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MEMBRANE ADENOSINE TRIPHOSPHATASE ACTIVITIES IN RAT PANCREAS

S.S. MARTIN and A.E. SENIOR

Department of Biochemistry, University of Rochester Medical Center, Rochester, NY 14642 (U.S.A.)

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Summary

The membrane ATPase activities present in rat pancreas were studied to investigate the possible role of ATPase enzymes in HCO_3^- secretion in the pancreas. It was found that all the HCO_3^--sensitive (anion-sensitive) ATPase activity was accountable as pancreatic mitochondrial ATPase, thus supporting the view that a distinct plasma membrane 'bicarbonate-ATPase' is not involved in HCO_3^- secretion in pancreas. A remarkably high Mg^{2+}- and Ca^{2+}-requiring ATPase activity (30 μmol ATP hydrolysed/min per mg) was found in the plasma membrane fraction (ρ = 1.10—1.13). This activity has been characterized in some detail. It is inhibited by *p*-fluorosulfonylbenzoyladenosine, an affinity label analogue of ATP and the analogue appears to label covalently a protein of $M_r \sim 35\,000$. The (Ca^{2+} + Mg^{2+})-ATPase activity did not form a 'phosphorylated-intermediate' and was vanadate-insensitive. These and other tests have served to demonstrate that the (Ca^{2+} + Mg^{2+})-ATPase activity is different in properties from (Na^+ + K^+)-ATPase, Ca^{2+}-ATPase, (H^+ + K^+)-ATPase or mitochondrial H^+-ATPase. Apart from the (Ca^{2+} + Mg^{2+})-ATPase of plasma membrane and mitochondrial ATPase, the only other membrane ATPase activities noted were (Na^+ + K^+)-ATPase, which occurred in the same fractions as the (Ca^{2+} + Mg^{2+})-ATPase at ρ = 1.10—1.13 and was of surprisingly low activity, and an ATPase activity in light membrane fractions (ρ = 1.08—1.09) derived from zymogen granule membranes.

Abbreviations: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; SDS, sodium dodecyl sulfate; FSBA, *p*-fluorosulfonylbenzoyl-5'-adenosine.

At this time, therefore, there is no obvious candidate for an ATPase activity at the luminal surface of pancreatic cells which is directly involved in ion transport, but the results presented here direct attention to the high activity (Ca^{2+} + Mg^{2+})-ATPase in the plasma membrane fraction.

Introduction

The exocrine pancreas plays a major role in digestion by secreting a juice rich in $NaHCO_3$ in response to hormonal stimulation. The mechanism of secretion of HCO_3^- and Na^+ is unknown, but the facts that in human about 1200 ml of juice containing up to 140 mM HCO_3^- are secreted daily underscore that this is an important transport process.

Ridderstrap and Bonting [1,2] implicated the (Na^+ + K^+)-ATPase in secretory function of pancreas, and further work has confirmed and extended this view [3,4]. However, the (Na^+ + K^+)-ATPase has a basolateral plasma membrane location in the pancreas [5] as it does elsewhere and this, together with studies on Na^+ and H^+ fluxes in isolated pancreas [4,6,7], suggests that a separate active transport process at the luminal surface also occurs. Several workers have suggested that a 'bicarbonate-ATPase' is directly involved in pumping HCO_3^- in pancreas and evidence has been provided that such a plasma-membrane enzyme activity exists [8—10]. Others, however, have seriously questioned these findings [11].

It is certainly reasonable to hypothesize that a membrane ATPase other than (Na^+ + K^+)-ATPase may be involved in pancreatic ion transport. Analogies may be drawn, for instance, with the (H^+ + K^+)-ATPase thought to be involved in H^+ secretion in stomach [12,13] with the Ca^{2+}-ATPase of red blood cells [14] and with the H^+-ATPase in chromaffin granules [15,16]. We have therefore taken the direct approach of characterizing the membrane ATPase activities which are present in rat pancreas membrane fractions in order to find out which are possibly involved in pancreatic transport functions.

Materials and Methods

Preparation of rat pancreas homogenate. Male Sprague Dawley rats weighing 250—450 g were given free access to food and water. They were killed by decapitation and exsanguination. The pancreas was removed and placed in ice-cold rinsing solution containing 300 mM sucrose, 10 mM Tris-HCl (pH 7.5) and 2 mM EDTA (sodium salt). After dissecting the fat from the pancreas, the tissue was weighed and then minced finely with scissors in a beaker on ice. All subsequent operations were performed on ice or in a cold room at 4°C. Either of two methods of homogenization was applied.

Teflon pestle homogenization. The minced pancreas was homogenized in 10 vols. of buffer containing 250 mM sucrose, 40 mM Tes-Na (pH 6.5), 3 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 0.2 mg/ml soybean trypsin inhibitor protein and 0.1 mg/ml bovine serum albumin. 80-ml batches were homogenized in a glass homogenizing tube with a Teflon pestle (clearance 0.004 inch, 10 strokes

up and down). The suspension was then filtered through two layers of cheesecloth.

Polytron homogenization. Minced pancreas was mixed with homogenizing buffer as above. 50-ml batches were placed in a 125 ml square homogenizing vessel on ice and dispersed by treatment for 5 s at full power with a Brinkman Polytron Homogenizer using a PT10ST generator. The resulting suspension was filtered through two layers of cheesecloth.

Fractionation of homogenate by differential centrifugation. The filtrate obtained by either of the above methods was centrifuged for 10 min at 500 × *g* to remove cell debris. The supernatant was centrifuged again (2000 × *g* for 15 min) and a pellet consisting of two well defined layers was obtained. The white lower layer (zymogen granules) was resuspended in 15 vols. of 100 mM Tes-Na (pH 7.8), 100 mM NaCl, 100 mM KCl, 0.5 mg/ml soybean trypsin inhibitor, as described previously [17] to lyse the granules. The beige upper layer (mitochondria) was resuspended in 40 mM Tes-Na (pH 7.5), 1 mM EGTA, 1 mM EDTA, 0.1 mg/ml soybean trypsin inhibitor, 0.1 mg/ml bovine serum albumin, 50% (w/v) sucrose (called 'suspending buffer'). The supernatant was centrifuged at 5000 × *g* for 15 min, yielding a mitochondrial pellet which was resuspended in suspending buffer. The two mitochondrial pellets were pooled. Finally, the supernatant was centrifuged at 100 000 × *g* for 90 min, yielding a microsomal pellet and post-microsomal supernatant. The microsomal pellet was resuspended in suspending buffer. Zymogen granule membranes were obtained from the lysed zymogen granule fraction after 1 h of lysis at 4°C by centrifugation (100 000 × *g* for 90 min). This pellet was resuspended in suspending buffer.

