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Redistribution and characterization of $(H^+ + K^+)$-ATPase membranes from resting and stimulated gastric parietal cells

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When isolated from resting parietal cells, the majority of the $(H^+ + K^+)$-ATPase activity was recovered in the microsomal fraction. These microsomal vesicles demonstrated a low K^+ permeability, such that the addition of valinomycin resulted in marked stimulation of $(H^+ + K^+)$-ATPase activity, and proton accumulation. When isolated from stimulated parietal cells, the $(H^+ + K^+)$-ATPase was redistributed to larger, denser vesicles: stimulation-associated (s.a.) vesicles. S.a. vesicles showed an increased K^+ permeability, such that maximal $(H^+ + K^+)$-ATPase and proton accumulation activities were observed in low K^+ concentrations and no enhancement of activities occurred on the addition of valinomycin. The change in subcellular distribution of $(H^+ + K^+)$-ATPase correlated with morphological changes observed with stimulation of parietal cells, the microsomes and s.a. vesicles derived from the intracellular tubulovesicles and the apical plasma membrane, respectively. Total $(H^+ + K^+)$-ATPase activity recoverable from stimulated gastric mucosa was 64% of that from resting tissue. Therefore, we tested for latent activity in s.a. vesicles. Permeabilization of s.a. vesicles with octyl glucoside increased $(H^+ + K^+)$-ATPase activity by greater than 2-fold. Latent $(H^+ + K^+)$-ATPase activity was resistant to highly tryptic conditions (which inactivated all activity in gastric microsomes). About 20% of the non-latent $(H^+ + K^+)$-ATPase activity was also resistant to trypsin digestion. We interpret these results as indicating that, of the s.a. vesicles, approx. 55% have a right-side-out orientation and are impermeable to ATP, 10% right-side-out and permeable to ATP, and 35% have an inside-out orientation.

INTRODUCTION

The gastric parietal cell secretes HCl into the lumen of the stomach at concentrations in excess of 150 mM. A central role has been established for the gastric $(H^+ + K^+)$-ATPase (EC 3.6.1.36) in acid secretion (Ganser & Forte, 1973; Forte & Lee, 1977). When isolated, the $(H^+ + K^+)$-ATPase is found in vesicles orientated with their ATP-hydrolysing site exposed on the exterior face, such that, in the presence of Mg^{2+} and internal K^+, addition of ATP results in the accumulation of protons (Lee *et al.*, 1974; Sachs *et al.*, 1976; Lee & Forte, 1978).

Stimulation of acid secretion is associated with extensive morphological changes within the parietal cell. There is a 5–10-fold increase in the apical surface of the parietal cell with the concomitant depletion of the extensive intracellular tubulovesicular membranes. Withdrawal of the stimulus results in a cessation of acid secretion, accompanied by a reversion of the parietal cell ultrastructure back to the resting state. A membrane recycling hypothesis has been offered to account for these changes (Forte *et al.*, 1977, 1981). Subcellular fractionation of gastric mucosa in the resting and stimulated state reveals that the ultrastructural changes are accompanied by biochemical and functional alternations. Fractionation of resting gastric mucosa yields the majority of the $(H^+ + K^+)$-ATPase in low density, microsomal membrane vesicles. In contrast, the $(H^+ + K^+)$-ATPase from stimulated gastric mucosa is redistributed to larger, denser membrane vesicles (Wolosin & Forte, 1981*a*). The membrane vesicles from stimulated tissue show a greater permeability to KCl than those from resting tissue (Wolosin & Forte, 1981*b*).

In the present study, we have carried out a detailed comparison of the distribution of the $(H^+ + K^+)$-ATPase in subcellular fraction from both resting and stimulated gastric mucosa of the rabbit. In carrying out these studies, we noted a reduction in total $(H^+ + K^+)$-ATPase activity recovered from stimulated, as compared with resting, mucosa. This observation led us to test for latent $(H^+ + K^+)$-ATPase activity in membrane vesicles from stimulated tissue. We provide evidence that the membrane vesicles isolated from stimulated gastric mucosa show a mixture of right-side-out (rso) and inside-out (iso) orientation.

MATERIALS AND METHODS

The procedure for preparation of $(H^+ + K^+)$-ATPase-enriched vesicles was based on that previously described by Wolosin & Forte (1981*a*).

Abbreviations used: s.a., stimulation-associated; rso, right-side-out; iso, inside-out; pNPPase, *p*-nitrophenylphosphatase. The density gradient fractions are designated according to the starting material (P_1, P_2, or P_3), and the Ficoll layer (5%, 10%, or 16%). In addition, a superscript S (stimulated) or R (resting) indicates the physiological state of the stomach. Thus $P_2{}^S$ 16% indicates the fraction of P_2 from a stimulated stomach that layers on top of the 16% Ficoll. As described in the Materials and methods section, the pellets resulting from the density gradient centrifugation of P_1, P_2, and P_3 are designated P_4, P_5, and P_6, respectively.

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Treatment of animals and preparation of tissue

New Zealand white rabbits (1.5–3.0 kg) of either sex were used. Animals were fasted overnight with free access to water. To achieve stimulation of the stomachs, the animals were allowed to feed freely for 20 min, and then given sequentially and subcutaneously 0.8–1.2 ml of a sedative mixture [ketamine hydrochloride (Ketaset; Bristol Laboratories, Syracuse, NY, U.S.A.), 60 mg/ml; xylazine (Rompun; Miles Laboratories, Shawnee, KA, U.S.A.), 60 mg/ml; acepromazine maleate (Aveco, Fort Dodge, IA, U.S.A.), 1.2 mg/ml], 0.3 ml of chlorpheniramine maleate (15 mg/ml) and 0.2 ml of histamine (100 mM), followed 10 min later by a further 0.2 ml of histamine subcutaneously. The animals were then anaesthetized with sodium pentobarbital (Nembutal; Abbott Laboratories, Chicago, IL, U.S.A.; approx. 1.2 ml intravenously of a 50 mg/ml solution) and given an additional 0.2 ml of the histamine solution intravenously. For resting stomachs, the animals were not fed, and the chlorpheniramine and histamine injections were replaced by 0.3 ml of cimetidine (150 mg/ml; Tagamet; Smith Kline & French Laboratories, Carolina, PR, U.S.A.). The rest of the procedures were identical for both secretory states. The stomachs were removed 10 min after the final histamine or cimetidine injection, divided longitudinally and immersed in ice-cold saline solution. The stomach was washed free of contents and the antrum was discarded. The fundic mucosa was blotted to remove the mucous layer, and the mucosae were scraped from the muscle layers with a glass slide and placed in a small volume (approx. 20 ml) of ice-cold homogenizing medium (125 mM-mannitol, 40 mM-sucrose, 1 mM-EDTA, 5 mM-Pipes, brought to pH 6.7 with Tris). The tissue was then minced with surgical scissors, diluted with 25 vol. of homogenizing medium, and triturated in a Potter–Elvehjem homogenizer by 15 passes of a loose-fitting Teflon pestle at 200 cycles/min.

