) of the mitochondrial marker succinic dehy-

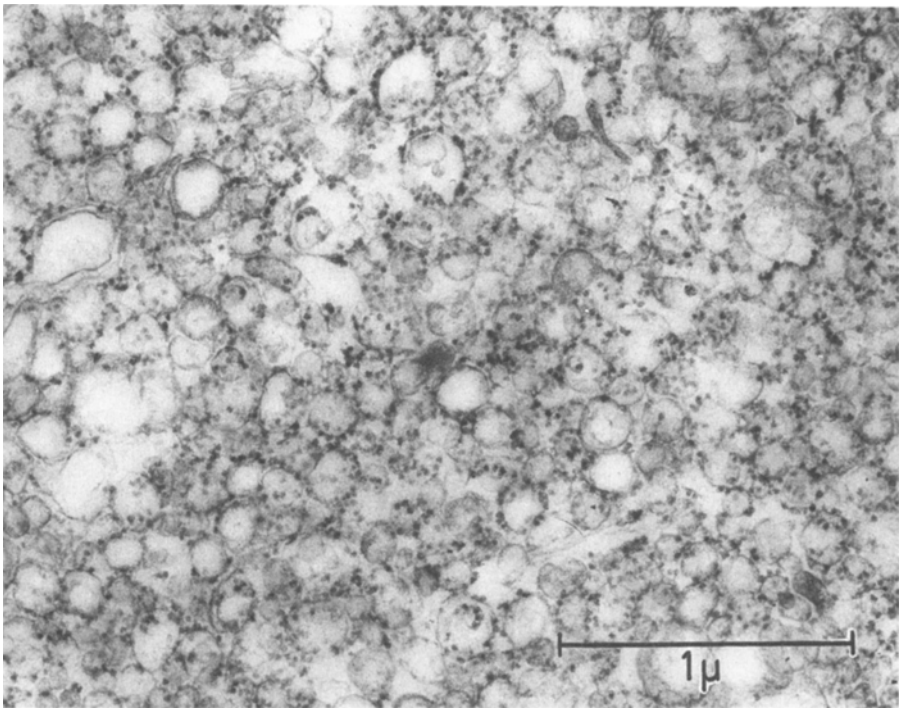

Fig. 4*B*

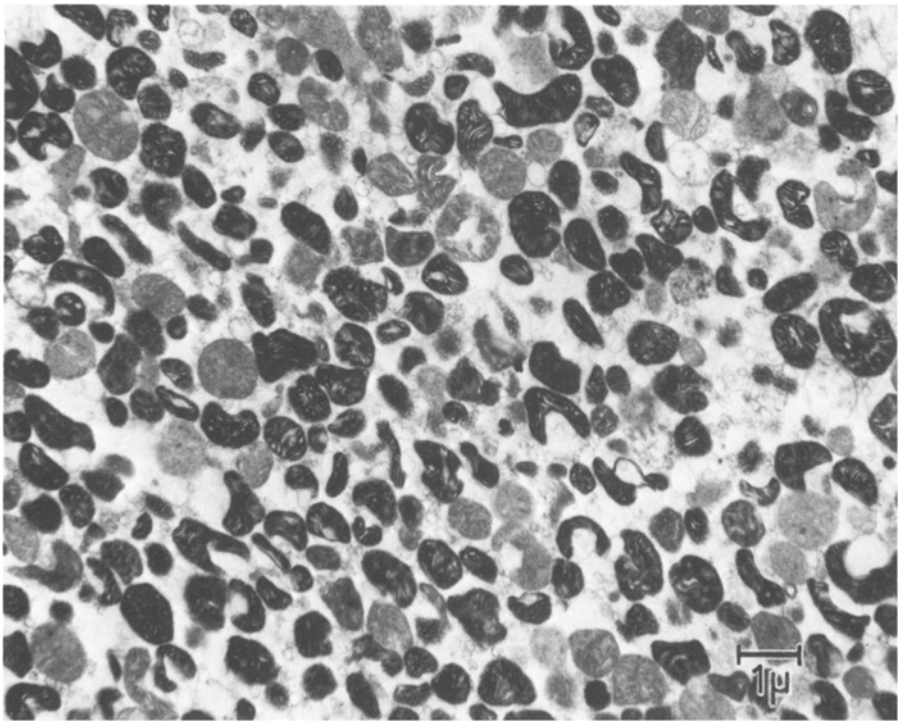

Fig. 4*C*

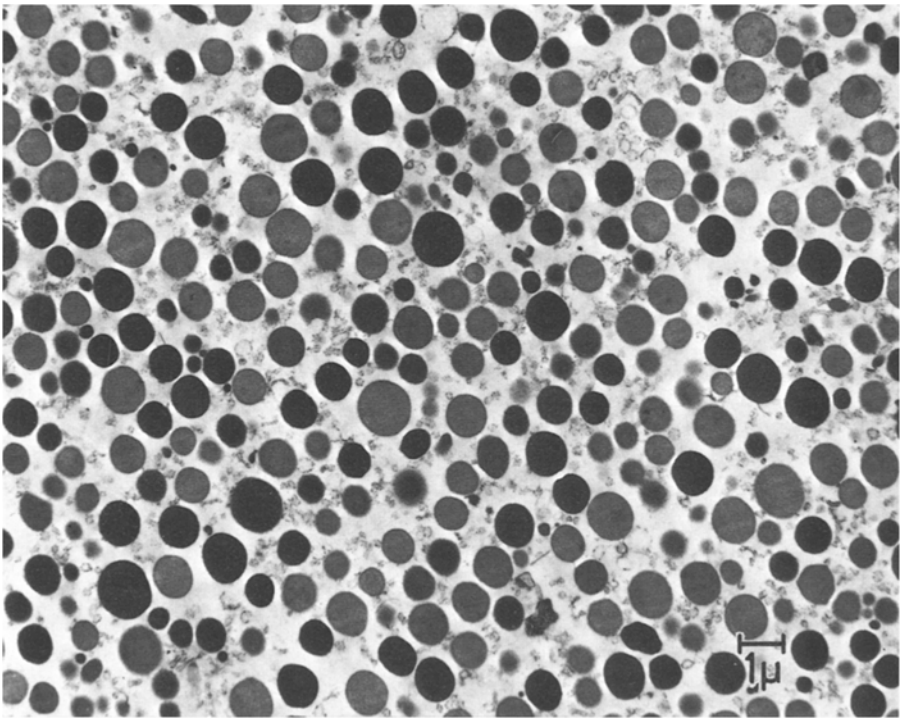

Fig. 4*D*

drogenase recovered from the gradient. It is free of plasma membrane markers (hormone-stimulated adenylate cyclase, $(Na^+ + K^+)$-ATPase, 5′-nucleotidase) or it contains only traces of them (basal adenylate cyclase, alkaline phosphatase). Only small amounts of ADPase are associated with this fraction but it is enriched in HCO_3^--stimulated-Mg^{++}-dependent, Ca^{++}- and Mg^{++}-ATPases and contains also some NADH-cytochrome-*c*-reductase (Figs. 3*A* and 3*B*). The electron microscopic picture (Fig. 4*C*) shows that this fraction consists mainly of mitochondria.

D. Fraction IV. This contains the bulk of the zymogen granule markers trypsin and amylase (Fig. 3*B*). No enrichment of other enzyme markers is present in this fraction. However, it shows some contamination with RNA. Fig. 4*D* shows that this fraction consists primarily of zymogen granules, recognizable by their circular shape and the homogeneity of their dense content. Consistent with the biochemical data some rough endoplasmic reticulum is also present in this fraction. This fraction could be further purified to obtain zymogen granules less contaminated by RNA.

While the "de Duve plot" gives a better survey of the enzyme marker distribution among the four fractions, an exact quantitative description of fractionation allowing direct comparison with the data reported in the literature is given in Table 1. The data summarized in this Table allow comparison of all fractions with total homogenate and each other in terms of specific activity, enrichment of enzyme activity over total homogenate and recovery in per cent of the total homogenate. The highest enrichment of plasma membrane markers compared to homogenate is 9 to 15 times, found in Fraction I. It is noteworthy that the putative plasma membrane markers showed a very similar distribution as a group among the four fractions recovered from the zonal gradient concerning both enrichment and recovery. Thus, the enrichment of $(Na^+ + K^+)$-ATPase, basal adenylate cyclase, secretin-stimulated and pancreozymin-stimulated adenylate cyclase and alkaline phosphatase is 9- to 12-fold in Fraction I and four- to fivefold in Fraction II. The total recoveries for these enzymes are also similar, being 23 to 28% for Fraction I and 24 to 28% for Fraction II. 5′-nucleotidase shows a small deviation from this distribution pattern having a slight preference for Fraction I in comparison with other plasma membrane markers and alkaline phosphatase is associated with Fraction III more than would be expected on the basis of other membrane markers. Further comparison between Fractions I and II reveals that, although they contain approximately the same total amount (recovery) of plasma membrane markers, Fraction II is about 3 times richer in protein indicating a higher contribution of material derived from other sources than plasma membranes, i.e. from rough endoplasmic reticulum. This is demonstrated by a higher enrichment of RNA and by electron microscopical picture (Fig. 4*B*). Fraction II contains slightly higher amounts (recovery) of the endoplasmic reticulum markers ADPase and rotenone-insensitive NADH-cytochrome-*c*-reductase than Fraction I; however, both these enzymes are more enriched in Fraction I (four- to fivefold versus 1.5-fold) showing their association with smooth rather than rough endoplasmic reticulum. Owing to the high recovery of plasma membrane markers the Fraction II is (in addition to Fraction I) a valuable source of plasma membranes for further purification (*see below*). Mitochondrial contamination is estimated from the relative specific activity of succinic dehydrogenase to be 1.5 and 2.5 per cent for Fractions I and II, respectively. The presence of small amounts of trypsin and amylase in Fractions I and II (relative enrichment between 0.2 and 0.5) may originate from the content of the vesicles derived from rough and smooth endoplasmic reticulum which have not been

Table 1. Enzyme distribution in Fractions I–IV recovered from zonal density gradient (step I) (Figures represent mean values ± SD of 12–20 tissue preparations and 12–40 determinations for each enzyme)