Fractionation of the resuspended pellets by sucrose density gradient centrifugation. Linear sucrose density gradients were formed at 20°C using a Beckman Density Gradient Former. The gradients contained 20—45% (w/v) sucrose in a buffer medium containing 40 mM Tes-Na, pH 7.5 at 4°C, 1 mM EDTA, 1 mM EGTA, 0.1 mg/ml soybean trypsin inhibitor and 0.1 mg/ml bovine serum albumin. The zymogen granule membrane, mitochondrial, or microsomal pellet suspensions, prepared as described above, were layered under the density gradients, The tubes were then centrifuged to equilibrium in a Beckman SW 27 rotor at 27 000 rev./min for 12—16 h. Fractions were collected by pumping 60% sucrose through the bottom of the tubes and collecting the gradient solution from the top. Fractions were stored frozen at —20°C and assayed within 2 weeks of preparation.

To expedite the preparation of plasma membrane fractions, a discontinuous sucrose gradient procedure was also developed. Discontinuous gradients containing layers of 10, 27 and 34% sucrose in buffer medium as above were prepared. The suspended pellets (40% (w/v) in sucrose instead of 50% (w/v)) were layered under the discontinuous gradients and centrifuged as described above. The band forming at the 27—34% interface was collected, diluted with 2 or more vols. of the suspending buffer containing no sucrose and sedimented to a pellet by centrifugation at 100 000 × *g* for 90 min. The pellets were resuspended in the suspending buffer containing 250 mM sucrose and were stored frozen at —20°C.

Enzyme assays. ATPase activity was measured in a final volume of 1.0 ml

containing 75 mM Tes-Tris (pH 7.5), 10 mM $MgCl_2$, 5 mM Na_2ATP and 0.1 mg/ml bovine serum albumin at 37°C. The reaction was started by the addition of 5—20 μg of membrane suspension, and was terminated by the addition of 1.0 ml of 10% (w/v) sodium dodecyl sulfate (SDS). The amount of P_i liberated was determined by using the method of Taussky and Shorr [18]. Specific activity was expressed as μmol P_i released/min per mg.

The effect of the specific mitochondrial ATPase inhibitor protein was measured by preincubating the purified inhibitor protein prepared from beef heart mitochondria [19] with the sample at a concentration of 300 μg inhibitor protein/unit of ATPase activity (1 unit = 1 μmol P_i/min) at 22°C in a buffer containing 15 mM Tes-Tris (pH 6.7), 0.5 mM $MgSO_4$, 0.5 mM ATP, 0.1 mg/ml bovine serum albumin, and 250 mM sucrose, final volume 1 ml. After 15 min, 200 μl of this preincubated material were added to 0.8 ml of ATPase assay medium such that the final concentrations of Tes, $MgCl_2$, ATP and bovine serum albumin were the same as in the ATPase assay (above) at pH 7.5 and 37°C.

Oligomycin was added in 2 μl ethanol when required (final concentration 1.25 μM). $NaHCO_3$ was added to 20 mM final concentration from a 200 mM stock solution (pH 7.5) just before use. $(Na^+ + K^+)$-activated ATPase was measured by adding NaCl to 100 mM and KCl to 20 mM (with and without 1 mM ouabain in ethanol) to the ATPase medium as above. Controls contained ethanol as required.

Adenylate cyclase activity was determined in a final volume of 0.20 ml containing 40 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1.0 mM Na_2ATP, 40 μg/ml soybean trypsin inhibitor protein, 10 mM NaF, 10 mM theophylline, 0.2 mg/ml bovine serum albumin, and an ATP-regenerating system consisting of 50 μg/ml pyruvate kinase and 10 mM phospho*enol*pyruvate. After bringing the reaction mixture to 37°C, the membranes were added in a volume of 25—50 μl. The reaction was allowed to proceed for 15 min at 37°C and then stopped by placing the tube in boiling water for 2 min. Aggregated proteins were removed by centrifugation at 8700 × *g* for 2 min in a Beckman Microfuge B. The supernatant was stored frozen until a 50 μl aliquot of each was assayed for cyclic AMP content using an Amersham Cyclic AMP Assay Kit, TRK 432. Specific activity was expressed as pmol cyclic AMP formed/min per mg. This procedure is a modification of that described by Poirier et al. [20].

Phosphatase activity was estimated by following the production of *p*-nitrophenol from *p*-nitrophenyl phosphate. In a final volume of 1.0 ml, the assay medium contained 10 mM $MgCl_2$, 5 mM *p*-nitrophenyl phosphate and 0.1 mg/ml bovine serum albumin. In addition, the medium contained 40 mM citrate-Tris (pH 4.5) for acid phosphatase, 40 mM Tris-HCl (pH 7.4) for neutral phosphatase, 40 mM Tris-HCl (pH 9.4) for alkaline phosphatase activity and 20 mM KCl for K^+-stimulated phosphatase. Reactions were initiated by the addition of 5—50 μg of sample, and were terminated after 30 min at 37°C by the addition of 1.0 ml of 0.2 M NaOH. The amount of *p*-nitrophenol formed was determined by measuring the absorbance of this solution at 410 nm, with known amounts of *p*-nitrophenol serving as standards.

Glucose-6-phosphatase and 5'-nucleotidase were assayed as suggested by Solyom and Trams [21]. Phosphate released from glucose-6-phosphate and

adenosine 5′-monophosphate was determined as described for the ATPase reaction.

Cytochrome *c* oxidase activity was assayed essentially as described by Yonetani [22]. Samples were added to a buffer containing 0.2 M potassium phosphate (pH 6.0), 1.0 mM EDTA and 1 mg/ml Tween 80 at room temperature (22°C). Upon addition of reduced cytochrome *c* to 15 μM, the decrease in absorbance at 550 nm was recorded. An estimate of initial velocity was made from this record using an extinction coefficient of 19.6 $mM^{-1} \cdot cm^{-1}$ for reduced minus oxidized cytochrome *c*.

SDS-polyacrymide gel electrophoresis. Polyacrylamide gels in SDS were run as previously described [23]. Radioactivity in the gels was determined by slicing the gel, eluting the protein and counting as described previously [23].

Routine procedures. Radioactivity was measured in Fluor containing 500 ml of toluene, 500 ml Triton X-114 and 4 g of 2,5-diphenyloxazole per l. Samples were dissolved in 2 ml of 3% SDS and added to 10 ml Fluor. Protein was estimated using the method of Miller [24] using dry bovine serum albumin as standard. Lipid analyses were carried out as described by Crain and Marinetti [25]. Densities of sucrose-containing solutions were measured using a Bausch and Lomb refractometer at 20°C.

