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CHARACTERIZATION OF PROTON-TRANSPORTING MEMBRANES FROM RESTING PIG GASTRIC MUCOSA

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Membrane vesicles were purified from resting corpus mucosa of pig stomachs by velocity-sedimentation on a sucrose-Ficoll step gradient. Two vesicular fractions containing the $(H^+ + K^+)$-ATPase were obtained. One fraction was tight towards KCl, the other was leaky. At 21°C maximal $(H^+ + K^+)$-ATPase activities of 0.8 and 0.4 $\mu mol \cdot mg^{-1} \cdot min^{-1}$, respectively, were observed in lyophilized vesicles. The vesicles contained a membrane-associated carbonic anhydrase, the activity of which was in 100-fold excess of the maximal ATPase activity. Both vesicular fractions were rich in phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and cholesterol. The characteristics of ion permeability and transport in the tight vesicles were in agreement with corresponding data for vesicles of a tubulovesicular origin in the parietal cell. Measurement of the rate of K^+ uptake into the vesicles was based on the ability of K^+ to promote H^+ transport. The uptake was slow and dependent on the type of anion present. The effectiveness in promoting uptake of K^+ by anions was $SCN^- > NO_3^- > Cl^- \gg HCO_3^- > SO_4^{2-}$. Uptake of K^+ was much more rapid at alkaline pH than at neutral or at acidic pH. Addition of CO_2 at alkaline pH strongly stimulated the rate of H^+ accumulation in the vesicles. The initial part of this stimulation was sensitive to acetazolamide, an inhibitor of carbonic anhydrase. A model how the $(H^+ + K^+)$-ATPase and the carbonic anhydrase may co-operate is presented. It is concluded that membrane vesicles of a tubulovesicular origin can produce acid.

Introduction

The parietal cells of the gastric mucosa secrete hydrochloric acid. In the resting state, these cells contain a large number of tubulovesicles which upon stimulation are transformed into secretory canaliculi in communication with the plasma membrane on the luminal side [1–3]. Isolated vesicular membranes from the gastric mucosa contain the $(H^+ + K^+)$-ATPase [4–8]. The ATPase constitutes the hydrogen-ion pump of the stomach. The secretory state of stomachs used as starting material for preparation of gastric vesicles had not been well defined until Wolosin and Forte recently purified stimulation-associated vesicles derived from secretory canaliculi and their microvilli [9]. These vesicles exhibit a high permeability for KCl as compared with vesicles of a tubulovesicular origin. One specific aim of the present investigation was to characterize gastric vesicles which had been purified from resting pig stomachs as defined from electron microscopic studies. The

Abbreviations: Mes, 4-morpholineethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; SDS, sodium dodecyl sulphate.

present report describes a simple and rapid purification on a large scale of gastric membrane vesicles, which accumulate acid in an ATP- and K^+-dependent process. The ability of the vesicles to accumulate H^+, their permeability for K^+ and various anions is described, as well as their protein and phospholipid composition. The vesicles contain a carbonic anhydrase of high activity. This activity has previously been demonstrated histochemically in the secretory canaliculi of the parietal cell [10]. The present investigation demonstrates the presence of carbonic anhydrase in membranes of tubulovesicular origin. The kinetic results suggest a co-operative action of the $(H^+ + K^+)$-ATPase and the carbonic anhydrase.

Materials and Methods

Materials. Tris salt of ATP was prepared by passing its disodium salt (Sigma) over a Dowex 50W-X8 column in the Tris form [11]. Aminopyrine was obtained from Aldrich-Europe (Belgium). Acridine orange was from Merck. ^{14}C-labeled aminopyrine and [γ-^{32}P]ATP were purchased from New England Nuclear. Ficoll 70 was obtained from Pharmacia Fine Chemicals. Fluoroinert 70 was from 3M Company. Other chemicals were of analytical grade and commercially available. Destilled, deionized water was used throughout the experiments.

Preparation of vesicular membranes from gastric mucosa. Pigs were fasted overnight before slaughter at a local slaughter house. Vesicular membranes were prepared from their stomachs which were obtained fresh and put on ice. Part of the preparation is based on a previously described procedure [7]. In a typical preparation the gastric mucosa of four stomachs was flooded with cold saturated NaCl for 3–5 min. The superficial cells, cell debris plus the mucus was wiped off with the edge of a plastic ruler and with paper towels. The mucosa was scraped off. About 100 g scrapings was divided into portions of 10 g and homogenized in 0.25 M sucrose with seven strokes in a Potter-Elvehjem Teflon-glass homogenizer. Total volume was 600 ml. The homogenate was centrifuged at $20\,000 \times g$ for 40 min. The pellet was discarded. The supernatant was centrifuged at $75\,000 \times g$ for 1 h. The resulting microsomal pellet was homogenized in 30 ml of 0.25 M sucrose. Aliquots of 15 ml of this were transferred to 100 ml centrifuge tubes and layered on top of step gradients, from the bottom comprising 25 ml 37% sucrose (w/v) and 45 ml 7.5% Ficoll (w/v) in 0.25 M sucrose. The tubes were centrifuged at $75\,000 \times g$ for 1 h in a 6×100 ml MSE angle rotor at 4°C. The gradient was then fractionated by pumping Fluoroinert 70 through a narrow tubing in a fractionating cap down to the bottom of the tube. Fractions were collected from top through a center hole in the fractionating cap. The yield of vesicles in a typical preparation was about 50–75 mg of protein. The whole preparation procedure was performed within 5 h from slaughter to the final vesicles. In order to maintain a stable vesicular structure for a long period of time, vesicles were frozen at −70°C under nitrogen. They could then be kept for several months without decrease of $(H^+ + K^+)$-ATPase activity or loss of their proton transporting ability. Addition of 1.3% (v/v) glycerol to the vesicle preparation before freezing prevented loss of the proton transporting ability even after repeated freezing and thawing. No change in the kinetics for the uptake of KCl into these vesicles was detectable.