Subcellular fractionation

The mucosae were fractionated by differential centrifugation followed by density gradient centrifugation. The homogenate was centrifuged at 120 $g_{max.}$ for 10 min to yield a pellet (P_0); the supernatant was recentrifuged at 1000 $g_{max.}$ for 10 min to yield the nuclear pellet (P_1). The supernatant was adjusted to pH 7.4 with 1 M-Tris, and recentrifuged at 14500 $g_{max.}$ for 11 min to give the mitochondrial pellet (P_2). The supernatant was recentrifuged at 142800 $g_{max.}$ for 60 min to yield the microsomal pellet (P_3) and cytosol (S_3). The pellets were resuspended in 300 mM-sucrose buffered with 5 mM-Tris/HCl, pH 7.4. P_1, P_2, and P_3 in a total volume of 18 ml were layered on top of a discontinuous gradient made of 5%, 10%, and 16% Ficoll 400 (Sigma) in 300 mM-sucrose/5 mM-Tris/HCl, pH 7.4, and centrifuged at 27000 rev./min (135000 $g_{max.}$) for 16 h in a Beckman SW27 rotor. The material layered on top of the 5%, 10%, and 16% Ficoll bands was harvested, diluted with 300 mM-sucrose/5 mM-Tris/HCl, pH 7.4, and centrifuged at 48000 $g_{max.}$ for 30 min (P_1 and P_2 fractions) or 142800 $g_{max.}$ for 60 min (P_3 fractions) to free them of Ficoll. The resulting pellets were resuspended in 300 mM-sucrose/5 mM-Tris/HCl, pH 7.4. The pellets from the Ficoll gradients (P_4, P_5, and P_6 from P_1, P_2, and P_3 respectively) were resuspended in 300 mM-sucrose/5 mM-Tris/HCl, pH 7.4, in a Ten Broeck (glass/glass) homogenizer. All fractions were used immediately, or stored at 4 °C for a maximum of 3 days before use.

Analytical methods

ATPase. ATPase activity was measured by the liberation of inorganic phosphate at 37 °C in 1 ml of medium containing 5–70 μg of protein, 10 mM-Pipes buffer, pH 6.7, 1 mM-$MgSO_4$, 1 mM-ouabain with 0, 30 or 140 mM-KCl (K^+ replaced by Na^+ to a total cation concentration of 140 mM), and initiated by the addition of 1 mM-Na_2ATP (equine; Sigma). Valinomycin (10 μM) was added to some tubes before the ATP. The reaction was stopped by the addition of 1 ml of ice-cold 14% (w/v) trichloracetic acid, and inorganic phosphate was determined by extraction of the phosphomolybdate complex into butyl acetate (Sanui, 1974). Mg^{2+}-ATPase activity is defined as the ATPase activity in the absence of K^+. $(H^+ + K^+)$-ATPase activity is defined as the ATPase activity in the presence of 30 or 140 mM-K^+, or 140 mM-K^+ plus 10 μM-valinomycin as described, after subtraction of the Mg^{2+}-ATPase activity in the same sample. Ouabain was included to inhibit $(Na^+ + K^+)$-ATPase activity.

***p*-Nitrophenylphosphatase.** pNPPase activity was measured at 37 °C in 1 ml of medium containing 5–70 μg of protein, 20 mM-Tris/HCl (pH 7.5), 4 mM-$MgSO_4$, 0.2 mM-EDTA, 1 mM-ouabain, 10 mM-NaCl or KCl, made iso-osmotic with sucrose, and initiated by the addition of 5 mM-sodium *p*-nitrophenyl phosphate (Calbiochem). The sucrose was omitted to measure pNPPase activity under hypo-osmotic conditions. K^+-pNPPase activity is defined as the activity in the presence of 10 mM-K^+ after subtraction of the rate in 10 mM-Na^+.

Cytochrome *c* oxidase. Cytochrome *c* oxidase was measured spectrophotometrically using the conditions described by Smith (1955) with the addition of 0.1% Triton X-100 to reveal total activity (Culp & Forte, 1981), and absorbance at 550 nm monitored continuously.

RNA and DNA. Samples were digested with 0.3 M-KOH at 37 °C for 60 min, and acidified to 0.2 M-$HClO_4$ to yield the acid-soluble RNA products (Munro & Fleck, 1966). The acidified alkaline digest pellets were washed with 0.2 M-$HClO_4$, and DNA was digested in 0.5 M-$HClO_4$ at 70 °C for 20 min (Davidson, 1957). Both RNA and DNA were measured at 260 nm.

Protein. Protein was assayed by the method of Bradford (1976) with γ-globulin (Cohn fraction II; Sigma) as standard.

H^+ uptake

Vesicular acidification was followed by the quenching of Acridine Orange fluorescence (Lee & Forte, 1978). Experiments carried out at 37 °C were started by the addition of 0.625 mM-Mg–ATP to a small aliquot of the vesicles in the uptake medium, consisting of 1.2 ml of 30 or 140 mM-KCl made up to 300 mosM with sucrose, 2.5 mM-Tris/Tes (pH 7.2), 0.05 mM-EDTA, and 2 μM-Acridine Orange (Eastman Kodak). Fluorescence measurements were made with a Perkin–Elmer MPF-44a spectrofluorimeter with wavelengths of 493 and 530 nm (excitation and emission).