Materials. Soybean trypsin inhibitor protein, ATP (Grade I, sodium salt), *p*-fluorosulfonylbenzoyl-5′-adenosine and bovine serum albumin (Fraction V essentially fatty acid-free) were obtained from Sigma Chemical Co. The [γ-^{32}P]ATP was purchased from Amersham Corp. *p*-Fluorosulfonyl[^{14}C]-benzoyl-5′-adenosine was the generous gift of Professor William S. Allison. Sodium orthovanadate was purchased from Fisher Scientific. All other materials used were of the highest grade available.

Results

ATPase activities in pellets obtained by differential centrifugation. Typical ATPase activities from differential centrifugation fractions prepared after Polytron homogenization are shown in Table I. High bicarbonate- and oligomycin-insensitive ATPase activity was present in membrane fractions from the pancreas prepared in this way. The microsomal pellet ATPase activity was almost completely insensitive to either of these agents and the mitochondrial

TABLE I

ATPase ACTIVITIES IN FRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION OF PANCREAS HOMOGENATES

	ATPase activity (μmol/min per mg)		
	+20 mM NaCl	+20 mM $NaHCO_3$	+20 mM $NaHCO_3$ + 1 μg/ml oligomycin
Homogenate	0.21	0.21	0.19
Zymogen granule membranes	0.18	0.18	0.14
Mitochondria	0.43	0.47	0.33
Microsomes	0.58	0.57	0.56
Post-microsomal supernatant	0.02	0.03	0.02

pellet was relatively insensitive. Similar results were obtained using Teflon pestle homogenization but the ATPase activities were lower (30—50% of the activities seen in Table I). Experiments were also performed to measure the increase in activity when Na^+ and K^+ were added, or the decrease in activity on addition of 1 mM ouabain (see Materials and Methods). K^+-stimulated *p*-nitrophenylphosphatase (±1 mM ouabain) was also estimated as a possible measure of ($Na^+ + K^+$)-activated ATPase. By these criteria, the ($Na^+ + K^+$)-activated ATPase was very low compared to the total ATPase, having an activity less than 0.01 μmol/min per mg in homogenate, mitochondrial and microsomal pellets.

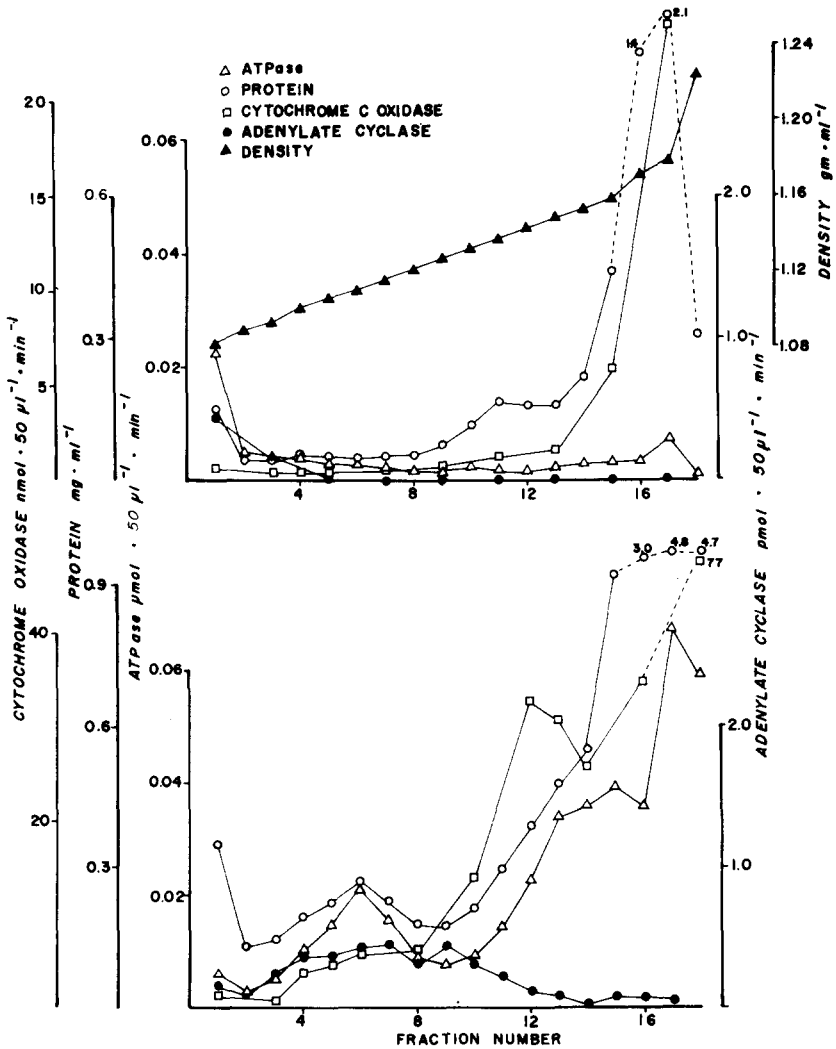

Fig. 1. Linear sucrose density gradient centrifugation of pellets obtained from rat pancreas homogenate by differential centrifugation. The techniques are described in detail in Materials and Methods. Upper, zymogen granule membrane pellet (obtained after lysis of zymogen granules) layered under gradients. Lower, mitochondrial pellet layered under gradients.

Continuous sucrose density gradient centrifugation of pellets obtained by differential centrifugation. Fig. 1 shows the results of experiments in which zymogen granule membrane pellets and mitochondrial pellets were layered under linear continuous sucrose density gradients and centrifuged to equilibrium. In these experiments, the homogenates were obtained by Teflon pestle homogenization. The total ATPase activity in zymogen granule membrane preparations (Fig. 1, upper) was low and separated into two peaks, one at the very top and one at the bottom of the gradient. The pooled fractions from the top of the gradient (ρ = 1.08—1.09) had specific ATPase activities of 1.8 and were completely insensitive to oligomycin and the specific mitochondrial ATPase inhibitor protein. The ATPase at the bottom of the gradient was stimulated by HCO_3^- and sensitive to oligomycin. In the gradients to which mitochondrial pellets were applied (Fig. 1, lower), two peaks of ATPase activity were seen, one at the bottom of the gradients (density greater than 1.15) and one in the middle, at a density of 1.10—1.13. The ATPase activity at the bottom of the gradient was inhibited 50—70% by oligomycin, 88% by the specific mitochondrial ATPase inhibitor protein and stimulated 50% by $NaHCO_3$ (20 mM). The ATPase activity in the middle of the gradients (ρ = 1.10—1.13) was unaffected by these agents. In experiments in which zymogen granule membrane pellets and mitochondrial pellets were derived by Polytron homogenization and applied to continuous sucrose gradients, results were generally similar to those in Fig. 1.