Electron microscopy. Directly after slaughter, samples of about 1 cm^2 of fresh gastric corpus mucosa were put in 2.5% glutaraldehyde in 0.125 M cacodylate buffer (pH 7.2) and fixed overnight at 4°C. Vesicles isolated from fresh gastric mucosa were diluted 10-fold in the same glutaraldehyde-containing buffer as above and then centrifuged at $75\,000 \times g$ for 30 min. The vesicles were washed twice and fixing was continued overnight at 4°C. They were then dehydrated in ethanol and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate [12]. The electron microscope was a JEOL-100B.

Assay of ATPase activity. ATPase was assayed as the release of $^{32}P_i$ from [γ-^{32}P]ATP [13]. Incubation volume was 1 ml. The standard assay medium comprised 2 mM $MgCl_2$/10 mM KCl/1 mM [γ-^{32}P]ATP/10 mM Hepes adjusted with Tris (pH 7.4).

Phosphoenzyme intermediate of $(H^+ + K^+)$-ATPase. Maximal amount of phosphoenzyme intermediate of $(H^+ + K^+)$-ATPase was measured at 5 μM ATP/2 mM $MgCl_2$/40 mM Tris-

HCl, (pH 7.4) [14]. In a typical preparation this was out 1.5 nmol/mg of vesicular protein.

Assay of carbonic anhydrase. Carbonic anhydrase was assayed by a modification of the method described by McIntosh [15]. The rate of hydration of CO_2 (5% CO_2/95% O_2), constantly bubbling through the reaction mixture, was measured at 0°C in the presence of 50 mM NaCl/5 mM sodium phosphate (pH 7.0). Constant pH was maintained by means of a pH-stat (Metrohm).

Hydrogen ion transport in gastric vesicles. The ability of the vesicles to accumulate acid in the presence of ATP was measured by either a weak base accumulation technique with amino [^{14}C]pyrine (pK_a 5.0) and subsequent filtration [16], or with a spectrophotometric method using the dye acridine orange [17]. With the former method about 50 μg of the vesicular protein was incubated in 450 μl 2 mM $MgCl_2$/10 mM Hepes adjusted to pH 7.4 with Tris. The vesicles were preincubated with KCl as indicated. The reaction was started by the addition of 50 μl buffer comprising of 10 mM ATP/50 μM amino[^{14}C]pyrine. Isoosmotic pressure was maintained with sucrose unless otherwise indicated. The reaction was stopped by the addition of 5 ml of an ice-cold solution comprising 3 mM EDTA/150 mM choline chloride/5 mM Hepes-Tris (pH 7.4). The vesicles were then collected on a Millipore filter of 0.45 μm pore size. The filter was washed once in another 5 ml of the stopping solution. The stopping-washing procedure was completed within 1 min. The radioactivity retained on the filter was counted in a liquid scintillation counter.

In the spectrophotometric assay of hydrogen-ion transport, a standard spectrophotometer equipped with a recorder was used. In a cell about 10 μg of vesicular protein per ml was mixed with acridine orange at the final concentration of 10 μM and ionic composition as indicated. The reaction was started by the addition of 0.5 mM ATP and the decrease of absorbance at 493 nm was registered. Acridine orange (A) responds to ΔpH across membranes and the plot of log (A_{in}/A_{out}) vs. ΔpH gives a straight line. The non-ideal calibration curves for acridine orange makes this technique non-quantitative [17]. The initial rate of change in absorbance reflects the rate of proton transport, and since one extravesicular proton is exchanged for one intravesicular potassium ion [18] the maximal change in absorbance is related to the initial concentration of intravesicular potassium ions.

Requirement of intravesicular K^+ for H^+ transport; uptake of KCl in vesicles. The uptake of KCl in gastric vesicles was very slow ($t_{1/2}$ was 1.5 h at pH 7.5 and 21°C). The permeation of K^+ across the vesicular membranes was measured by means of its ability to stimulate the rate and extent of acid accumulation in the vesicles. The more KCl present in the vesicles, the more acid was accumulated. Rb^+ is as potent as K^+ in activating the ($H^+ + K^+$)-ATPase [19]. The results obtained with the present indirect technique using acid accumulation as a measure of the K^+ content, and uptake into the vesicles, are in agreement with the kinetics of $^{86}Rb^+$ uptake into gastric vesicles [19]. The technique for measuring K^+ uptake appears therefore suitable for the aims of the present investigation.

Lipid analysis. The lipids were extracted with chloroform/methanol (1 : 1, v/v). A chloroform phase containing the lipids was obtained after the addition of 0.5 vol. 0.15 M NaCl. Phospholipids were separated on thin layers of silica gel impregnated with magnesium acetate [20]. The plates were developed in two dimensions, first in chloroform/methanol/25% ammonia/water, 65 : 35 : 5 : 2 (v/v) and then in chloroform/methanol/acetic acid/water, 60 : 30 : 8 : 5 (v/v). After development, the plates were sprayed with dichlorofluorescein and the lipid spots were located under ultraviolet light. Identification of the lipid fractions and determination of their phosphate content was done as previously described [21]. Cholesterol was determined enzymatically [22].

Protein analysis. Protein was assayed according to the method of Lowry et al. [23], or according to the method of Bradford [24]. The protein was dissolved in 1 M NaOH before analysis. The sample was neutralized before the addition of Bradford's reagent. The two methods gave identical results. Human serum albumin was used as a standard. The content of nitrogen of this albumin was determined with a micro-Kjeldahl method.

Results

Electron microscopy of corpus gastric mucosa. Pig stomachs freshly obtained at a local slaughter house were the starting material for isolation of gastric vesicles. Several stomachs were examined in order to gain information about their morphology and the secretory state. Part of a representative parietal cell of these stomachs is shown in Fig. 1. The morphology indicated that the parietal cells were in a resting, unstimulated state. The cells contained a large number of tubulovesicular structures and mitochondria.