Table 1. Distribution of markers after differential centrifugation

Values are presented as the average total activity in the homogenate and individual fractions, normalized on the basis of the wet weight of mucosal scrapings for each tissue. The distribution of markers from resting (R) and stimulated (S) tissues are compared. The numbers in parentheses indicate the number of preparations in which markers were measured. Results are expressed as mean ± S.E.M. The total marker activity in fractions P_1 and P_2, expressed as a ratio of that in the microsomal fraction P_3, is given in the final column (P_1+P_2/P_3). $(H^+ + K^+)$-ATPase activity was measured in the presence of 140 mM-K^+ and 10 μM-valinomycin. Conditions for the other assays are given in the Materials and methods section.

Marker		Total homogenate	Fractions					P_1+P_2
			P_0	P_1	P_2	P_3	S_3	P_3
Protein (mg/g)	R (2)	135±3	45±1	15±1	16±1	11±0	46±8	2.82
	S (5)	146±4	45±2	17±1	16±1	10±1	51±3	3.30
$(H^+ + K^+)$-ATPase (μmol/h per g)	R (2)	717±163	148±55	59±31	126±8	263±10	0	0.70
	S (4)	457±116	101±25	129±13	140±12	51±19	0	5.27
K^+-pNPPase (μmol/h per g)	R (2)	325±46	91±32	17±3	45±21	181±5	4±4	0.34
	S (4)	264±29	108±18	61±2	65±11	40±12	1±1	3.15
Mg^{2+}-ATPase (μmol/h per g)	R (2)	644±134	341±32	74±10	125±8	150±4	1±1	1.33
	S (4)	708±50	361±16	135±19	154±34	124±15	8±4	2.33
DNA (mg/g)	R (1)	2.5	1.3	0.2	0.01	0.01	0	
	S (2)	2.4	1.9	0.2	0.03	0.1	0.2	
RNA (mg/g)	R (1)	11.3	3.6	0.6	0.6	1.2	4.2	
	S (2)	10.4	3.3	0.6	0.7	1.3	4.2	
Cytochrome *c* oxidase (ΔA_{550}/min per mg)	R (1)	37.0	12.7	30.5	24.7	1.9	0	
	S (2)	52.3	11.4	23.9	18.3	0.6	0	

Trypsin digestion

Treatment with trypsin was carried out at 30 °C in 300 mM-sucrose/5 mM-Tris/HCl buffer, pH 7.4, with a weight ratio of trypsin (bovine, type 1, twice crystallized; Sigma) to membrane protein of 1:10. At selected times, aliquots were taken and added to soya-bean trypsin inhibitor (Sigma) to give a weight ratio of inhibitor to trypsin of 10:1. Controls were carried out where trypsin inhibitor was added before trypsin.

Octyl glucoside latency

Latent $(H^+ + K^+)$-ATPase and K^+-pNPPase activities were investigated by pretreatment of the vesicles with *n*-octyl glucoside (Calbiochem). Vesicles were incubated for 5 min at room temperature with 1–21 mM-octyl glucoside in 300 mM-sucrose/5 mM-Tris/HCl buffer, pH 7.4. The concentration of membrane protein was in the range of 1.0 mg/ml. The detergent was diluted with 6 vol. of ice-cold 300 mM-sucrose/5 mM-Tris/HCl buffer, pH 7.4. Of the resulting sample, 50 μl was used in the enzyme assays, producing a further 20-fold dilution of the detergent.

RESULTS

Purification of plasma membranes enriched in $(H^+ + K^+)$-ATPase

Differential centrifugation. Table 1 summarizes the distribution of markers for a number of different stomachs after the initial separation procedure by differential centrifugation. Values are presented as total activity in the individual fractions and the total homogenate, normalized on the basis of the wet weight of mucosal scrapings for each tissue. The patterns of distribution for protein, Mg^{2+}-ATPase, DNA, RNA, and cytochrome *c* oxidase were similar in tissue from resting and stimulated gastric mucosae. In contrast, there were major redistributions in the H^+ pump marker enzymes, $(H^+ + K^+)$-ATPase and K^+-pNPPase. In resting tissue, the largest proportion of these latter enzymes (as well as the highest specific activity) appeared in P_3, such that the pellet distribution ratio, expressed as $(P_1+P_2)/P_3$ for $(H^+ + K^+)$-ATPase and K^+-pNPPase activities was 0.70 and 0.34, respectively. In stimulated stomach, there was a large decrease in both of these activities in P_3 with complementary increases in P_1 and P_2; the $(P_1+P_2)/P_3$ ratio being 5.27 and 3.15, respectively, for the two K^+-stimulated enzymes. The redistribution of $(H^+ + K^+)$-ATPase and K^+-pNPPase activities in stimulated stomach was accompanied by a reduction in the total activities, as compared with the resting tissue (the significance of this latter point will be taken up later).

The differential centrifugation procedure resulted in plasma membrane fractions with various contaminants. The very low speed, P_0, fraction contained a great deal of connective tissue as well as many unbroken cells (microscopic observation). P_0 included significant quantities of the various marker enzymes. This was in large part due to the relatively gentle procedure of homogenization that was intended to prevent excessive fragmentation of the apical plasma membranes. A major contaminant of both P_1 and P_2 is mitochondrial, as judged by the activity of cytochrome *c* oxidase. In addition, a second major contaminant of P_1 is nuclear, as judged by the DNA content, and by direct examination by phase microscopy. The major contaminant of P_3 is ribosomal, as judged by the RNA content. A previous study (Wolosin & Forte, 1981*a*) with an identical centrifugation procedure of whole mucosal homogenates has shown P_1, P_2, and P_3 to be relatively uncontaminated by basolateral plasma membranes [i.e. $(Na^+ + K^+)$-ATPase was predominantly in P_0] and pepsinogen granules (i.e. pepsin was localized to the supernatant).