Fig. 2 upper and lower, shows the results obtained when microsomal pellets obtained after Teflon pestle homogenization were run on continuous gradients. Total (upper) and specific (lower) enzyme activities are shown. Fig. 2, upper, shows two peaks of ATPase activity separating from microsomes, one at the bottom of the gradient ($\rho > 1.15$) and the other in the middle of the gradient (ρ = 1.10—1.13). The ATPase activity in the heavy fractions at the bottom of the gradient was sensitive to HCO_3^- (+38%) oligomycin (—72%) and the specific mitochondrial ATPase inhibitor protein (—72%). The ATPase activity in the middle of the gradient was insensitive to these agents. Fig. 3, upper, shows the data for microsomal pellets obtained after Polytron homogenization. The same peak of ATPase activity, which was insensitive to HCO_3^-, oligomycin and the specific mitochondrial ATPase inhibitor protein, again appeared in the middle of the gradient (ρ = 1.10—1.13). Fig. 3, lower, shows the specific enzyme activities seen when microsomal pellets obtained after Polytron homogenization were run on the gradients, and it is seen that very high ATPase activity is present in the region of density 1.10—1.13. From the data on ATPase activities, taken together with assays of cytochrome oxidase, 5′-nucleotidase and adenylate cyclase which are also shown in Figs. 1—3, we have made the following conclusions. Based on the behavior in sucrose density gradients, and using two different homogenization techniques, there appear to be the following types of membrane ATPase activity in rat pancreas homogenate. A low-density (ρ = 1.08—1.09) membrane ATPase activity was derived from zymogen granule membrane fractions only. It was not associated with cytochrome oxidase, and was not sensitive to inhibitors or activators of mitochondrial ATPase. We tentatively identify this activity with zymogen granule membrane ATPase as recently described by Harper et al. [17]. Membrane

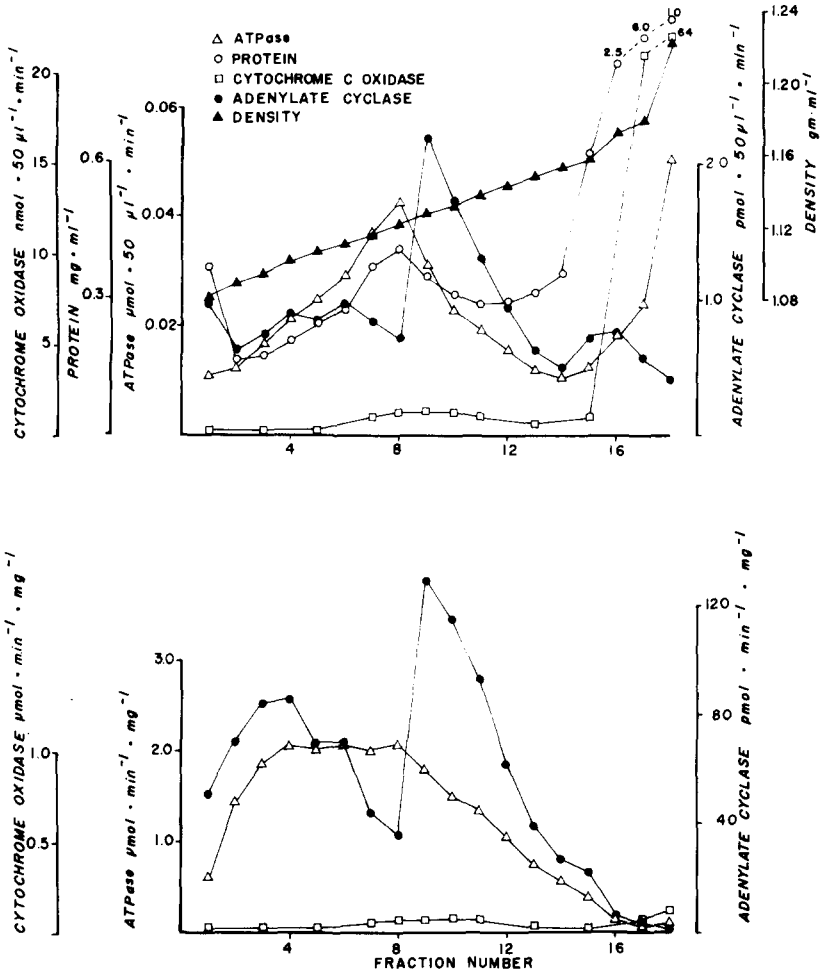

Fig. 2. Linear sucrose density gradient centrifugation of pellets obtained from rat pancreas homogenate by differential centrifugation. The techniques are described in detail in Materials and Methods. Microsome pellets obtained from homogenate made by Teflon pestle homogenization were layered under gradients. Upper, total activities (enzyme units per unit of volume). Lower, specific enzyme activities (units per mg protein).

ATPase activity of density 1.10—1.13 was seen in mitochondrial fractions to some extent, but was present more strikingly in microsomal fractions, and was not associated with cytochrome oxidase activity. It was not activated by HCO_3^-, nor inhibited by inhibitors of mitochondrial ATPase. It was present at high total and very high specific activity in microsomal gradients, especially those prepared by Polytron homogenization and for this reason we have characterized it more extensively (see later). There appeared to be two peaks of adenylate cyclase activity in the microsomal gradients (Figs. 2 and 3, upper) perhaps representing two sub-populations of plasma membrane in the microsomal pellets. The high ATPase activity (density 1.10—1.13) was associated with one of the adenylate cyclase peaks. Assays of 5′-nucleotidase activities (Fig. 3, upper) showed that this ATPase activity and 5′-nucleotidase activities