	Protein yield (absolute and per cent)		Basal activity of adenylate cyclase[a]	Secretin-stimulated adenylate cyclase[b]
Total homogenate	510 ± 135 mg	Specific activity	7.84 ± 1.60	19.37 ± 3.95
Fraction I	13.27 ± 3.3 mg	specific activity[1]	70.56 ± 12.39	202.20 ± 23.05
		enrichment[2]	9.01 ± 1.2	10.43 ± 0.62
	2.60 ± 0.50 (%)	recovery[3]	24.03 ± 3.17	27.14 ± 2.5
Fraction II	32.14 ± 9.60 mg	specific activity[1]	35.85 ± 5.45	89.69 ± 15.91
		enrichment[2]	4.70 ± 0.63	4.19 ± 0.98
	6.30 ± 1.07%	recovery[3]	28.19 ± 3.77	24.29 ± 6.69
Fraction III	18.00 ± 4.68	specific activity[1]	3.42 ± 0.98	6.50 ± 2.23
		enrichment[2]	0.44 ± 0.09	0.28 ± 0.07
	3.53 ± 0.68(%)	recovery[3]	2.29 ± 0.29	1.06 ± 0.20
Fraction IV	51.24 ± 11.74 mg	specific activity[1]	0.54 ± 0.10	2.12 ± 0.30
		enrichment[2]	0.07 ± 0.05	0.11 ± 0.04
	10.04 ± 2.67(%)	recovery[3]	0.7 ± 0.11	1.09 ± 0.17

Specific activity expressed in:
[a] pmoles cAMP formed per mg protein per min.
[b] pmoles cAMP formed per mg protein per min, basal adenylate cyclase activity substracted.
[c] μmoles of P_i released per mg protein per hr.
[d] mU/mg protein.
[e] nmoles cytochrome *c* reduced at 22 °C per mg protein per min.
[f] μmoles per mg protein per hr.
[g] μg per mg protein.
[1] Expressed as enzyme activity per mg protein, for RNA: μg RNA per mg protein.
[2] Specific activity divided by specific activity of total homogenate.
[3] Total enzyme activity recovered in the fraction as a per cent of total activity in homogenate.

completely extracted by the low ionic strength buffer. It will be shown (*see below*) that this contamination will decrease in further separation always together with NADH-cytochrome *c*-reductase activity.

The enrichment of SDH in Fraction III is about eightfold and that of trypsin and amylase in Fraction IV about threefold. As seen in Table 1 the group of enzymes comprising Ca^{++}-ATPase, HCO_3^--stimulated-Mg^{++}-dependent and Mg^{++}-ATPase shows a broader distribution among the three first fractions being absent in Fraction IV. Although their highest enrichment is associated with Fraction I, comprised mainly

Pancreozymin-stimulated adenylate cyclase[b]	$(Na^+ + K^+)$-ATPase[c]	5′-nucleotidase[c]	Alkaline phosphatase[d]	Ca^{++} ATPase[c]	HCO_3^- ATPase[c]
20.01 ± 4.02	0.294 ± 0.095	0.670 ± 0.038	0.563 ± 0.067	1.90 ± 0.31	0.253 ± 0.029
208.42 ± 31.22	3.750 ± 0.873	11 ± 1.08	5.40 ± 0.68	14.51 ± 1.56	2.68 ± 0.71
10.50 ± 1.44	11.60 ± 2.19	15.10 ± 0.98	9.12 ± 2.39	4.51 ± 0.71	10.3 ± 2.57
28.3 ± 4.90	27.61 ± 5.24	39.93 ± 3.47	23.41 ± 2.90	9.06 ± 1.37	11.76 ± 1.29
88.92 ± 12.12	1.43 ± 0.25	2.39 ± 0.27	2.77 ± 0.61	6.69 ± 0.70	1.01 ± 0.18
4.39 ± 0.93	4.86 ± 0.67	3.57 ± 0.38	4.86 ± 0.81	3.30 ± 0.56	4.07 ± 1.69
24.29 ± 2.60	27.93 ± 2.5	19.23 ± 1.56	28.19 ± 2.93	12.79 ± 2.14	12.95 ± 2.59
4.62 ± 0.40	0.60 ± 0.050	0.09 ± 0.04	0.81 ± 0.03	6.32 ± 0.58	1.41 ± 0.16
0.23 ± 0.08	0.20 ± 0.05	0.15 ± 0.08	1.44 ± 0.11	2.97 ± 0.32	6.01 ± 0.89
1.41 ± 0.31	1.20 ± 0.51	0.36 ± 0.21	5.46 ± 0.70	12.60 ± 1.94	23.11 ± 3.17
2.97 ± 0.95	0	0	0.773 ± 0.090	0.72 ± 0.20	0.25 ± 0.07
0.15 ± 0.03	–	–	1.37 ± 0.35	0.38 ± 0.10	0.98 ± 0.15
1.39 ± 0.09	–	–	13.76 ± 2.87	3.80 ± 0.92	9.86 ± 1.49

by plasma membranes, these enzymes cannot be considered as exclusive plasma membrane markers, as demonstrated in many other tissues.