Fractionation of gastric vesicles. Methods of purification of membrane vesicles of various homogeneity from the gastric mucosa have been described previously [4–8] but the secretory state of the mucosa was not reported. The aim of the present investigation was to obtain homogeneous vesicles consisting of as unmodified membranes as possible with respect to possible regulatory proteins and interacting membrane bound enzyme systems. An angle rotor in an ultracentrifuge provided the means of easy and rapid isolation of a vesicular fraction (Fig. 2A). This fraction contained $(H^+ + K^+)$-ATPase and was collected from the top of a sucrose-Ficoll cushion in the centrifuge tube. A second vesicular fraction, containing $(H^+ + K^+)$-ATPase, could be obtained at the interphase between the sucrose-Ficoll and a 37% sucrose cushion (Fig. 2B). The former vesicular fraction will be referred to as peak I and the latter as peak II. The vesicular fraction of peak II was only partly characterized. Membranes pelleted at the bottom of the tube exhibited low ATPase activity

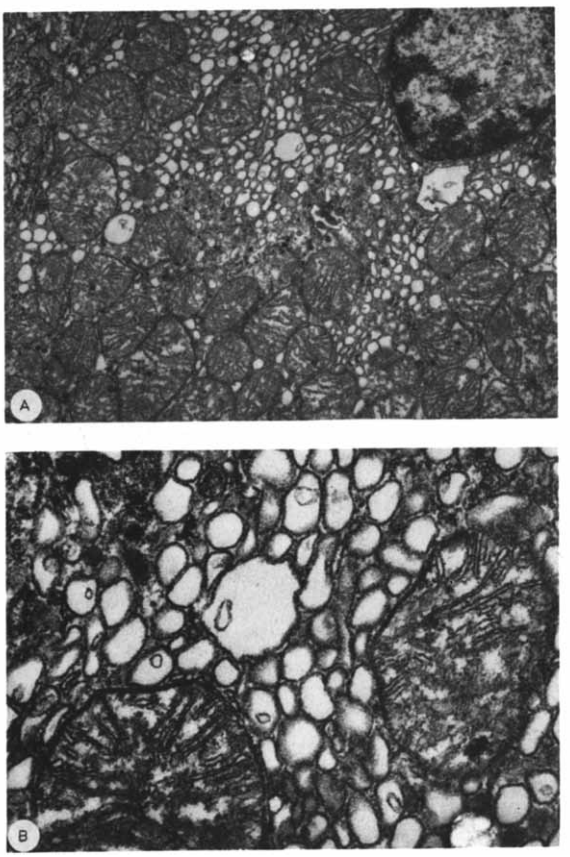

Fig. 1. A parietal cell of pig gastric mucosa. (A) An overall view of a representative parietal cell containing nucleus, mitochondria and tubulovesicles (magnification 5600×); (B) a detail of a parietal cell with two mitochondria and tubulo-vesicular structures (magnification 9000×). For details see Materials and Methods.

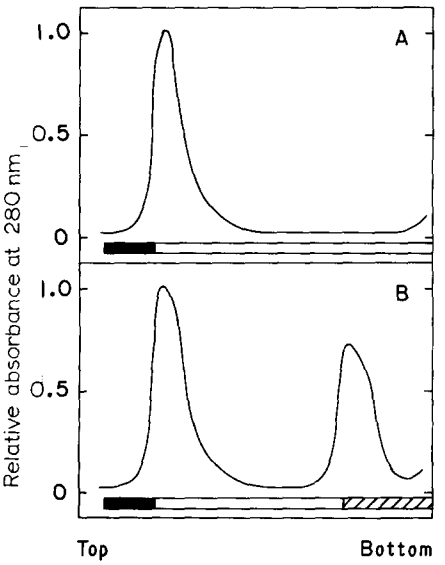

Fig. 2. Fractionation of gastric vesicular membranes on a sucrose-Ficoll step gradient. Microsomal membranes (black bar) were layered on top of 70 ml 7.5% Ficoll (w/v)/0.25 M sucrose (open bar) (A). Alternatively, microsomal membranes were layered on top of 45 ml sucrose/Ficoll solution and in addition there was 25 ml 37% sucrose (hatched bar) in the bottom of the centrifuge tube (B). Fractions were collected from the top of the gradient by pumping high density Fluoroinert 70 through a capillary tubing to the bottom of the centrifuge tube. Protein was measured continuously with a Pharmacia Dual Path Monitor UV-2 at 280 nm.

TABLE I

K^+ – STIMULATION OF THE ATPase ACTIVITY IN FRESH AND IN LYOPHILIZED FRACTIONS FROM A REPRESENTATIVE SUCROSE-FICOLL STEP GRADIENT

The ATPase was assayed at 21°C in the presence of 2 mM $MgCl_2$/10 mM Hepes-Tris (pH 7.4), in the absence or presence of 10 mM KCl. Isoosmotic pressure was maintained with sucrose. Maximal activity was obtained with lyophilized membranes from peak I. The activity was 0.8 $\mu mol \cdot mg^{-1} \cdot min^{-1}$. This activity was taken as unity.

Treatment of membrane fractions	Relative ATPase activity		
	Peak I	Peak II	Pellet
Fresh	0.08	0.08	0.09
Fresh + K^+	0.10	0.24	0.12
Lyophilized	0.16	0.13	0.14
Lyophilized + K^+	1.00	0.49	0.16

only and were not characterized further. The (H^+ + K^+)-ATPase activity of fresh peak I was low compared to lyophilized peak I (Table I). Lyophilization apparently increased the permeability for KCl 10-times. Fresh peak II exhibited only about 50% of the activity of lyophilized peak II. An electron microscopic picture of the vesicular preparation (peak I) is presented in Fig. 3A. Essentially all the membranes appeared to exist in vesicular form.

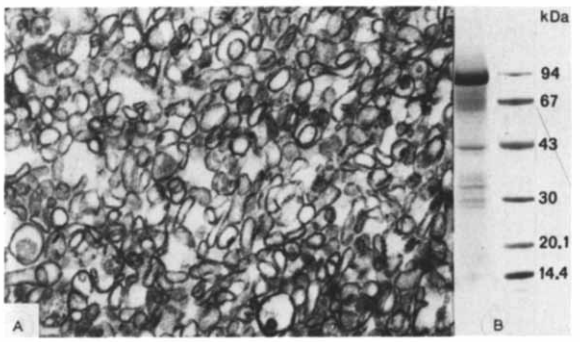

Fig. 3. Vesicles from pig gastric mucosa. (A) Vesicles (peak I) from a sucrose/Ficoll gradient were fixed in 2.5% glutaraldehyde and stained as described under Materials and Methods (magnification 8500×); (B) polyacrylamide gel electrophoresis of gastric vesicles. About 50 μg of vesicular protein was dissolved in 2% SDS and 50 μM dithiothreitol in an electrophoresis buffer comprising 25 mM Tris base/192 mM glycine/0.1% SDS. Reference proteins were phosphorylase *b* (94 kDa); albumin (67 kDa); ovalbumin (43 kDa) carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa). Electrophoresis was run in a 2.5–15% polyacrylamide gradient gel for 18 h at 10 mA.