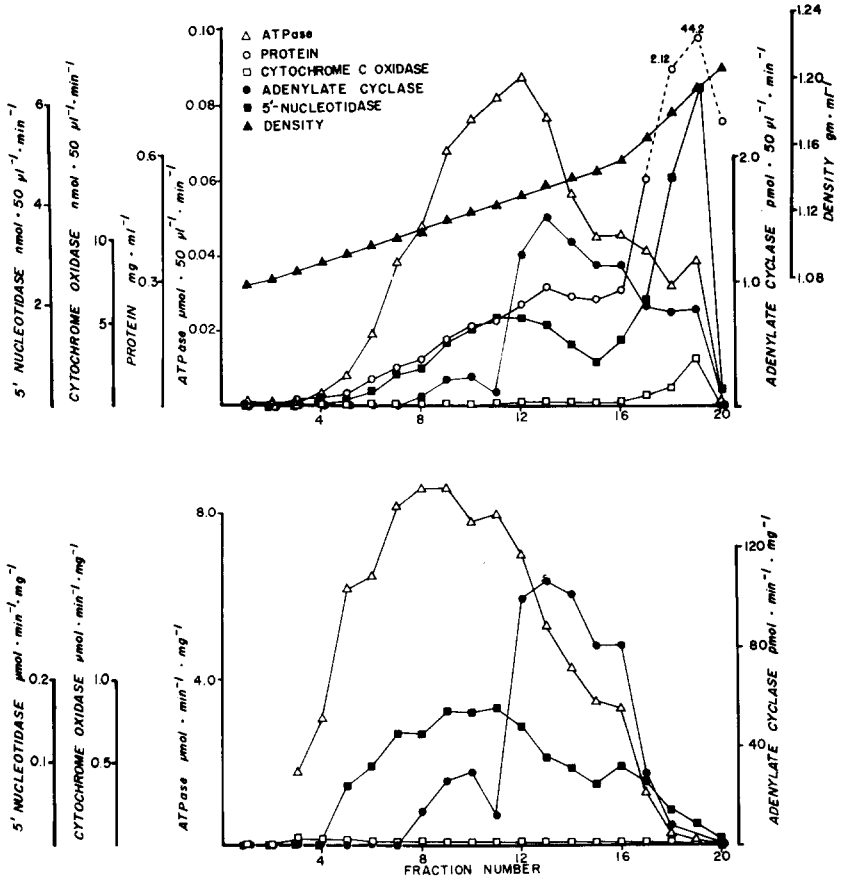

Fig. 3. Linear sucrose density gradient centrifugation of pellets obtained from rat homogenate by differential centrifugation. The techniques are described in detail in Materials and Methods. Microsome pellets obtained from homogenate made by Polytron homogenization were layered under gradients. Upper, total activities (enzyme units per unit of volume). Lower, specific enzyme activities (units per mg protein).

were similarly distributed in the gradient. Assays of alkaline phosphatase activities (not shown) showed similar patterns. The relative specific activities of four enzymes in the fractions obtained by differential centrifugation and in the fractions of $\rho = 1.10—1.13$ from discontinuous density gradients (see below) are shown in Fig. 4 and demonstrate enrichment of 5′-nucleotidase and adenylate cyclase, but not of cytochrome oxidase in the fractions of $\rho = 1.10—1.13$. Therefore, we conclude that the high membrane ATPase activity in fractions of density 1.10—1.13 is of plasma membrane origin. ATPase activity seen at the bottom of zymogen granule membrane gradients (Fig. 1, upper), mitochondrial gradients (Fig. 1, lower) and microsomal gradients (Fig. 2, upper) with density greater than 1.15 was deduced to be mitochondrial ATPase on account of its association with cytochrome oxidase and its sensitivity to HCO_3^-, oligomycin and the specific mitochondrial ATPase inhibitor protein. Dicyclohexylcarbodiimide (10 µM) also strongly inhibited this activity. When Polytron homogenization was used, only small amounts

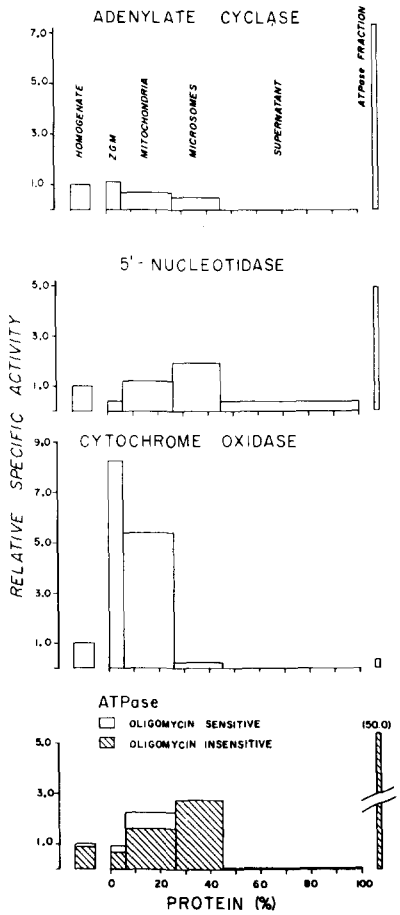

Fig. 4. Relative specific activities of marker enzymes in homogenate, fractions obtained by differential centrifugation, and in the plasma membrane fraction obtained by discontinuous sucrose density gradient centrifugation. The activity in the homogenate (obtained by Polytron homogenization) is set at 1.0. ZGM, zymogen granule membrane pellet; ATPase fraction, plasma membrane fraction collecting at 27—34% interface in the discontinuous gradients.

of mitochondrial membranes apparently ended up in the microsomal pellet, since there was low cytochrome oxidase activity in this gradient (Fig. 3, upper).

$(Na^+ + K^+)$-activated ATPase was assayed in the fractions from the continuous sucrose gradients. As reported above, very low activity of this enzyme was seen in the pellets which were applied to the gradients. On the gradients the activity was found at a density of 1.10—1.13 and had specific activity of 10% or less of the total ATPase (Teflon pestle homogenization) or 3% of total ATPase (Polytron homogenization).

Discontinuous sucrose density gradient centrifugation. Because the plasma membrane pooled fraction had high ATPase activity, we wished to characterize it thoroughly in order to ascertain its properties and to compare it with other known membrane ATPase enzymes. In order to simplify the preparative procedure, discontinuous gradients were substituted for the continuous gradients (see Materials and Methods) and the material which accumulated at the

27--34% interface was collected. This corresponded to the plasma membrane ATPase activity seen in the continuous gradients at ρ = 1.10—1.13.

Properties of the plasma membrane ATPase activity. The data from here on describe the material obtained from discontinuous sucrose gradients. Material obtained after either homogenization method behaved in essentially the same way in experiments to be described. We use the term 'ATPase activity' in a collective sense, it is important to stress that different kinds of ATPase enzymes could be contributing to the total activity measured, indeed the data cited above showed that the ($Na^+ + K^+$)-activated ATPase enzyme is a discrete although minor component of total measurable ATPase activity in the plasma membrane fraction.

General. The specific ATPase activity (initial rate over 1 min) of plasma membrane fractions was 6—14 μmol ATP hydrolyzed/min per mg (Teflon pestle homogenization) and 30 μmol/min per mg (Polytron homogenization). This difference in specific activity was the only major difference between the preparations obtained after the two homogenization procedures. The time-course of ATP hydrolysis was not linear, but fell off over 15 min to a rate of one-third the initial rate. Inclusion of an ATP-regenerating system consisting of phospho*enol*pyruvate plus pyruvate kinase did not change the time-course.

Layering the microsomal pellet under the gradients before centrifuging was a critical feature of the procedure. Very much lower specific ATPase activities were seen in the 27—34% interface material when the microsomal pellets were layered on top and then centrifuged. For example, in a test experiment, 'top' loaded material gave a specific ATPase activity of 3.6 at the 27—34% interface, 'bottom' loaded material gave a specific activity of 31.0.