Table 2. Distribution of markers after density gradient centrifugation of P_2

Values are presented as the average specific activity markers, and the average protein content, for resting (R) and stimulated (S) tissue. Material was recovered from the top of the corresponding Ficoll gradient, and the pellet (P_5). Results are expressed as mean ± S.E.M., with number of preparations in parentheses. $(H^+ + K^+)$-ATPase activity was measured in the presence of 140 mM-K^+ and 10 μM-valinomycin. Conditions for the other assays are given in the Materials and methods section.

Marker		Crude P_2	Density gradient fractions			
			$P_2$5%	$P_2$10%	$P_2$16%	P_5
$(H^+ + K^+)$-ATPase (μmol/mg per h)	R	8.0±0.2 (2)	5.3	3.2	3.8	0.4
	S	8.8±0.5 (4)	15.1±3.9 (4)	11.8±1.4 (4)	25.1±2.4 (13)	4.8±0.5 (4)
K^+-pNPPase (μmol/mg per h)	R	2.8±1.2 (2)	2.8	2.0	5.0	0.1
	S	3.8±0.5 (5)	11.2±1.9 (5)	8.4±1.2 (5)	14.8±1.0 (9)	0.5±0.0 (5)
Mg^{2+}-ATPase (μmol/mg per h)	R	7.9±0.1 (2)	19.1	4.2	9.6	2.0
	S	9.6±1.9 (4)	25.6±5.2 (4)	9.8±1.6 (4)	16.8±1.7 (13)	4.1±0.8 (4)
DNA (μg/mg)	S	1.5±0.2 (2)	6.4±4.9 (2)	1.2±0.7 (2)	0.7±0.6 (5)	1.6±1.1 (2)
RNA (μg/mg)	S	40.1±8.8 (2)	50.3±1.9 (2)	46.8±1.4 (2)	45.6±30.5 (5)	20.7±1.7 (2)
Cytochrome *c* oxidase (ΔA_{550}/min per mg)	S	1.1±0.3 (2)	0.1±0.1 (2)	0.1±0.1 (2)	0.6±0.2 (5)	2.7±1.0 (2)
Protein (mg)	R	139.0±10 (2)	1.9	5.4	1.2	85.6
	S	138.4±9.5 (5)	2.2±0.4 (5)	7.2±1.1 (5)	5.0±0.8 (12)	93.7±9.4 (5)

Density gradient centrifugation. Fractions P_1, P_2 and P_3 were further purified by centrifugation on a Ficoll density step gradient. Only the bands of material that layered on top of the 5%, 10%, and 16% Ficoll, and the pellet, were collected as it was felt this might result in fractions with more uniform characteristics. The specific activities of the enzyme markers in P_2, as well as the density gradient subfractions, are summarized in Table 2. Upon centrifugation of P_2 from resting mucosa, no fraction showed enrichment of $(H^+ + K^+)$-ATPase or K^+-pNPPase activities. On the other hand, the 16% fraction from stimulated mucosa (i.e. P_2^S16%) showed a 2.9-fold enrichment of $(H^+ + K^+)$-ATPase, and 3.9 fold enrichment of K^+-pNPPase. This fraction also showed a reduction in DNA and cytochrome *c* oxidase contamination. The majority of the cytochrome *c* oxidase activity was pelleted in P_5, along with the majority of the protein. Although P_5 also contained significant $(H^+ + K^+)$-ATPase and K^+-pNPPase activities, it was felt that increasing the concentration of Ficoll in the lower layer to capture more of the activity would also result in greater mitochondrial contamination. The low level of mitochondrial contamination in P_2^S16% was also ascertained by the absence of submitochondrial particles in fractions observed by negative-staining electron microscopy.

The microsomal fraction, P_3, has routinely been used for the preparation of gastric microsomes rich in $(H^+ + K^+)$-ATPase activity (Forte *et al.*, 1975). For these earlier studies, the microsomes were subfractionated using sucrose gradients. However, in the present study, the same Ficoll gradient as was used for P_2 was chosen to subfractionate P_3 so that the resulting fractions might be more comparable. It should be noted that there was a considerable loss of protein with this protocol. The results are summarized in Table 3. P_3 from stimulated stomachs showed a lower specific activity for $(H^+ + K^+)$-ATPase and K^+-pNPPase than P_3 from resting stomachs, and the Ficoll gradient produced very little enrichment. In the resting stomach, fractionation of P_3 resulted in two layers enriched in $(H^+ + K^+)$-ATPase and K^+-pNPPase: P_3^R5% and P_3^R16%. P_3^R5% showed a 3.6–4.3-fold enrichment of these activities, whilst the enrichment was 2.0–2.5-fold with P_3^R16%. However, as will be described later, these two fractions showed important functional differences. P_3^R5% and P_3^R16% showed reduced ribosomal contamination as judged by RNA.

Fractionation of gastric mucosa from stimulated stomachs resulted in a redistribution of $(H^+ + K^+)$-ATPase and K^+-pNPPase activities to P_1 as well as P_2, as compared with the pattern achieved from resting tissue (Table 1). Fractionation of P_1 on the same Ficoll gradient as used for P_2 and P_3 did result in a band of high specific activity material in the same layer as observed with P_2, i.e., P_1^S16% (results not shown). P_1^S16% showed very similar specific activity to P_2^S16%, and identical characteristics (i.e. a lack of stimulatory effect of valinomycin; see below). P_1^S16% contained only about half the total activity of P_2^S16%, and, as with P_2, a large proportion of the total activity (81%) was found in the pellet, P_4. Better yield of P_1 was achieved by separation on a discontinuous density gradient made of 20%, 27%, and 40% sucrose. With the sucrose gradient, 70% of the total activity banded on top of the 40% sucrose layer, and showed no valinomycin stimulation. Separation of P_2 on the same sucrose gradient was not as successful, with only about 40% of the total activity banding above the 40% sucrose layer. Separation of P_1 and the use of sucrose gradients were not carried further.