Phospholipid composition of gastric vesicles. The phospholipid composition of gastric vesicles is presented in Table II. Phosphatidylethanolamine, phosphatidylcholine and sphingomyelin were the most dominating phospholipids in both peak I and II. Peak I contained less phosphatidylcholine and more sphingomyelin than peak II, although the sum of these two choline-containing lipids was the same in the two membrane fractions. Lysophosphatidylethanolamine and lysophosphatidylcholine were found in highest amounts in peak II, while phosphatidylinositol was highest in peak I. The total lipid phosphorus content of the membranes was 0.8 μmol lipid phosphorus per mg protein in both peak I and II. The phospholipid/cholesterol molar ratio was 1.53 ± 0.04 (mean ± S.D., $n = 4$) in peak I and 1.98 ± 0.04 (mean ± S.D., $n = 3$) in peak II.

Assay of ($Na^+ + K^+$)-ATPase in gastric vesicles. The lipid composition of the vesicles was similar to that of plasma membranes [25,26]. Therefore, several attempts were made to detect a ($Na^+ + K^+$)-ATPase activity as a marker of plasma membranes in the gastric vesicles. Its activity was mea-

TABLE II

PHOSPHOLIPID COMPOSITION OF GASTRIC VESICLES

Vesicular membranes (peak I and II, Fig. 2) in different preparations were analyzed for phospholipids as described in Materials and Methods. Cardiolipin was not detectable (less than 1%). Total amount of lipid-P was 0.83 ± 0.05 μmol/mg protein for peak I (mean ± S.D., $n = 6$) and 0.80 ± 0.06 μmol/mg protein for peak II (mean ± S.D., $n = 6$).

Phospholipid	Percentage of lipid P (mean ± S.D.)	
	Peak I ($n = 5$)	Peak II ($n = 3$)
Phosphatidylcholine	22.1 ± 1.5	29.0 ± 2.6
Phosphatidylethanolamine	31.2 ± 1.0	30.0 ± 0.7
Phosphatidylserine	12.8 ± 1.2	12.7 ± 4.3
Phosphatidylinositol	4.2 ± 0.6	1.2 ± 0
Sphingomyelin	27.1 ± 1.8	20.8 ± 0.4
Lysophosphatidylcholine	0.7 ± 0.4	1.9 ± 0.6
Lysophosphatidylethanolamine	1.9 ± 0.4	3.8 ± 0.9

sured as a Na^+- plus K^+-stimulated and ouabain-inhibitable ATPase, or as an ouabain-inhibitable *p*-nitrophenylphosphatase. No $(Na^+ + K^+)$-ATPase was detected (less than 5 nmol/mg per min).

Protein composition of gastric vesicles. Gastric vesicular membranes are reported to contain mainly one protein band of about 110 [7] or of 100 kDa [27] which contains the $(H^+ + K^+)$-ATPase. These values of molecular mass appear to be slightly overestimated. The present preparation of vesicles contains one major protein band corresponding to a molecular mass of 92–94 kDa, but in addition there are several other proteins which are visualized by overloading a high-resolving polyacrylamide gel (Fig. 3B). No difference between the protein patterns of peak I and II was detected.

Carbonic anhydrase of gastric vesicles. The gastric mucosa contains high amounts of carbonic anhydrase [10,28]. The gastric vesicles (peak I and II) described in this paper contain a carbonic anhydrase of high activity ranging from 70–120 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ at 0°C. Part of the carbonic anhydrase might have been trapped inside the vesicles during the preparation, or was adsorbed to the vesicular membrane. Therefore vesicles (peak I) were diluted 20-times with distilled water and subsequently frozen in order to disrupt the vesicular structure. After thawing the sample and sedimenting the membranes by centrifugation, about 80% of the original activity was released in the supernatant. The activity remaining in the membranes was not released by washing of the membranes in 0.3 M KCl or 0.1% Triton X-100. Thus carbonic anhydrase with an activity of 20 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ at 0°C appeared to be firmly associated with the vesicular membrane. Both the firmly associated as well as the soluble enzyme was inhibited by acetazolamide with a K_i of $2 \cdot 10^{-8}$ M (Fig. 4). SCN^-, a classical inhibitor of carbonic anhydrase, gave a K_i of $2 \cdot 10^{-3}$ M.

Acid accumulation in gastric vesicles. Maximal accumulation of aminopyrine in vesicles (peak I) preloaded overnight with 150 mM KCl was ob-

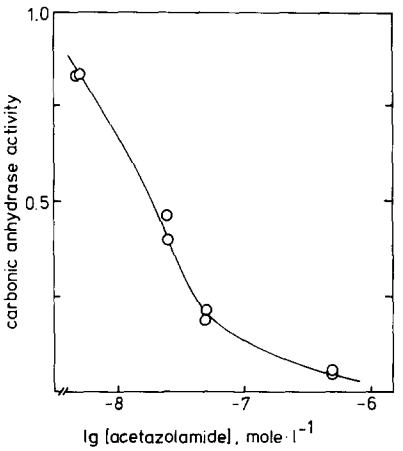

Fig. 4. Inhibition of carbonic anhydrase activity in gastric vesicles by acetazolamide. The hydration of CO_2 was measured at 0°C by means of a pH-stat technique. About 100 μg of vesicular protein (peak I) was incubated in a volume of 5 ml. The reaction mixture was comprised of 50 mM NaCl/5 mM sodium phosphate (pH 7.0). The mixture was continuously bubbled with 5% CO_2 plus 95% O_2. The H^+ produced was titrated with KOH in order to keep the pH constant. Unity represents the carbonic anhydrase activity in the absence of inhibitor.