II. Further Fractionation by Differential Centrifugation (Step II)

Further purification of plasma membrane fractions derived from the zonal density gradient could be obtained by differential centrifugation of the material resuspended in hypotonic buffer. Table 2 shows that 80–95% of basal adenylate cyclase, hormone-stimulated adenylate cyclase and $(Na^+ + K^+)$-ATPase, but only 31% of the protein is associated with the material pelleting at $11{,}000 \times g$ for 15 min. This results in a further purification of plasma membranes in the pellet. It can be seen from Table 2 that the bulk ($\sim$90%) of the reductase stays in the supernatant with only small amounts of $(Na^+ + K^+)$-ATPase and adenylate cyclase. The same holds for the RNA and trypsin. This separation of vesicles carrying plasma membrane markers from the vesicles derived from the endoplasmic reticulum applies for both Fractions I and II.

After this step the final enrichment over total homogenate for basal adenylate cyclase, secretin-stimulated adenylate cyclase and pancreozy-

Table 1 (continued)

	Protein yield (absolute and per cent)		Mg^{++}-ATPase[c]	NADH-cyt-*c* reductase (rotenone-insensitive)[e]
Total homogenate	510 ±135 mg	Specific activity	1.85±0.22	44 ± 6.05
Fraction I	13.27± 3.3 mg	specific activity[1]	9.20±1.37	183 ±18
		enrichment[2]	4.97±0.502	4.07± 0.38
	2.60± 0.50(%)	recovery[3]	7.39±1.43	10.59± 1.50
Fraction II	32.14± 9.60 mg	specific activity[1]	4.61±0.63	61.50± 5
		enrichment[2]	2.64±0.19	1.37± 0.22
	6.30± 1.07(%)	recovery[3]	10.97±1.63	10.80± 1.46
Fraction III	18.00± 4.68 mg	specific activity[1]	5.86±0.88	84 ± 9.50
		enrichment[2]	3.17±0.73	1.79± 0.20
	3.53± 0.68(%)	recovery[3]	11.14±1.24	6.29± 1.46
Fraction IV	51.24± 11.74 mg	specific activity[1]	1.01±4.03	0
		enrichment[2]	0.55±0.10	–
	10.04± 2.67(%)	recovery[3]	5.49±1.44	–

min-stimulated adenylate cyclase as well as for $(Na^+ + K^+)$-ATPase is about 25- and 16-fold for Fractions I and II, respectively. No obvious difference in the distribution pattern of $(Na^+ + K^+)$-ATPase and adenylate cyclase was observed (Table 2).

In spite of a good separation between the rotenone-insensitive NADH-cytochrome-*c*-reductase and the plasma membrane markers adenylate cyclase and $(Na^+ + K^+)$-ATPase obtained in this step, the purified plasma membranes still contain NADH-cytochrome-*c*-reductase activity but now about two- to fivefold less than Fraction I from the zonal gradient. Since it was not possible to improve separation between different vesicles by changing the conditions of differential centrifugation (time and centrifugal force), an additional step of gradient centrifugation was used.

III. Fractionation in Sucrose-Step-Gradient (Step III)

Additional purification of pancreatic plasma membranes to that obtained in step II could be achieved by centrifugation of the $11{,}000 \times g$ pellet in a discontinuous sucrose gradient. Following 3 hr of centrifugation at $100{,}000 \times g$ the material was distributed into three bands on

ADPase[c]	RNA[g]	Succinic dehydrogenase[f]	Trypsin[d]	Amylase[d]
1.87±0.34	45.45± 6.55	0.195±0.062	337 ± 54	352 ±81
9.27±0.95	25 ± 5.45	0.021±0.010	129 ± 24	84 ±11
5.27±0.69	0.55± 0.12	0.11 ±0.09	0.39± 0.07	0.24± 0.20
13.70±6.84	1.43± 0.32	0.290±0.05	1.01± 0.04	0.61± 0.29
2.99±0.18	118.47±27.61	0.055±0.016	197 ± 50.2	39 ±14
1.60±0.19	2.58± 0.46	0.28 ±0.11	0.57± 0.10	0.15± 0.06
8.66±1.01	16.07± 0.66	1.76 ±0.60	3.59± 0.81	0.49± 0.21
0.83±0.19	64.75± 5.13	1.50 ±0.14	1.02± 2.47	144 ±35
0.44±0.19	1.43± 0.01	7.75 ±1.24	0.30± 0.12	0.30± 0.04
1.50±0.44	5.03± 0.91	27.74 ±4.57	1.06± 0.13	0.97± 0.19
0	20 ± 7.01	0.013±0.094	1086 ±108	1031 ±98
–	0.44± 0.14	0.07 ±0.01	3.22± 0.99	2.93± 0.72
–	4.41± 1.36	0.70 ±0.14	32.34± 9.30	35.71± 6.64

a sucrose step gradient and recovered at the interface between 15 and 27% (band I), between 27 and 32% (band II), and between 32 and 40% of sucrose (w/w) (band III).