Functional characteristics of gastric vesicles

As has previously been described (Wolosin & Forte, 1981*a*, *b*), the redistribution of the $(H^+ + K^+)$-ATPase into larger, more dense vesicles upon stimulation is accompanied by changes in their functional characteristics. In particular, the $(H^+ + K^+)$-ATPase vesicles from stimulated stomachs no longer have restricted K^+ permeability as judged by the lack of requirement for valinomycin to achieve maximum rates of ATPase activity or H^+ uptake. We examined the functional characteristics in more detail.

$(H^+ + K^+)$-ATPase. ATPase activity in the subcellular fractions was assayed under four experimental conditions; no added K^+ (the Mg^{2+}-ATPase activity), low [K^+]

Table 3. Distribution of markers after density gradient centrifugation of P_3

Values are presented as the average specific activity of markers, and the average protein content, for resting (R) and stimulated (S) tissue. Material was recovered from the top of the corresponding Ficoll gradient, and the pellet (P_6). Results are expressed as mean ± S.E.M., with number of preparations in parentheses. $(H^+ + K^+)$-ATPase activity was measured in the presence of 140 mM-K^+ and 10 μM-valinomycin. Conditions for the other assays are given in the Materials and methods section.

Marker		Crude P_3	Density gradient fractions			
			$P_3$5%	$P_3$10%	$P_3$16%	P_6
$(H^+ + K^+)$-ATPase (μmol/mg per h)	R	24.3±1.0 (2)	108.9±33.4 (2)	26.9±10.4 (2)	47.7±5.9 (2)	3.3±0.8 (2)
	S	5.7±1.6 (3)	8.1±5.2 (2)	4.3±2.1 (2)	10.0±3.5 (2)	0 ±0 (2)
K^+-pNPPase (μmol/mg per h)	R	16.7±0.4 (2)	60.9±14.0 (2)	17.1±1.3 (2)	42.1±15.3 (2)	1.8±0.2 (2)
	S	4.5±0.9 (5)	3.8±0.4 (2)	3.5±0 (2)	8.9±0 (2)	1.4±0.3 (2)
Mg^{2+}-ATPase (μmol/mg per h)	R	13.8±0.3 (2)	30.0±0.4 (2)	19.7±5.3 (2)	16.4±1.0 (2)	11.2±2.3 (2)
	S	12.8±0.3 (3)	49.3±8.2 (2)	15.6±7.0 (2)	18.2±4.5 (2)	0 ±0 (2)
DNA (μg/mg)	R	0.5	0.4	1.1	0.9	0
	S	11.8	13.5	8.6	11.4	6.6
RNA (μg/mg)	R	110	52.4	89.4	54.7	79.2
	S	111	81.5	78.0	61.1	82.8
Cytochrome *c* oxidase (ΔA_{550}/min per mg)	R	0.2	0	0.1	0.3	1.1
	S	0.1±0.04 (2)	0	0.1	0.3	0.6
Protein (mg)	R	95.5±5.5 (2)	6.0±1.2 (2)	8.3±0.2 (2)	4.7±0.8 (2)	4.5±0.0 (2)
	S	88.2±10.4 (4)	2.4±0.8 (2)	7.8±2.3 (2)	3.1±1.6 (2)	6.3±3.4 (2)

Table 4. Characteristics of ATPase activities in subcellular fractions

Values are presented as the average specific activity of ATPase for resting (R) and stimulated (S) tissue, in the presence of 0, 30, and 140 mM-K^+, or 140 mM-K^+ with 10 μM-valinomycin. Results are expressed as mean ± S.E.M., with the number of preparations in parentheses.

Fraction	ATPase activity (μmol/mg per h)							
	0 mM-K^+ (Mg^{2+})		30 mM-K^+		140 mM-K^+		140 mM-K^+ + 10 μM-valinomycin	
	R	S	R	S	R	S	R	S
P_0	7.7±0.6 (2)	8.2±0.8 (4)	1.4	0.7±0.7 (2)	2.3±0.8 (2)	3.0±0.8 (4)	3.4±1.3 (2)	2.3±0.6 (4)
P_1	5.1±0.4 (2)	8.8±1.8 (4)	2.5	2.4±0.2 (3)	4.1±2.3 (2)	7.8±1.3 (4)	3.9±1.9 (2)	8.2±1.1 (4)
P_2	7.9±0.1 (2)	9.6±1.9 (4)	1.8±0.3 (2)	3.7±0.2 (4)	5.5±0.1 (2)	7.7±0.4 (4)	8.0±0.2 (2)	8.8±0.5 (4)
P_3	13.8±0.3 (2)	12.2±0.6 (4)	2.3±0.3 (2)	1.7±1.2 (4)	4.3±0.1 (2)	3.7±0.7 (4)	24.3±1.0 (2)	4.8±1.5 (4)
$P_2$5%	19.1	25.6±5.2 (4)	1.1	14.1±2.9 (4)	1.5	12.9±1.7 (4)	4.8	15.1±3.9 (4)
$P_2$10%	4.2	9.8±1.6 (4)	1.4	8.1±1.1 (4)	1.8	10.6±0.8 (4)	3.2	11.8±1.4 (4)
$P_2$16%	9.6	16.8±1.7 (13)	2.4	23.9±3.0 (13)	6.3	27.1±2.5 (13)	3.8	25.1±2.4 (13)
P_5	2.0	4.1±0.8 (4)	0.1	1.2±0.2 (4)	0.5	4.7±0.5 (4)	0.4	4.8±0.5 (4)
$P_3$5%	30.0±0.4 (2)	49.3±8.2 (2)	3.9±0.3 (2)	4.1±0.7 (2)	4.7±0.7 (2)	3.7±1.7 (2)	108.9±33.4 (2)	8.1±5.2 (2)
$P_3$10%	19.7±5.3 (2)	15.6±7.0 (2)	4.3±2.4 (2)	1.1±0.1 (2)	4.7±1.5 (2)	2.5±1.1 (2)	26.9±10.4 (2)	4.3±2.1 (2)
$P_3$16%	16.4±1.0 (2)	13.2±4.5 (2)	17.4±1.3 (2)	9.1±2.3 (2)	24.4±0.8 (2)	13.5±2.7 (2)	47.7±5.9 (2)	10.0±3.5 (2)
P_6	11.2±2.3 (2)	0 ±0 (2)	2.7±0.5 (2)	0 ±0 (2)	5.9±0.6 (2)	0 ±0 (2)	3.3±0.8 (2)	0 ±0 (2)

(30 mM), high [K^+] (140 mM), and high [K^+] (140 mM) in the presence of the K^+ ionophore, valinomycin. The results are summarized in Table 4.