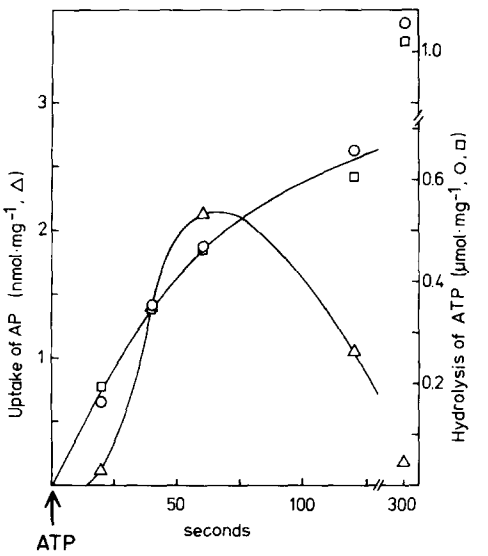

Fig. 5. Accumulation of acid in gastric vesicles. Vesicles (peak I) were incubated overnight at 4°C in the presence of 2 mM $MgCl_2$/150 mM KCl/10 mM Hepes-Tris (pH 7.4). Acid accumulation was measured at 21°C. At zero time, 50 μl of 10 mM ATP/50 μM amino[^{14}C]pyrine was added to 450 μl of vesicles (50 μg of protein) (△). In a separate experiment under identical conditions, [γ-^{32}P]ATP was added and its hydrolysis was measured as described in Materials and Methods; (○) hydrolysis of ATP in the absence of aminopyrine; (□) hydrolysis of ATP in the presence of 5 μM aminopyrine.

tained 60 s after the addition of ATP (Fig. 5). At longer times of incubation a progressive decrease of the aminopyrine accumulation was observed. The hydrogen ion pump is exchanging one external hydrogen ion for one internal potassium ion [18]. Therefore, the decrease of aminopyrine in the vesicles probably was a result of a leak of hydrogen ions out of the vesicles at a point when the intravesicular potassium ion concentration was too low to stimulate the ATPase and to maintain the H^+-gradient. The hydrolysis of ATP was most rapid during the first 10–15 s (first-order rate constant was 0.043 min^{-1}). At 60 s the rate constant was decreased to less than 1/10 of its initial value. In control experiments the hydrolysis of ATP was measured in the absence of aminopyrine. The hydrolysis was identical to that found in its presence.

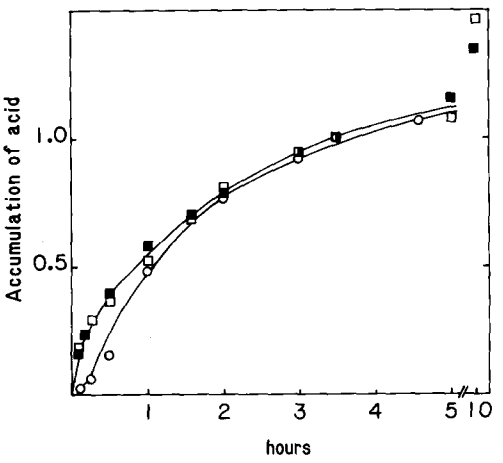

Fig. 6. Accumulation of acid in gastric vesicles. The ability of intravesicular KCl to stimulate the accumulation of acid was used as a measure of the uptake of KCl. Incubations were performed at 21°C in 150 mM KCl/2 mM $MgCl_2$/10 mM Hepes-Tris (pH 7.5). After mixing vesicles (peak I) with the incubation medium, samples were withdrawn at various times. Acid accumulation was measured by two different techniques: either by amino[^{14}C]pyrine accumulation (○) or by a spectrophotometric method using acridine orange (■ and □). The accumulation of amino[^{14}C]pyrine into the acid compartment was measured 40 s after the simultaneous addition of amino[^{14}C]pyrine and ATP to the sample. About 20% of the total amino[^{14}C]pyrine was trapped into vesicles which had been incubated with KCl for 3.5 h. Acid accumulation was also determined by measuring the change of absorbance at 493 nm after the addition of acridine orange and ATP to the sample. Assays with this technique were carried out in two different media. One medium was identical to that used in the aminopyrine assay (□). The other medium contained 150 mM choline chloride instead of 150 mM KCl (■). Due to inhibition of hydrogen-ion transport by extravesicular KCl, the change of absorbance measured in the presence of KCl was about 30% lower than in the presence of choline chloride (cf. Ref. 32). Relative values are given in order to make possible a comparison of experimental data obtained with the two different techniques. The uptake of KCl obtained after 3.5 h incubation with 150 mM KCl was given the value of one.

Time-dependence of the uptake of KCl in gastric vesicles. In order to characterize the uptake of KCl, vesicles were mixed with 150 mM KCl at 21°C and at pH 7.5. Samples were withdrawn at various times and tested for the ability of the vesicles to accumulate hydrogen ions. The accumulation of hydrogen ions was dependent on the time of preincubation in KCl and appeared to be independent of the type and conditions for the assay (Fig. 6). At short incubation times with KCl the aminopyrine technique gave slightly lower values than did the acridine orange technique. These indirect measurements of the uptake of KCl into the vesicles indicated that KCl penetrates the vesicular membrane slowly, with a half-time of 1.5 h.

Uptake of potassium ions into gastric vesicles and its dependence on anions. Vesicles were incubated

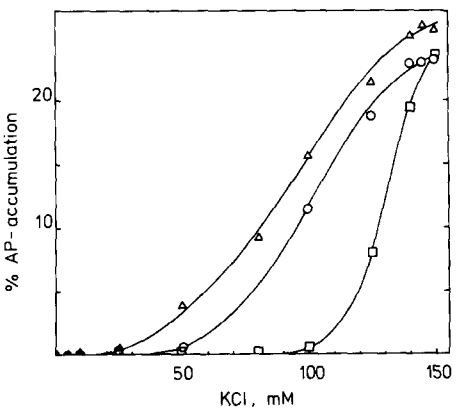

Fig. 7. Accumulation of amino[^{14}C]pyrine in gastric vesicles and its dependence on KCl. Gastric vesicles (peak I) were incubated for 3 h at 21°C in 10 mM Hepes-Tris/2 mM $MgCl_2$/various concentrations of KCl up to 150 mM (pH 7.4). Iso-osmotic pressure was maintained by additions of sucrose (□), NaCl (△), or by addition of choline chloride (○). Uptake of K^+ was measured by determining acid accumulation 40 s after the addition of 1 mM ATP plus 5 μM amino[^{14}C]pyrine.