The activity was stable for months when frozen in suspending buffer (see Materials and Methods) plus 0.25 M sucrose at —20°C, and for days at 4°C.

pH optimum. The pH optimum was 7.5—7.7. A plot of activity vs. pH showed a symmetrical, broad curve, with 50% of activity observable at pH 6.0 and 9.5.

Effects of anions on ATPase activity. $NaHCO_3$, $NaHSO_3$ (both 20 mM), NaSCN (10 mM), and NaI (5 mM) had no effect on ATPase activity. NaN_3 (1 mM) inhibited by 8%. NaF (10 mM) inhibited by 40%. The anion-channel inhibitors, SITS (acetamidoisothiocyanostilbene-2,2'-disulfonic acid) and pyridoxal phosphate (100 μM and 5 mM, respectively) inhibited ATPase activity only by 3 and 9%.

Requirement of divalent cations for ATPase activity. Ca^{2+} or Mg^{2+} was equally effective in promoting ATPase activity in the presence of 5 mM ATP (Fig. 5). At a saturating level of either Mg^{2+} or Ca^{2+}, addition of the other cation had no effect. Mn^{2+} would not substitute for Mg^{2+} or Ca^{2+}. At 10 mM Mg^{2+}, 1 mM Zn^{2+} and 10 mM Zn^{2+} caused 70 and 90% inhibition, respectively.

Tests of possible activating effects of cation. Enzyme was first dialyzed exhaustively at 4°C to remove Na^+ and K^+. NaCl (100 mM), KCl (20 mM) added in combination gave 3—5% stimulation of ATPase activity. This increment was fully inhibited by ouabain, and was therefore referable to ($Na^+ + K^+$)-ATPase. K^+ alone had no effect. EGTA (1 mM), ruthenium red (10 or 100 μM) or $LaCl_3$ (10 or 100 μM) gave zero or low inhibition. The experiments showed, therefore, that ($Na^+ + K^+$)-ATPase, ($H^+ + K^+$)-ATPase and Ca^{2+}-acti-

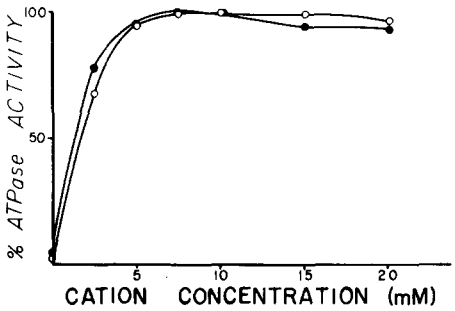

Fig. 5. Cation dependence of plasma membrane ATPase activity. $MgCl_2$ (○———○) or $CaCl_2$ (●———●) was present together with other assay constituents as described in Materials and Methods.

vated ATPase are minor components or absent from this pancreas plasma membrane fraction. The bulk of the activity may be referred to as (Ca^{2+} + Mg^{2+})-ATPase activity.

Effects of known ATPase enzyme inhibitors. A series of inhibitors with well characterized effects on known membrane ATPases were tested on the pancreatic plasma membrane ATPase activity (Table II). This activity was not inhibited by oligomycin or the specific mitochondrial ATPase inhibitor protein under conditions where pancreatic mitochondrial ATPase was inhibited strongly (see above). Data in Table II serve further to distinguish the pancreatic plasma membrane ATPase from mitochondrial ATPase. Thus, dicyclohexylcarbodiimide (10 μM), 4-chloro-7-nitrobenzofurazan, tributyl tin chloride and quercetin all give strong inhibition of mitochondrial ATPase. Furthermore, 4-chloro-7-nitrobenzofurazan strongly inhibits (Na^+ + K^+)-ATPase [26], as do *N*-ethylmaleimide and vanadate [27,28]. Ca^{2+}-ATPase of sarcoplasmic reticulum and red blood cells is also strongly inhibited by *N*-ethylmaleimide [29,30].

TABLE II

EFFECTS OF INHIBITORS ON PANCREATIC PLASMA MEMBRANE ATPase ACTIVITY

Samples of enzyme (5—20 μg) were preincubated at 37°C for 10 min with the concentration of inhibitor shown. The preincubation medium contained 75 μmol of Tes-Tris (pH 7.5) and 100 μg bovine serum albumin in a volume of 0.9 ml. After preincubation, the assay was started by addition of 100 μl containing 5 μmol ATP and 10 μmol $MgCl_2$. Other conditions were as described in Materials and Methods.

Inhibitor	Concentration (μM)	% inhibition of ATPase activity
Dicyclohexylcarbodiimide	10	0
	200	70
p-Chloromercuribenzoate	500	65
N-Ethylmaleimide	2×10^3	0
4-Chloro-7-nitrobenzofuran	10	0
	100	15*
Tributyl tin chloride	0.1	0
	1.0	6
	20	22
	50	9
	100	0
Quercetin	66	12
Sodium orthovanadate	10	0
	100	2

* Preincubated for 30 min.

The most potent inhibitors of the plasma membrane (Ca^{2+} + Mg^{2+})-ATPase activity were *p*-chloromercuribenzoate and dicyclohexylcarbodiimide at 200 μM.

Ionophores and uncoupling agents. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), gramicidin, and valinomycin (in the presence or absence of 10 mM KCl) were without effect on ATPase activity at concentrations of up to 10 μM. These results may be due to the membrane preparation being leaky to small ions, however. Electron micrographs of stained sectioned samples showed smooth membranous bags and sheets with gap junctions present. Negatively stained samples appeared as unsealed vesicles (Martin, S.S. and Perrachia, C., unpublished observations).

Kinetics and nucleotide specificity. The K_m for ATP at 10 mM $MgCl_2$ (constant concentration) was 0.3 mM as calculated from linear Lineweaver-Burk plots. At a fixed Mg : ATP ratio of 0.5, double-reciprocal plots of $1/v$ against 1/total [ATP] were curved. Double-reciprocal plots of $1/v$ against 1/[MgATP] (calculated as in Ref. 31) again gave a straight line from which a K_m value of 0.1 mM for MgATP was calculated. ATP, GTP, UTP, ITP, (5 mM) were hydrolyzed at approximately equal rates. ADP was hydrolyzed at 27% of the ATP hydrolysis rate *. AMP was hydrolyzed at only 0.5% of the ATP hydrolysis rate, and *p*-nitrophenyl phosphate was hydrolyzed at less than 0.5% of the ATP hydrolysis rate at acid, neutral or alkaline pH. Glucose 6-phosphate was hydrolyzed at negligible rates. Therefore, the high rate of ATP hydrolysis in the plasma membrane fraction is due to nucleoside triphosphatase activity and is not referable to contaminating phosphatase activity.