The general observation may be made that valinomycin had relatively little enhancing effect on $(H^+ + K^+)$-ATPase activity in fractions from stimulated stomachs. In addition, the activities in the presence of 30 and 140 mM-K^+ were similar in $P_2{}^S$5%, $P_2{}^S$10%, and $P_2{}^S$16%. In contrast, addition of valinomycin increased $(H^+ + K^+)$-ATPase in the fractions from the resting stomach, this stimulation being particularly evident in P_3 and its subfractions. Both $P_3{}^R$5% and $P_3{}^R$16% showed high specific activities for $(H^+ + K^+)$-ATPase. However, $P_3{}^R$5% showed almost complete dependence on valinomycin, with a 23-fold stimulation in ATPase activity upon addition of the ionophore. $P_3{}^R$16% already showed relatively good activity in the presence of 140 mM-K^+ alone, and only a 2-fold stimulation by valinomycin. Thus the $(H^+ + K^+)$-ATPase from resting stomachs was concentrated in small, low density vesicles which are relatively impermeable to K^+ as exemplified by the P_3 fractions. The $(H^+ + K^+)$-ATPase from stimulated stomachs was redistributed to larger, denser vesicles which are freely permeable to K^+, and are exemplified by the P_2 fractions.

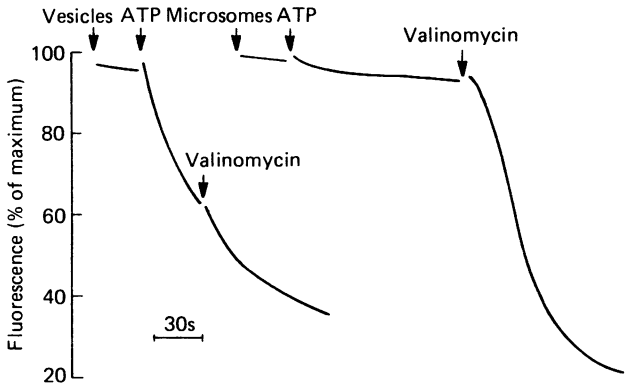

Fig. 1. H^+ uptake by gastric vesicles

H^+ uptake was measured by quenching of Acridine Orange fluorescence. S.a. vesicles ($P_2^S16\%$; vesicles) and gastric microsomes ($P_3^R5\%$; microsomes) were incubated in a buffered 140 mM-KCl solution (see the Materials and methods section) with 2 μM-Acridine Orange, and 0.625 mM-ATP added as indicated. S.a. vesicles showed rapid H^+ uptake, and this was not appreciably enhanced by 10 μM-valinomycin. In contrast, microsomes did not accumulate H^+ until the addition of 10 μM-valinomycin.

H^+ uptake. The H^+ uptake properties of the gastric vesicles, as monitored by quenching of Acridine Orange fluorescence, are illustrated in Fig. 1. Vesicles from stimulated stomachs (e.g. $P_2^S16\%$) showed rapid H^+ accumulation in the presence of KCl, with no difference in uptake rates whether 30 or 140 mM-KCl was used. Addition of valinomycin did not appreciably enhance H^+ uptake. Vesicles from resting stomachs (e.g. $P_3^R5\%$) were unable to accumulate H^+ until the addition of valinomycin. The dependence of H^+ uptake on valinomycin in resting vesicles, and the independence in stimulated vesicles, correlated with the $(H^+ + K^+)$-ATPase activities in these two conditions. $P_2^S16\%$ and $P_3^R5\%$ exemplified these characteristics. Other fractions from stimulated stomachs, while often showing good H^+ uptake in the presence of KCl alone, showed further enhancement with valinomycin. This may be due to contamination with 'resting-type' vesicles. Similarly, some fractions from resting tissue were capable of some H^+ uptake without valinomycin, although the addition of ionophore always resulted in an increased rate.

For further characterization of the $(H^+ + K^+)$-ATPase vesicles from stimulated and resting stomachs, two fractions were chosen, $P_3^R5\%$ and $P_2^R16\%$. These fractions were chosen on the basis of (*a*) demonstrating the highest specific activities of the $(H^+ + K^+)$-ATPase under the two physiological states (Table 4), (*b*) reduced levels of contaminating organelles (Tables 2 and 3), and (*c*) characteristic functional properties: i.e. stimulated vesicles, high specific activity of $(H^+ + K^+)$-ATPase and H^+ uptake in 30 mM-K^+, and no stimulatory effect of valinomycin; resting vesicles, low specific activity $(H^+ + K^+)$-ATPase and H^+ uptake in 30 and 140 mM-K^+, with marked stimulation by valinomycin (Fig. 1 and Table 4). These criteria are met by $P_3^R5\%$ from resting tissue, and $P_2^S16\%$ from stimulated tissue. For simplicity, these two vesicular populations will be called microsomes ($P_3^R5\%$) and s.a. vesicles ($P_2^S16\%$).

Orientation of gastric $(H^+ + K^+)$-ATPase vesicles

The total $(H^+ + K^+)$-ATPase and K^+-pNPPase activities apparent in gastric mucosae from stimulated stomachs were only about 64% and 80%, respectively, of those measured in resting tissue (Table 1). The lower activities in the stimulated tissue could be the result of (*a*) reduced numbers of the $(H^+ + K^+)$-ATPase enzyme, (*b*) the same number of $(H^+ + K^+)$-ATPase enzymes, but with reduced activity, or (*c*) isolation of a proportion of the $(H^+ + K^+)$-ATPase in an orientation inaccessible to ATP and *p*-nitrophenyl phosphate. The first two explanations would appear to be teleologically unsound, since maximal $(H^+ + K^+)$-ATPase activity is required when the stomach is in the stimulated, rather than the resting, state. Therefore, evidence for latent $(H^+ + K^+)$-ATPase enzyme was investigated.