As shown in Table 3 the highest activity of the plasma membrane markers is associated with the peak banding at 27 to 32% sucrose interface (band II). In contrast the higher activity of NADH-cytochrome-*c*-reductase is found in the lightest fraction at 15 to 28% sucrose interface (band I). Further purification of plasma membranes in this step as compared to the original pellet is 1.5- to twofold for the material derived from Fraction I and 2.4- to threefold of that derived from Fraction II. The final enrichment of basal and hormone-stimulated adenylate cyclase and $(Na^+ + K^+)$-ATPase as compared to total homogenate is about 40 times for both fractions. The activity of NADH-cytochrome-*c*-reductase is decreased by 10-fold in comparison with the supernatant from step II. Considering the hormone-stimulated adenylate cyclase as a typical plasma membrane marker enzyme and the NADH-cytochrome-*c*-reductase as a typical marker for endoplasmic reticulum the contamination of the final plasma membrane preparation by the vesicles derived from endoplasmic reticulum is estimated to be about 10% of that in Fraction I.

Table 2. Distribution of enzymes in pellet and supernatant obtained by differential centrifugation (step II) of Fractions I and II from zonal gradient (step I) (Values give the mean ± SD of 10 experiments and 20 determinations)

	Protein yield (absolute and per cent)		Basal adenylate cyclase[a]	Secretin-stimulated adenylate cyclase[b]
Fraction I (start. material)	12 mg	specific activity	70.56	202.20
pellet 11,000 × *g*	3.72 mg	specific activity[1]	148.18 ± 25.03	593.55 ± 60.43
		enrichment[2]	2.1	2.85
	31 (%)	recovery[3]	80	95
supernatant	8.28 mg	specific activity[1]	16.42 ± 3.20	12.50 ± 5.14
		enrichment[2]	0.23	0.06
	69 (%)	recovery[3]	20	5
Fraction II (start. material)	25 mg	specific activity	35.85	89.69
pellet 11,000 × *g*	6.5 mg	specific activity[1]	91.09 ± 14.56	398.20 ± 67.66
		enrichment[2]	2.54	4.44
	26 (%)	recovery[3]	82	85
supernatant	18.5 mg	specific activity[1]	10.30 ± 2.32	25.49 ± 6.92
		enrichment[2]	0.29	0.28
	74 (%)	recovery[3]	18	15

Specific activity expressed in:
[a] pmoles cAMP formed per mg protein per min.
[b] pmoles cAMP formed per mg protein per min, basal adenylate cyclase activity subtracted.
[c] μmoles of P_i released per mg protein per hr.
[d] mU/mg protein.
[e] nmoles cytochrome *c* reduced at 22 °C per mg protein per min.
[f] μg per mg protein.
[1] Enzyme activity per mg protein, for RNA: μg RNA per mg protein.
[2] Specific activity divided by specific activity in the starting material (Fraction I or II from zonal rotor step).
[3] Total enzyme activity recovered in the fraction as a per cent of total activity of the starting material.

IV. Polyacrylamide Gel Electrophoresis

Fig. 5 (*A* and *B*) shows electropherograms of SDS solubilized protein material derived from membrane Fractions I and II. It can be seen that each fraction is characterized by its distinctive gel pattern. The gel peptide band distribution of supernatant and pellet obtained in this step is shown

Pancreozymin-stimulated adenylate cyclase[b]	$(Na^+ + K^+)$-stimulated ATPase[c]	NADH-cyt-*c* reductase (rotenone-insensitive)[e]	RNA[f]	Trypsin[d]
208.42	3.75	183	69	129
584.92 ± 72.40	7.47 ± 0.92	98.82 ± 15	13 ± 4	55 ± 7
2.81	1.99	0.54	0.19	0.43
94	95	17	4	6
14.00 ± 2.21	0.22 ± 0.03	336 ± 62	99 ± 11	175 ± 31
0.07	0.06	1.84	1.45	1.36
6	5	86	96	94
88.92	1.43	61.5	118	197
370.5 ± 50.48	4.24 ± 12	43 ± 12	61 ± 14	58 ± 6
4.16	2.97	0.70	0.52	0.29
82	71	20	14	7
23.05 ± 3.11	0.62 ± 0.12	109 ± 13	159 ± 29	280 ± 31
0.26	0.43	1.77	1.35	1.42
18	29	80	86	93

for Fraction II, Fig. 5 (*C* and *D*). Two different gel patterns for the lighter and heavier parts of Fraction II can be distinguished.

V. Assessment of Membrane Degradation

Since pancreatic homogenates contain active digestive enzymes (e.g. amylase and lipase) and proteolytic zymogens that could get activated during the course of the 9-hr duration of tissue preparation, it appeared to be important to assess the degree of possible inactivation of enzymes. Estimation of adenylate cyclase activity in the total homogenate, measured either subsequently to preparation or after 9 hr of storage at +2 °C, showed that in the latter case about 50% of the basal, catalytic (NaF-stimulated) and hormone-stimulated activities were lost. Prior treatment of purified membranes (fraction F_I from the zonal rotor) for 15 min at 30 °C resulted in a fall of basal and of pancreozymin- and secretin-

Table 3. Distribution of enzyme activities in three bands recovered from sucrose density step gradient (step III) (Values represent the mean ± SD of five experiments and 10 determinations)