in the presence of various concentrations of KCl up to 150 mM for 3 h at 21°C. Isoosmotic pressure was maintained with NaCl, choline chloride or with sucrose (Fig. 7). Formation of acid measured as aminopyrine accumulation occurred only at external concentrations of KCl higher than 20 mM. Substituting NaCl for KCl resulted in slightly higher aminopyrine accumulation than with choline chloride as substitute. With sucrose, more than 90 mM external KCl was required before any aminopyrine accumulation was observed. The presence of KCl was essential; neither NaCl nor choline chloride was able to stimulate acid accumulation. In addition, the poor effect produced by KCl in the presence of sucrose suggested that there was an effect of anions on the uptake of K^+. In order to test this hypothesis, vesicles were incubated with 150 mM of various salts of potassium (Fig. 8). The presence of 150 mM SCN^- gave the most rapid uptake of K^+. NO_3^- was slightly less effective than was SCN^-, but it was more effective than Cl^- was. In the presence of HCO_3^- the uptake of K^+ was only about 1–2% of that with SCN^- (not shown). SO_4^{2-} was even less effective. This is in agreement with a report by Lee et al. [29].

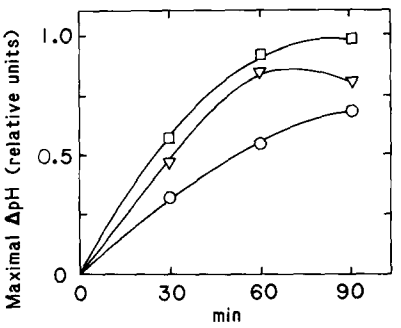

Fig. 8. Maximal ΔpH across vesicular membranes after incubation with various salts of potassium. Gastric vesicles (peak I) were mixed at 30°C with 150 mM of various salts of potassium in the presence of 2 mM $MgCl_2$/10 mM Hepes-Tris (pH 7.0). The potassium salts were KSCN (□); KNO_3 (▽); and KCl (○). Acid accumulation was measured at 21°C by the acridine orange technique in a standard assay mixture comprising 2 mM $MgCl_2$/150 mM choline chloride/10 mM Hepes-Tris (pH 7.0). Vesicles were diluted 40-times during the assay, thus reducing the various salts of potassium to about 4 mM on the outside of the vesicles. Maximal pH was observed in vesicles which had been incubated with KSCN for 90 min. This value represents unity.

Uptake of K^+ into gastric vesicles and its dependence on pH. The intracellular pH of the parietal cell increases after stimulation by secretagogoues, since H^+ disappears from the cytoplasm to the luminal side of the secreting membrane [30]. The presence of K^+ on the luminal side seems to be of crucial importance for continuous formation of acid. Therefore, the effect of pH on the uptake of K^+ into gastric vesicles was investigated. Vesicles were pre-incubated with 150 mM KCl or 150 mM KSCN at various pH levels. Samples were tested after various times by the acridine orange method. The uptake of K^+ was at all pH levels tested faster in the presence of SCN^- as compared with Cl^-. The uptake of K^+ was strongly enhanced at an alkaline pH (Fig. 9). A reduction of the pH from 8.0 to 7.0 reduced the uptake with 13% in the presence of KSCN and with 42% in the presence of KCl after 180 min pre-incubation. Again, the present results indicate that the permeability for Cl^- is considerably lower than that for K^+.

A possible role of carbonic anhydrase in the formation of hydrochloric acid. The $(H^+ + K^+)$-ATPase is inhibited by K^+ and Na^+ at neutral or slightly

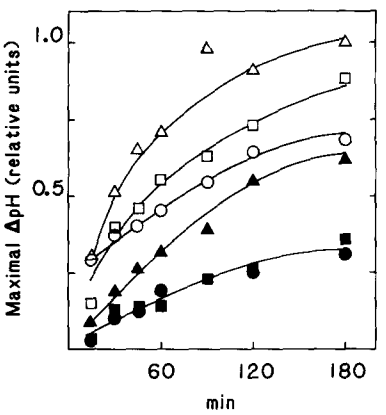

Fig. 9. Maximal ΔpH across vesicular membranes after preincubation with K^+ and its dependence on extravesicular pH. Gastric vesicles (peak I) were mixed at 21°C with 150 mM KCl (filled symbols) or KSCN (open symbols) in the presence of 2 mM $MgCl_2$ and buffers of various pH. The buffers used were: 10 mM Mes adjusted with Tris (pH 6.0) (○; ●); 10 mM Hepes adjusted with Tris (pH 7.0) (□; ■); 10 mM Tris adjusted with HCl (pH 8.0) (△; ▲). The ability of acid accumulation was measured by the acridine orange technique at standard conditions at pH 7.0 (see legend of Fig. 8). Maximal change of pH was observed in vesicles which had been pre-equilibrated with KSCN for 180 min at pH 8.0. This value represents unity.

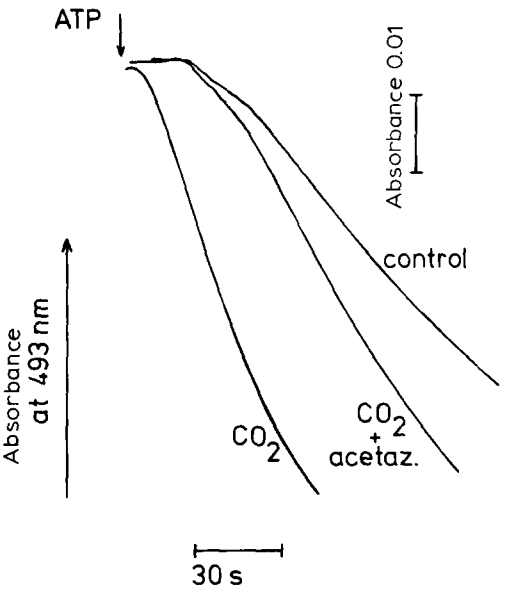

Fig. 10. Accumulation of acid in gastric vesicles (peak I) equilibrated with 150 mM KCl. The decrease of absorbance of acridine orange was monitored in the presence of 0.5 mM ATP/2 mM $MgCl_2$/150 mM KCl/10 mM Hepes adjusted with Tris (pH 8.0). When indicated, 100 µl of buffer saturated with a gas mixture of 5% CO_2 and 95% O_2 at 0°C was added together with the ATP. The final concentration of CO_2 was 0.4 mM as calculated from the Henderson-Hasselbalch equation. In one experiment, acetazolamide was added to give a final concentration of $5 \cdot 10^{-6}$ M. Incubation volume was 1 ml. Only ATP was added to the control.