Latency expressed by octyl glucoside. Permeabilization of s.a. vesicles by octyl glucoside resulted in increased $(H^+ + K^+)$-ATPase and K^+-pNPPase activities. Fig. 2 illustrates that 12 and 15 mM-octyl glucoside increased both activities, 15 mM producing the greater increase. Concentrations of greater than 15 mM-octyl glucoside reduced activities, and at 21 mM, the activities were reduced to less than 10% as compared with the control. Basal pNPPase activity was slightly enhanced by 12 and 15 mM-octyl glucoside, and an even greater increase was observed in the basal Mg^{2+}-ATPase activity (Fig. 2). The maximum $(H^+ + K^+)$-ATPase activity after treatment with 15 mM-octyl glucoside, 68.4 ± 9.4 ($n = 6$) μmol/h per mg, was 224% of that observed without octyl glucoside, 30.6 ± 5.3 ($n = 6$) μmol/h per mg. The increased activity as a result of octyl glucoside permeabilization was not due to increasing the intravesicular availability of K^+; $(H^+ + K^+)$-ATPase activity was similar when measured in 30 mM-K^+, 140 mM-K^+, or 140 mM-K^+ plus 10 μM-valinomycin, indicating K^+ permeability was not a limiting factor (Table 4). K^+-pNPPase activity was increased to a lesser degree than $(H^+ + K^+)$-ATPase activity; activity of 36.8 ± 1.6 ($n = 2$) μmol/h per mg after 15 mM-octyl glucoside was 155% of that without octyl glucoside [23.7 ± 3.9 ($n = 2$) μmol/h per mg].

Hypo-osmotic shock latency. As an alternative approach to investigate latent activity in the s.a. vesicles, a small volume (10 μl) of the vesicular suspension was added to 0.9 ml of a hypo-osmotic solution (approx. 70 mosM) for measurement of pNPPase activity. Enzyme activity in this hypo-osmotic solution was compared with that in an iso-osmotic solution (see the Materials and methods section). K^+-pNPPase activity in iso-osmotic conditions was 14.8 ± 1.0 ($n = 9$) μmol/h per mg, and this was increased by 55% in hypo-osmotic conditions to 23.0 ± 1.8 ($n = 9$) μmol/h per mg. Basal K^+-pNPPase activity was similar in iso- and hypo-osmotic solutions [1.7 ± 0.3 and 1.1 ± 0.2 ($n = 9$) μmol/h per mg, respectively]. The latent K^+-pNPPase activity measured with osmotic shock [8.3 ± 1.0 ($n = 9$) μmol/h per mg] and octyl glucoside permeabilization were of similar magnitude; 36% of the total activity was latent as determined by both techniques.

Tryptic sensitivity of $(H^+ + K^+)$-ATPase as a test of vesicular orientation. To complement the evidence

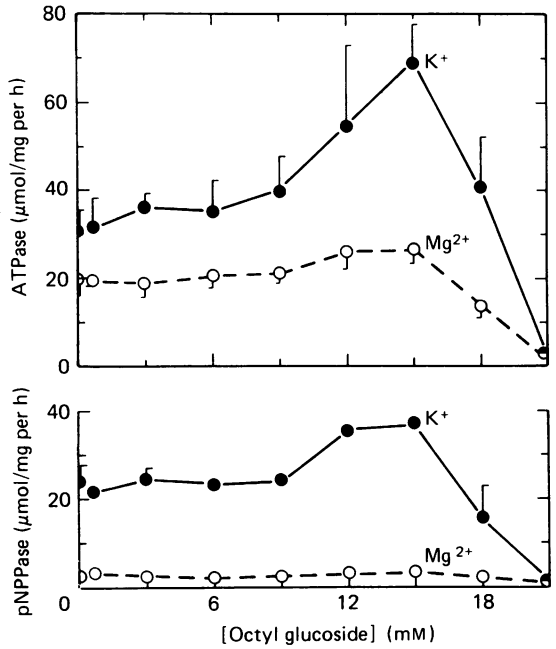

Fig. 2. Octyl glucoside permeabilization of s.a. vesicles

ATPase and pNPPase specific activities were measured after treatment of s.a. vesicles with octyl glucoside. ATPase activity was measured in the absence (Mg^{2+}, ○) and presence (K^+, ●) of 30 mM-K^+. pNPPase activity was measured in the absence (Mg^{2+}, ○) and presence (K^+, ●) of 10 mM-K^+. K^+-stimulated ATPase and pNPPase activities are illustrated after subtraction of activities in the absence of K^+. Each point illustrates the mean ± S.E.M. for six (ATPase) or two (pNPPase) preparations. Octyl glucoside treatment was as described in the Materials and methods section.

presented above for the orientation of the s.a. vesicles, $(H^+ + K^+)$-ATPase and K^+-pNPPase activities were determined after incubation with trypsin. Highly tryptic conditions were chosen, with a trypsin to membrane protein ratio of 1:10. In practice, these conditions resulted in inactivation of virtually all of the $(H^+ + K^+)$-ATPase and K^+-pNPPase activity in microsomes after as little as 5 min at 30 °C (Fig. 3). Similar incubation of s.a. membranes showed approx. 10% residual non-latent activity even after 60 min incubation. In contrast, Fig. 3 illustrates that the latent $(H^+ + K^+)$-ATPase and K^+-pNPPase activities were unaffected by tryptic digestion for periods up to 60 min. Expressed as activity relative to control samples, trypsin treatment for 30 min showed no significant reduction in $(H^+ + K^+)$-ATPase [0.87 + 0.10 ($n = 5$)] nor K^+-pNPPase [1.05 + 0.20 ($n = 3$)] latent activities. At the same time, non-latent $(H^+ + K^+)$-ATPase and K^+-pNPPase activities were reduced by 70–80%; 0.20 + 0.03 ($n = 5$) and 0.29 ± 0.10 ($n = 3$), respectively. The data on the tryptic sensitivity of the non-latent $(H^+ + K^+)$-ATPase activity would suggest that approx. 20% of the non-latent activity may be localized in rso vesicles that are inaccessible to trypsin but are leaky to ATP. The remainder non-latent activity is present in iso orientated apical vesicles, similar to the microsomes. The latent activity is situated in rso vesicles, and the tryptic sensitive sites are thus not accessible.