	Protein yield (absolute and per cent)		Basal activity of adenylate cyclase[a]	Secretin-stimulated adenylate cyclase[b]	Pancreozymin-stimulated adenylate cyclase[b]	$(Na^+ + K^+)$-stimulated ATPase[c]	NADH-cyt-*c* reductase (rotenone-insensitive)[d]
Fraction I (start. material) 11,000 × *g* pellet (step II)	3.5 mg	SA	148.18	593.55	584.92	7.47	117
Band I	0.91 mg	SA[1]	59.13	77.16	93.59	4.48	200.07
		E[2]	0.40	0.13	0.16	0.60	1.71
	26%	R[3]	11	3	4	16	42
Band II	1.7 mg	SA[1]	180.20	908.92	904.17	14.01	78
		E[2]	1.22	1.53	1.55	1.87	0.67
	53%	R[3]	72	87	84	91	34
Band III	0.74 mg	SA[1]	103.73	253.23	274.91	2.39	160.29
		E[2]	0.70	0.43	0.47	0.32	1.37
	21%	R[3]	15	9	10	7	27
Fraction II (start. material) 11,000 × *g* pellet (step II)	6 mg	SA[1]	91.09	398.20	370.5	4.24	43
Band I	2.1 mg	SA[1]	29.15	43.80	51.87	3.05	61
		E[2]	0.32	0.11	0.14	0.72	1.43
	35%	R[3]	11	4	5	17	49
Band II	2.28 mg	SA[1]	170.42	910.20	913.30	13.02	31
		E[2]	1.87	2.30	2.46	3.07	0.73
	38%	R[3]	71	87	92	78	28
Band III	1.62 mg	SA[1]	24.59	87.60	70.40	1.31	37
		E[2]	0.27	0.22	0.19	0.31	0.85
	27%	R[3]	7	6	4	5	23

Specific activity expressed in:

[a] pmoles cAMP formed per mg protein per min.

[b] pmoles cAMP formed per mg protein per min, basal adenylate cyclase activity subtracted.

[c] μmoles of P_i released per mg protein per hr.

[d] nmoles cytochrome *c* reduced at 22 °C per mg protein per min.

[1] Enzyme activity per mg protein.

[2] Specific activity divided by specific activity in the starting material (11,000 × *g* pellet of Fractions I and II, respectively).

[3] Total enzyme activity recovered in the fraction as a per cent of total activity of the starting material.

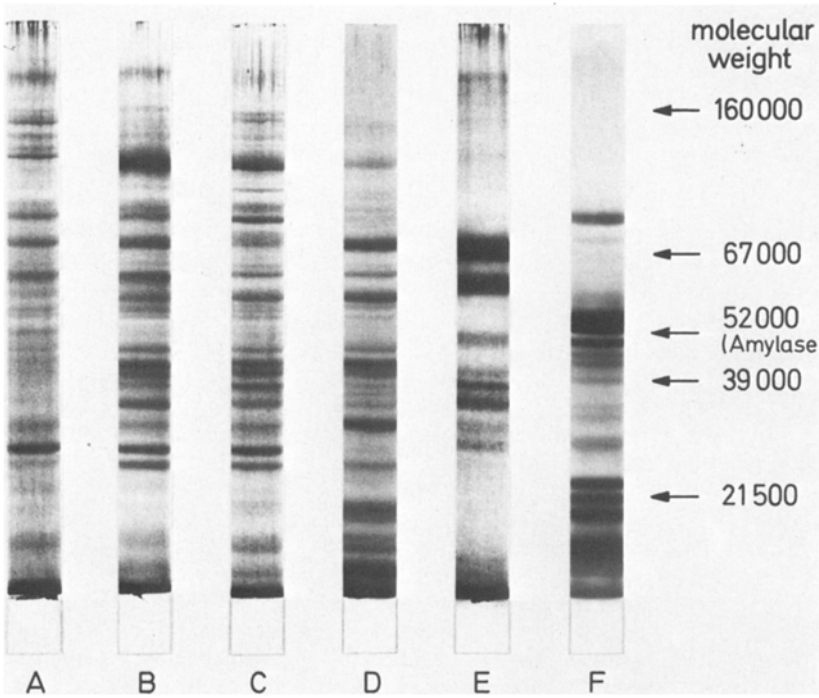

Fig. 5. Polyacrylamide gel patterns obtained after dissolution of the material in 1% SDS-5% mercaptoethanol. (*A*) Protein distribution of material derived from zone I from zonal ficoll-sucrose density gradient; (*B*) zone II from zonal ficoll-sucrose density gradient; (*C*) pellet derived from differential centrifugation (20,000 × *g*) of zone II; (*D*) supernatant derived from differential centrifugation (20,000 × *g*) of zone II; (*E*) membranes of zymogen granules; (*F*) content of zymogen granules (supernatant of lysed ZG)

stimulated activities by 50% and prior trypsinization (6 μg of trypsin/mg protein) for 5 min at 30 °C reduced basal and hormone-stimulated activities by 85–90%. However, in all cases the characteristics of hormonal activation of the residual activity had not changed since hormone concentrations giving half-maximal activation of the enzyme did not change (results not shown).

The same results were obtained in a comparative study using two different membrane preparations. The half-maximal stimulation of adenylate cyclase by various peptide hormones occurs virtually at the same hormone concentration in purified membranes (prepared in about 9 hr by zonal centrifugation) and in crude preparation (4,000 × *g* pellet) of pancreatic membranes (Table 4). The latter procedure is quick enough to allow enzyme determination within 2 hr after homogenization.