alkaline pH [31,32]. Under similar conditions the rate of hydrogen ion transport was slow (Fig. 10). Addition of 0.4 mM CO_2 to the incubation mixture markedly increased the rate of transport and decreased the lag phase before onset of the transport. The change of pH in the buffer by the addition of CO_2 was maximally 0.08 pH units. Control experiments testing the rate of hydrogen ion transport in buffers with pH of 8.00, 7.90 and 7.80 showed that the small change in pH of 0.08 pH units could not increase the uptake of hydrogen ions in the vesicles as did the addition of CO_2. Acetazolamide initially inhibited the increase of hydrogen ion transport produced by the CO_2 but the effect of the drug was poor or absent at longer incubation times, probably because the uncatalyzed hydration of CO_2 and the subsequent formation of H^+ was sufficiently rapid. A lag phase before the onset of the transport always was observed at alkaline pH but not at neutral or acidic pH. Partial denaturation of the $(H^+ + K^+)$-ATPase by storage of vesicles in diluted solutions (about 10 µg of protein per ml) at 21°C increased this lag phase but did not alter the amplitude of the acridine orange signal (not shown). The lag phase probably represents a condition where the concentration of H^+ at the cytoplasmic side of the membrane is limiting acid formation. Any condition that increases the H^+ concentration locally on the cytoplasmic side will enhance hydrogen-ion transport and thereby reduce the lag phase. Such conditions can be obtained by increasing p_{CO_2} or can be obtained from a general increase of the ATPase reaction, where hydrogen ions are formed as the ATP is hydrolyzed [32,33]. Thus, at alkaline pH of the cytoplasm in the parietal cell, a membrane-associated carbonic anhydrase might play an important role in the formation of hydrogen ions at the cytoplasmic side of the secretory membrane.

Discussion

This investigation presents a rapid procedure of purification of tight gastric vesicles. The vesicles were capable of accumulating H^+ in an energy-requiring process provided KCl was present intravesicularly. The ATPase activity in a lyophilized preparation was 0.8 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ at 21°C and maximal amount of the phosphoenzyme intermediate under standard conditions [14] was 1.5 nmol/mg, irrespective of whether the vesicles were lyophilized or not. Occasionally preparations with 80–85% of these values were obtained. The molar activity, however, was always 530 min^{-1} at 21°C and 1600 min^{-1} at 37°C. These figures are equal to or higher than those for previously reported purification procedures [4–8].

Many proteins were detected by a high-resolving polyacrylamide gel electrophoresis in SDS. One major protein band corresponded to a molecular mass of 92–94 kDa, this molecular mass being slightly less than previously reported [7,18]. The gastric vesicular membranes contained high amounts of cholesterol and sphingomyelin which are found in a normal plasma membrane of an animal cell [24,25]. A similar lipid composition was recently reported by Schrijen et al. [34]. These investigators, however, did not report any

lysophospholipids. The present results suggest that the tubulovesicular membranes of the parietal cell originate from the plasma membrane of the cell, despite their apparent intracellular location. A continuation between the plasma membrane and tubulovesicular structures is not readily demonstrated. Such a continuation, however, is present between the plasma membrane and the secretory canalicular membranes as judged from electron microscopic pictures [36]. The lipid composition may indicate that the vesicular membranes are reformed within the parietal cell from the secretory membrane in a membrane recycling process after each stimulatory cycle of acid secretion.

The permeability for anions appeared rate-limiting for the uptake of K^+ into the vesicles, but the permeability increased with an increase of pH. A dependence of the permeability of the membrane on pH may be of physiological significance for the regulation of acid production. The half-time for the uptake of KCl at pH 7.5 and at 21°C was 1.5 h, which is about twice the time previously reported for gastric vesicles incubated at the same temperature but at pH 6.1 [19]. This descrepancy may indicate some variability of the stomachs used for the preparation of vesicles. Alkalinization of the cytoplasm upon stimulation by secretagogues is likely to give a K^+ and Na^+ inhibition from the cytoplasmic side of the $(H^+ + K^+)$-ATPase [31,32]. This inhibition of the enzyme activity will be opposed by an increased transfer of KCl to the luminal side of the secretory membrane, thereby providing more potassium ions for activation of the ATPase and the hydrogen-ion pump. In the present investigation it is shown that in addition to the $(H^+ + K^+)$-ATPase a carbonic anhydrase activity is associated with the vesicular membranes. Addition of a small amount of CO_2 to the incubation medium at slightly alkaline pH produced a considerable increase in the rate of H^+ accumulation. In vivo, this type of stimulation might occur through a general stimulation of the metabolism of the parietal cell and the subsequent production of CO_2. The present results (cf. Fig. 10) indicate that the carbonic anhydrase in gastric vesicular membranes is able to provide more H^+ for the transport process than would be possible in its absence. An additional stimulatory effect of a membrane-bound carbonic anhydrase might be to accomplish a decrease of pH locally close to the membrane more than in the surrounding medium, thereby releasing an inhibition of the transport system produced by alkali metal ions on the cytoplasmic side [32]. Still another way to stimulate the $(H^+ + K^+)$-ATPase via a production of H^+ at the cytoplasmic side is from the hydrolysis of ATP per se. The stimulation of H^+ transport by cytoplasmic H^+ appears to be balanced by a concomittant inhibition produced by a decrease of permeability of Cl^- across the vesicular membrane as pH is decreased, thus providing less KCl on the luminal side.