Reaction products of ATP hydrolysis were ADP and P_i. Reaction products from ADP were AMP and P_i. No cyclic AMP or PP_i was discernible. This was found using Eastman TLC cellulose plates in a solvent system containing ethyl acetate/formic acid/ethanol/water/dimethylsulfoxide (15 : 10 : 15 : 50 : 10. v/v).

Phosphorylation of the plasma membrane fraction by γ-[^{32}P]ATP. An acid-stable phosphorylated form of the membrane fraction was prepared by incubating with γ-[^{32}P]ATP as described in the legend to Fig. 6, upper. Preliminary experiments showed that the acid-stable ^{32}P bound increased rapidly over the first 20 s of incubation and reached a stable plateau at 30 s. The amount of ^{32}P bound was similar in membrane fractions obtained from either Teflon pestle or Polytron homogenate. NaCl (100 mM) increased the amount of bound ^{32}P formed by 50% and KCl (20 mM) reduced the amount, but the effect of KCl was small (15%). Addition of non-radioactive ATP during the incubation (Fig. 6, lower) caused only a slight fall in the amount of bound ^{32}P. The results, therefore, suggest that the acid-stable bound ^{32}P in these experiments is not an intermediate compound in ATPase activity. Preparations of (Na^+ + K^+)-ATPase, (H^+ + K^+)-ATPase and Ca^{2+}-ATPase of similar or lower ATPase activity to the pancreatic plasma membrane ATPase fractions form far greater amounts of acid-stable ^{32}P intermediate and addition of non-radioactive ATP dissipates

* The adenylate kinase inhibitor, adenylyl(3′-5′)adenosine (Ap5A) did not affect ADP hydrolysis rates at 0.1—1 mM (Hamlyn, J.M., and Senior, A.E., unpublished observations).

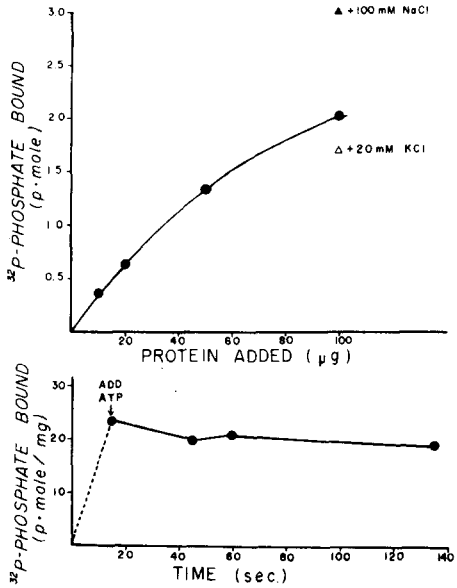

Fig. 6. Formation of acid-stable phosphorylated intermediate from [γ-^{32}P]ATP in plasma membrane preparations. Upper, 100 μl containing variable amounts of protein (Teflon pestle homogenization) was incubated for 30 s at 22°C in 1 ml containing 75 mM Tes-Tris (pH 7.3), 1.0 mM $MgCl_2$, 10 μM [γ-^{32}P]-ATP (4—20 · 10^5 cpm/nmol). The reaction was stopped by addition of 2 ml ice-cold 5% (w/v) $CHCl_3COOH$/10 mM H_3PO_4/1 mM ATP. Bovine serum albumin (1 mg) was added and the protein was sedimented by centrifugation for 10 min at 0°C (1800 X g). The pellet was resuspended and vortexed three times in the acid-quench solution centrifuging to sediment protein each time. Finally, the pellet was dissolved in 2 ml of 3% SDS and radioactivity and protein estimated. Controls of two kinds were run. One had additional ATP (1 mM, non-radioactive) present during the incubation. The other had 100 μl buffer substituted for 100 μl sample. They gave similar background results, equal to about 10% of the maximal amount of membrane phosphorylation. Lower, 50 μg of membrane protein (Polytron homogenization) were incubated as above but for 15 s, at which time non-radioactive ATP-Tris salt was added to give 0.1 mM final concentration. Samples were then analyzed for acid-stable ^{32}P bound at varying intervals after the addition of ATP-Tris.

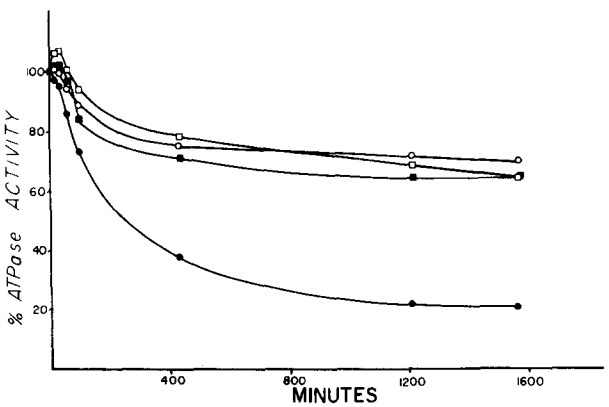

Fig. 7. Inactivation of plasma membrane ATPase activity by *p*-fluorosulfonylbenzoyl-5'-adenosine (FSBA). The reaction mixture contained 40 mM Tris-H_2SO_4 (pH 7.0), 1 mM EDTA, 0.25 M sucrose and 1 mg protein in 0.5—1.0 ml at 22°C. (○———○), control, 7 μl dimethysulfoxide (DMSO) added; (●———●), 2 mM (final) FSBA in 7 μl DMSO; (□———□), 10 mM ATP final concentration and 7 μl DMSO added; (■———■), 2 mM FSBA, 10 mM ATP and 7 μl DMSO. Samples were removed at times indicated for assay of ATPase activity.

the intermediate due to turnover. The acid-stable ^{32}P bound here is possibly bound to a protein kinase or other membrane protein. The lack of a 'phosphorylated intermediate' seems consistent with the ineffectiveness of vanadate as an inhibitor. Willsky [32] has recently pointed out that vanadate seems to inhibit only those ATPase enzymes which form a phosphorylated intermediate during turnover. We have also confirmed that vanadate has little effect on mitochondrial ATPase (4% inhibition at 10 μM, 18% at 100 μM) which does not form a phosphorylated intermediate, as far as is known.

Reaction of the pancreatic plasma membrane fraction with p-fluorosulfonylbenzoyl-5'-adenosine (FSBA). FSBA has been shown to be a suitable affinity label for ATP binding sites [33,34]. It inhibited plasma membrane ATPase activity markedly as shown in Fig. 7. Addition of excess ATP along with FSBA protected the enzyme against inactivation. 2-Mercaptoethanol added after inactivation did not reverse the inactivation. This, therefore, is further evidence that the bulk of the ATPase activity in these membranes is referable to a nucleotide triphosphatase. The membrane fraction was then incubated with *p*-fluorosulfonyl[^{14}C]benzoyl-5'-adenosine ([^{14}C]FSBA) as described in Fig. 8. The membrane fraction became radioactively labelled and SDS gel electrophoresis of the labelled membrane fraction showed that the radioactivity was localized primarily in one band of M_r approx. 35 000. The membrane fraction showed about ten prominent bands when similar gels were run and stained with Coomassie blue, with the bands having M_r values from 26 000 up to about 100 000.