Table 4. Comparison of sensitivity of pancreatic adenylate cyclase to the stimulation by various peptide hormones in two different tissue preparations (Values given are the mean ± SD; number of experiments are: for secretin 10, for each pancreozymin and the octapeptide 3, for VIP 2)

Hormone	Half-maximal stimulatory concentration	
	Crude membrane preparation (M)	purified plasma membranes (M)
synthetic secretin	$8.41 \pm 0.89 \times 10^{-9}$	$9.5 \pm 1.5 \times 10^{-9}$
vasoactive intestinal polypeptide	1.82×10^{-7} [a]	1.51×10^{-7} [a]
pancreozymin	$1.21 \pm 0.36 \times 10^{-6}$	$1.02 \pm 0.32 \times 10^{-6}$
octapeptide of pancreozymin	$3.46 \pm 0.76 \times 10^{-8}$	$3.12 \pm 0.62 \times 10^{-8}$

[a] Mean of two experiments.

Table 5. Enzyme activities in membranes isolated from zymogen granules (Values represent the mean ± SD of five experiments and 5–10 determinations)

	Basal activity of adenylate cyclase[a]	Secretin-stimulated adenylate cyclase[b]	Pancreozymin-stimulated adenylate cyclase[b]		$(Na^+ + K^+)$-stimulated ATPase[c]
specific activity[1]	6.44 ± 3.05	0	0		0
enrichment[2]	0.82	–	–		–
	5′-nucleotidase[c]	NADH-cyt-*c* reductase (rotenone-insensitive)[e]	Trypsin[d]	RNA[f]	Ca^{++}-ATPase[c]
specific activity[1]	1.81 ± 0.52	35 ± 4	81 ± 9	10 ± 0.9	7.01 ± 1.20
enrichment[2]	2.70	0.80	0.24	0.22	3.69

Specific activity expressed in:
[a] pmoles cAMP formed per mg protein per min.
[b] pmoles cAMP formed per mg protein per min, basal adenylate cyclase activity substracted.
[c] μmoles of P_i released per mg protein per hr.
[d] mU/mg protein.
[e] nmoles cytochrome *c* reduced at 22 °C per mg protein per min.
[f] μg per mg protein.
[1] Enzyme activity per mg protein for RNA: μg RNA per mg protein.
[2] Specific activity divided by specific activity in total homogenate.

VI. Preparation of Zymogen Granule Membranes

Membranes prepared from zymogen granules are enriched only in Ca^{++}-ATPase and 5′-nucleotidase activities (Table 5). Other plasma

membrane marker enzymes are either absent (hormone-stimulated adenylate cyclase, (Na−+K−)-ATPase) or present at very low levels (basal adenylate cyclase is, for example about 100 times less active than in plasma membranes). In contrast, specific activity of Ca^{++}-ATPase is comparable to the activity of this enzyme in plasma membranes (Table 1). As shown in Fig. 5*E* the zymogen granule membranes have a simple peptide gel pattern characterized by only two major bands, but several additional minor bands. For comparison the protein pattern of zymogen granule content is shown (Fig. 5*F*). Its main constituent, amylase, can be distinguished from the two major bands from zymogen granule membranes (Fig. 5*E*).

Discussion

The data presented show that density gradient centrifugation in a zonal rotor is an appropriate method for fractionation of a large amount of pancreatic homogenate in a single step. The distribution of fractionated material in a simple sucrose density gradient results in a separation of plasma membranes and endoplasmic reticulum from mitochondria and from zymogen granules. The addition of ficoll allows separation of membrane fragments into two fractions: (1) One banding at lower density containing the plasma membrane markers (hormone-stimulated adenylate cyclase and $(Na^{+}+K^{+})$-ATPase, enriched 10 and 12 times, respectively) and probable smooth endoplasmic reticulum markers ADPase and rotenone-insensitive NADP-cytochrome-*c*-reductase, enriched 5 and 4 times, respectively), but only small amounts of RNA. (2) The membrane fraction banding at higher density is rich in RNA (3 times enriched over total homogenate) and electron microscopy shows small vesicles with attached ribosomes which confirms that they originate from rough endoplasmic reticulum. In the same fraction the plasma marker enzymes are less enriched than in Fraction I (only 4 to 5 times higher than in total homogenate) but their quantity makes this fraction still suitable for further purification of plasma membranes. Both fractions of the ficoll-sucrose gradient contain only small mitochondrial contamination and low levels of trypsin and amylase. Since the separation between both fractions is not complete showing cross-contamination between plasma membranes and membranes from endoplasmic reticulum, an additional step of differential centrifugation was introduced. This results in a further separation of larger vesicles containing plasma membrane markers (i.e. adenylate cyclase and $(Na^{+}+K^{+})$-ATPase) from smaller vesicles carrying the bulk of the markers for endoplasmic reticulum

(NADH-cytochrome-*c*-reductase and RNA) in both fractions. Plasma membranes obtained after these two steps are about 20 times enriched with respect to the activity of plasma membrane markers. These two steps represent a relatively quick method for purification of plasma membranes with a yield of 50%.