SCN^- is a well-known inhibitor of acid secretion [36]. Diffusion of SCN^- and HSCN through lipid bilayer membranes was recently studied as a function of pH [37]. The authors suggested that SCN^- inhibits gastric acid secretion by combining with H^+ on the luminal side of the secretory membrane and then diffusing back into the cell as HSCN, thus dissipating the proton gradient. In the vesicular system, 150 mM KSCN was able to stimulate the formation of a H^+-gradient and to maintain an H^+-gradient upon addition of ATP. Thus, if a mechanism dissipating the H^+-gradient was present in the vesicles, its rate was much less than that of hydrogen ion transport via the ATPase. However, SCN^- inhibited the vesicular carbonic anhydrase with a K_i of $2 \cdot 10^{-3}$ M, which is in the same concentration range that inhibits gastric acid secretion [36]. The present results indicate that the role of carbonic anhydrase in maintaining an appropriate pH at the cytoplasmic side of the secretory membrane is important for the activity of the $(H^+ + K^+)$-ATPase. The intact parietal cell may, however, contain other elements that are inhibited by SCN^- than those present in a vesicular preparation, since a complete inhibition of carbonic anhydrase by azetazolamide still gave good H^+ transport across the vesicular membrane.

A morphological change of the parietal cell to include sectretory canaliculi and microvilli is essential for acid secretion to the lumen of the stomach [38–40]. The present investigation demonstrates that tubulovesicular membranes from resting parietal cells contain enzymes required for the formation and regulation of hydrochloric acid production.

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References

1 Sedar, A.W. (1965) Fed. Proc. 24, 1360–1367
2 Forte, J.G., Limlomwongse, L. and Kasbekar, D.K. (1969) J. Gen. Physiol. 54, 76–95
3 DiBona, D.R., Ito, S., Berglindh, T. and Sachs, G. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 6689–6693
4 Ganser, A.L. and Forte, J. (1973) Biochim. Biophys. Acta 307, 169–180
5 Ray, T.K. and Forte, J.G. (1976) Biochim. Biophys. Acta 443, 451–467
6 Chang, H., Saccomani, G., Rabon, E., Schackmann, R. and Sachs, G. (1977) Biochim. Biophys. Acta 464, 313–327
7 Saccomani, G., Stewart, B., Shaw, D., Lewin, M. and Sachs, G. (1977) Biochim. Biophys. Acta 465, 311–330
8 Saccomani, G., Helander, H., Crago, S., Chang, H.H., Dailey, D. and Sachs, G. (1979) J. Cell Biol. 83, 271–283
9 Wolosin, J.M. and Forte, J.G. (1983) J. Membrane Biol. 71, 195–207
10 Lönnerholm, G. (1977) Acta Physiol. Scand. 99, 53–61
11 Mårdh, S. and Post, R.L. (1977) J. Biol. Chem. 252, 633–638
12 Nilsson, B.O., Larsson, E., Sundström, P. and Widehn, S. (1981) Uppsala J. Med. Sci. 86, 225–232
13 Mårdh, S. (1975) Biochim. Biophys. Acta 391, 448–463
14 Wallmark, B. and Mårdh, S. (1979) J. Biol. Chem. 254, 11899–11902
15 McIntosh, J.E.A. (1968) Biochem. J. 109, 203–207
16 Berglindh, T., Helander, H.F. and Öbrink, K.J. (1976) Acta Physiol. Scand. 97, 401–414
17 Lee, H.C. and Forte, J.G. (1978) Biochim. Biophys. Acta 508, 339–356
18 Sachs, G., Chang, H.H., Rabon, E., Schackmann, R., Lewin, M. and Saccomani, G. (1976) J. Biol. Chem. 251, 7690–7698
19 Schackmann, R., Schwartz, A., Saccomani, G. and Sachs, G. (1977) J. Membr. Biol. 32, 361–381
20 Tolbert, M.E.M., White, A.C., Aspry, K., Cutts, J. and Fain, J.N. (1980) J. Biol. Chem. 255, 1938–1944
21 Arvidson, G.A.E. (1968) Eur. J. Biochem. 4, 478–486
22 Peters, W.H.M., Fleuren-Jakobs, A.M.M., De Pont, J.J.H.H.M. and Bonting, S.L. (1981) Biochim. Biophys. Acta 649, 541–549
23 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
24 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254
25 Jain, M.K. (1980) in Introduction to Biological Membranes (Jain, M.K. and Wagner, R.C., eds.), p. 36, John Wiley and Sons, New York
26 White, D.A. (1973) in Form and Function of Phospholipids (Ansell, G.B., Hawthorne, J.N. and Dawson, R.M.C., eds.), p. 464, Elsevier Scientific Publishing Company, Amsterdam
27 Peters, W.H.M., Fleuren-Jakobs, A.M.M., Scheijen, J.J., De Pont, J.J.H.H.M. and Bonting, S.L. (1982) Biochim. Biophys. Acta 690, 251–260
28 Maren, T.H. (1967) Physiol. Rev. 47, 595–781
29 Lee, H.C., Breitbart, H., Berman, M. and Forte, J. (1979) Biochim. Biophys. Acta 553, 107–131
30 Ekblad, E.B., (1980) Biochim. Biophys. Acta 632, 375–385
31 Stewart, B. Wallmark, B. and Sachs, G. (1981) J. Biol. Chem. 256, 2682–2690
32 Ljungström, M., Vega, F. and Mårdh, S. (1984) Biochim. Biophys. Acta 769, 220–230
33 Alberty, R.A. (1969) J. Biol. Chem. 244, 3290–3302
34 Schrijen, J.J., Omachi, A. Van Groningen-Luyben, W.A.H.M., DePont, J.J.H.H.M. and Bonting, S.L. (1981) Biochim. Biophys. Acta 649, 1–12
35 Helander, H.F. (1981) Int. Rev. Cytol. 70, 217–289
36 Hersey, S.J., Chew, C., Campbell, L. and Hopkins, E. (1981) Am. J. Physiol. 240, G232–G238
37 Gutknecht, J. and Walter, A. (1982) Biochim. Biophys. Acta 685, 233–240
38 Forte, J.G., Black, J.A., Forte, T.M., Machen, T.E. and Wolosin, J.M. (1981) Am. J. Physiol. 241, G349–G358
39 Wolosin, J.M. and Forte, J.G. (1981) J. Biol. Chem. 256, 3149–152
40 Gibert, A.J. and Hersey, S.J. (1982) J. Membrane Biol. 67, 113–124