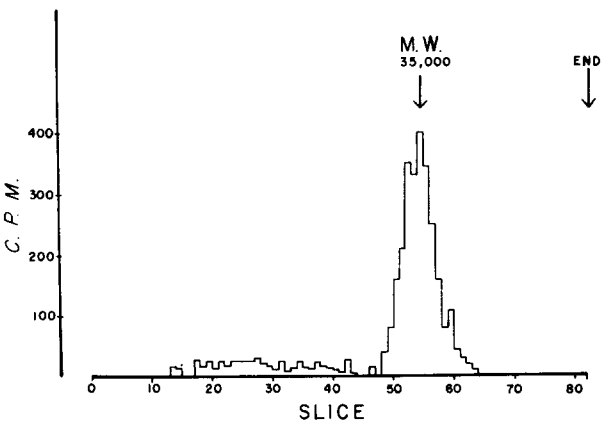

Fig. 8. SDS gel electrophoresis of plasma membrane ATPase activity after inactivation by *p*-fluorosulfonyl[^{14}C]benzoyl-5'-adenosine ([^{14}C]FSBA). Reaction conditions were similar to those in Fig. 7 except that [^{14}C]FSBA ($1.1 \cdot 10^6$ dpm/μmol) was used. Protein concentration was 2 mg/ml, total volume 0.5 ml. At 20 h the reaction was stopped by addition of 25 ml of ice-cold buffer containing 0.25 M sucrose, 40 mM Tris-H_2SO_4 (pH 7.0), 1 mM EDTA and 1% 2-mercaptoethanol. The samples were mixed and centrifuged (165 000 × 60 *g* · min). Pellets were redissolved in 0.4 ml of 1% SDS, 10 mM sodium phosphate (pH 7.1), 1% 2-mercaptoethanol and 0.2 M sucrose, heated at 100°C for 15 min and dialyzed 18 h against the same buffer, containing 0.1% 2-mercaptoethanol. 200-μg samples were run on 5% SDS gels. After electrophoresis, gels were sliced directly and counted (see Materials and Methods). The histograms shown are corrected for some smeared radioactivity seen in slices 20—38 in control samples containing [^{14}C]FSBA but no protein. Fixation of the gels in 10% trichloroacetic acid for 15 h before slicing removed all of this smeared radioactivity, but also caused appreciable loss (approx. 50%) of the radioactivity in slices 51—54.

Lipid composition of plasma membrane fractions. Extraction of membrane preparations by chloroform/methanol (1 : 1) showed that it contained 0.74 mg phospholipid per mg protein. The phospholipids contained (on a molar basis) 47% phosphatidylcholine, 24% phosphatidylethanolamine, 13% phosphatidylserine and 15% sphingomyelin. No cardiolipin was found.

Discussion

We have surveyed the ATPase activities present in the membranes derived from whole rat pancreas homogenate in order to relate the HCO_3^- transport function of this gland to possible ion pumps. We used two different, but commonly used, homogenization methods in order to obviate any error or bias. In fact, both homogenization methods yielded similar results.

In strong support of the earlier conclusions of van Amelsvoort et al. [11], we have found no HCO_3^--sensitive ATPase activity in any membrane fraction other than mitochondria. Therefore, models invoking a bicarbonate-ATPase as an ion pump in pancreas plasma membrane are contradicted by our evidence.

$(Na^+ + K^+)$-ATPase, known to be involved at least indirectly in HCO_3^- and fluid secretion in pancreas as discussed in Introduction, was present in the plasma membrane fractions, but its contribution to total ATPase activity was relatively minor. The major ATPase activity in plasma membrane fractions was a remarkably high Ca^{2+}- or Mg^{2+}-requiring activity. No distinct Ca^{2+}-activated or $(H^+ + K^+)$-activated ATPase activity was seen. Whether the $(Ca^{2+} + Mg^{2+})$-ATPase activity is referable to one distinct entity (a unique $(Ca^{2+} + Mg^{2+})$-ATPase enzyme) cannot be decided from our data. However, the experiments with *p*-fluorosulfonylbenzoyl-5′-adenosine suggest that this might be so, and that a component of M_r 35 000 contributes to the ATP-binding site. Besides the $(Ca^{2+} + Mg^{2+})$-ATPase and $(Na^+ + K^+)$-ATPase activities in the plasma membrane fraction, the other membrane ATPase activities which we found in pancreas were (a) the mitochondrial ATPase and (b) an ATPase activity in low-density (ρ = 1.08—1.09) membrane fractions derived from zymogen granule membrane pellets, but not seen in other pellets obtained after differential centrifugation. This activity is probably the same as that described by Harper et al. [17], and may be involved in zymogen granule membranes function.

Thus, in terms of possible transport functions across plasma membrane, the high $(Ca^{2+} + Mg^{2+})$-ATPase activity is of considerable interest. Similar activity has been noted before [35—37] but the activity in the preparation reported here is approx. 30-times higher. Previous workers, noting that Ca^{2+} may act as cofactor, have speculated that the ATPase activity may be linked to Ca^{2+} transport and may regulate calcium concentration in pancreas cells [38]. However, it should be pointed out that mitochondrial ATPase may use several divalent cations as cofactor, including Ca^{2+}, although the primary ion transported is H^+ [39,40]. Thus, such a speculation is not justified unless Ca^{2+}-activation or transport is seen. As yet, there is no firm evidence as to whether the $(Ca^{2+} + Mg^{2+})$-ATPase activity is linked to transport across the plasma membrane. Moreover, there is no evidence regarding the cellular origin of the plasma membrane fraction prepared here (e.g., acinar as compared to ductal;

endocrine as compared to exocrine). Current views favor the ductal cells as the primary site of HCO_3^- secretion.

However, these are problems which can now be attacked. Localization of the $(Ca^{2+} + Mg^{2+})$-ATPase activity, further characterization and purification of the activity, and examination of possible transport functions in sealed membrane preparations are all feasible objectives which we hope will be facilitated by the high activity preparation now available. So far, our data concerning the pattern of inhibitions by well known inhibitors of ATPase enzymes, the lack of a phosphorylated intermediate, the behavior towards cations and anions and the reaction with *p*-fluorosulfonylbenzoyl-5′-adenosine all suggest, albeit tentatively, that we are dealing here with a previously uncharacterized distinct membrane ATPase.

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