For the purpose of higher purification (but with smaller yield) it is possible to perform further fractionation by an additional separation of the material obtained in the differential centrifugation by distribution through a sucrose gradient. This results in further twofold purification. The final plasma membrane marker enrichment of about 40-fold was the same whether Fraction I or II from the zonal rotor was used.

To be able to monitor such a fractionation using very sensitive plasma membrane markers as hormone-stimulated adenylate cyclase and $(Na^+ + K^+)$-ATPase, it is necessary to prevent enzyme inactivation caused by lypolytic and proteolytic attack. This is a serious problem in fractionation of pancreatic tissue because of high levels of active or activatable phospholipolytic and proteolytic enzymes present in pancreatic homogenate. Rapid inactivation of hormone-stimulated adenylate cyclase and $(Na^+ + K^+)$-ATPase on aging and probably due to lipolytic and proteolytic degradation has already been reported [9, 23]. In preliminary experiments we had observed that fractionation of pancreatic homogenate in sucrose without any additions of protease inhibitors gave unsatisfactory results because of inactivation of hormone-stimulated adenylate cyclase during the course of fractionation procedure. Using buffered sucrose solutions with the addition of the protease inhibitor PMSF (phenylmethylsulfonylfluoride) and the Ca^{++}-chelator EGTA to block Ca^{++}-dependent phospholipolysis, the inactivation was minimized. Only under these conditions was both high recovery and specific activity of adenylate cyclase obtained. The high specific activities for hormone-stimulated adenylate cyclase (800 pmoles/mg · protein min) and for $(Na^+ + K^+)$-ATPase (14 μmoles/hr · mg protein) in this plasma membrane preparation in comparison to those reported in literature for pancreas are also due to relatively efficient separation of plasma membranes from other cell organelles. As in other tissues (for reference *see* [19]) smooth vesicles derived from endoplasmic reticulum represent the major contaminant and provide the most difficulty in purification of plasma membrane. This organelle is heterogeneous with respect to density, and its density partly overlaps with that of surface plasma membrane [19] rendering complete separation by density alone impossible. However, the larger size of the fragments derived from the surface membranes of pancreatic

cell as compared with those derived from endoplasmic reticulum allowed separation by rate sedimentation. Fifteen per cent of plasma membrane fragments of a smaller size are lost in endoplasmic reticulum supernatant in this step (Table 2). Combined with subsequent density separation this decreases endoplasmic reticulum contamination to about 10%.

As Table 1 shows, in only 9% of the total protein collected from the peaks of Fractions I and II about 50% of typical marker enzymes for plasma membranes was recovered. The recovery as well as enrichment factors for all plasma membrane markers (hormone-stimulated adenylate cyclase, $(Na^+ + K^+)$-ATPase, 5′-nucleotidase and alkaline phosphatase) are the same, suggesting that no selective inactivation of any of these enzymes took place. Moreover, pancreozymin- and secretin-stimulated adenylate cyclase showed the same enrichment and recovery as the basal activity, showing that receptors for both hormones were well preserved from inactivation during the fractionation procedure. In contrast to findings of others [25] who reported higher susceptibility to inactivation for pancreozymin-stimulated adenylate cyclase in comparison to secretin-stimulated adenylate cyclase, under conditions applied in this study, stimulation by both these hormones could be equally well preserved.

In contrast to 5′-nucleotidase and alkaline phosphatase which both copurified with the typical plasma membrane markers adenylate cyclase and $(Na^+ + K^+)$-ATPase, the distribution of Ca^{++}-ATPase and HCO_3^--stimulated-Mg^{++}-dependent ATPase is broader, showing highest enrichment in the first fraction but also occurrence in the second and mitochondrial fractions. It was shown already that the latter enzymes are located in mitochondrial fractions [6, 21, 24] but their presence in plasma membranes has been questioned. As this study shows, these two enzymes, although present also in mitochondria, are consistently enriched in the plasma membrane fraction to a degree comparable with other plasma membrane markers (10 times for HCO_3^--stimulated-Mg^{++}-dependent ATPase and 5 times for Ca^{++}- and Mg^{++}-ATPases). This finding supports the view that the HCO_3^--stimulated-Mg^{++}-dependent ATPase assumed to be involved in pancreatic HCO_3^- secretion [27, 28] is indeed located in the plasma membrane.

As seen from Table 1 zymogen granules were already purified in one step by zonal centrifugation as assessed by measurement of trypsin and amylase and by electron microscopy. However, they are contaminated by RNA five to six times more than the previous preparation of zymogen granules [15]. Zymogen granule membranes prepared from this fraction contained only Ca^{++}-ATPase in substantial amounts of

all the enzymes tested (Table 5). The absence of a complex enzyme pattern is reflected also in the typical gel pattern consisting of two major peptide bands at a molecular weight of ~100,000 (Fig. 5*E*). It is tempting to speculate that the simplicity of this membrane in terms of different protein species reflects its limited number of functions, i.e. storage and discharge of zymogens.

The availability of relatively pure pancreatic membrane fractions should allow an increased number of studies of pancreatic function at the subcellular level. One of these is described in the succeeding paper.

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