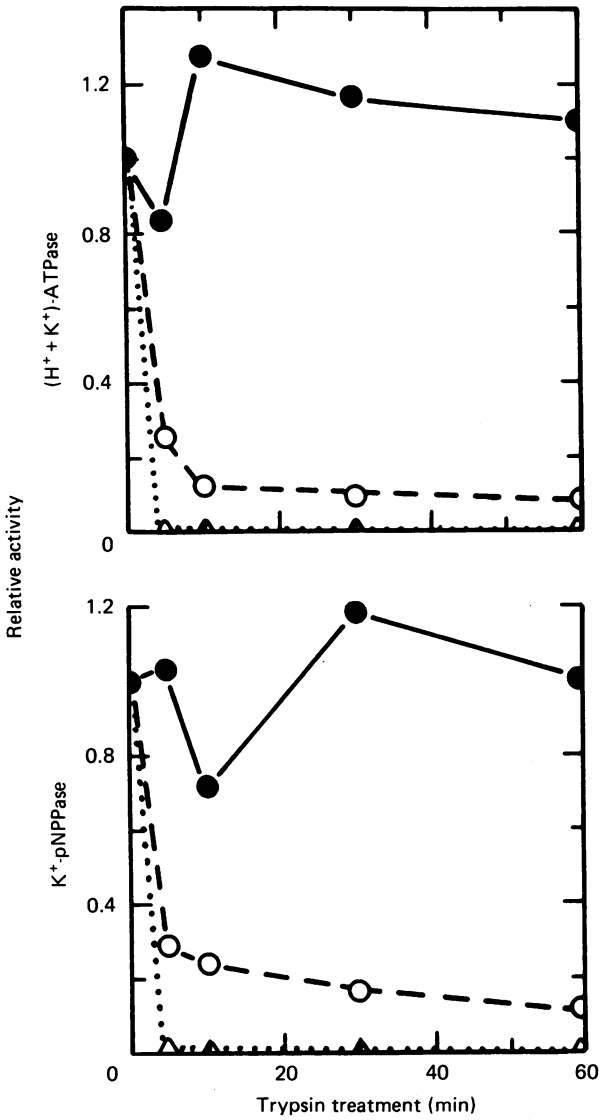

Fig. 3. Time course of trypsin digestion of s.a. vesicles and microsomes

S.a. vesicles and microsomes were incubated with trypsin at a trypsin/membrane protein ratio of 1:10 for up to 60 min at 30 °C. $(H^+ + K^+)$-ATPase activity was measured in 30 mM-K^+; K^+-pNPPase activity was measured in 10 mM-K^+. All enzyme activities are expressed relative to control values where soya-bean trypsin inhibitor was added before trypsin. Values are shown for gastric microsomes (△) and s.a. vesicles (○). Also shown are the latent activities for s.a. vesicles (●), which are the activities after permeabilization with 15 mM-octyl glucoside. These latter values were corrected for activities measured without octyl glucoside.

DISCUSSION

The molecular basis for gastric acid secretion is a proton pump, dependent on Mg^{2+} and K^+, and driven by ATP: the $(H^+ + K^+)$-ATPase (Ganser & Forte, 1973; Forte *et al.*, 1975; Sachs *et al.*, 1976; Berglindh *et al.*, 1980). Stimulation of the parietal cell results in structural and functional changes in the membranes in which the $(H^+ + K^+)$-ATPase is isolated (Wolosin & Forte,

1981*a*,*b*; Im *et al.*, 1984). The present detailed comparison of the distribution of the $(H^+ + K^+)$-ATPase in subcellular fractions from stimulated and resting tissue has confirmed the usefulness of the separation of $(H^+ + K^+)$-ATPase-rich membranes described by Wolosin & Forte (1981*a*). In the present study, subcellular fractionation of the rabbit gastric mucosa whilst in the resting (cimetidine-inhibited) state resulted in recovery of the majority of the $(H^+ + K^+)$-ATPase activity in the microsomal fraction (Table 1). These microsomes demonstrate a typical characteristic of gastric microsomal vesicles, i.e. a low K^+ permeability as indicated by the marked stimulation of $(H^+ + K^+)$-ATPase activity in the presence of valinomycin. Purification of the crude microsomal fractions (P_3) by Ficoll density gradient centrifugation yields a fraction ($P_3{}^R 5\%$) which shows very low rates of ATP hydrolysis in 30 and 140 mM-K^+, and a 23-fold stimulation of ATPase activity in the presence of valinomycin (Table 4). In addition, proton accumulation in these microsomes is almost entirely dependent on the addition of the K^+ ionophore (Fig. 1). Thus the microsomes, whilst containing the $(H^+ + K^+)$-ATPase required for acid secretion, are in an inactive state due to the exclusion of K^+ from their interior. There is convincing evidence that cellular correlates of the gastric microsomes are the tubulovesicles visible within resting parietal cells (Forte *et al.*, 1975; Lee *et al.*, 1979).

When isolated from stimulated tissue, the $(H^+ + K^+)$-ATPase is redistributed to larger, denser vesicles (Table 1). There is compelling evidence that these new s.a. vesicles are derived from the apical plasma membrane (Wolosin & Forte, 1981*a*, *b*, 1984). The change in subcellular distribution correlates with the morphological changes described in the Introduction, where the apical membrane is greatly expanded at the cost of the tubulovesicular membranes. Additional evidence with monoclonal antibodies indicates that this translocation of membrane includes the $(H^+ + K^+)$-ATPase (Smolka *et al.*, 1983). The redistribution of $(H^+ + K^+)$-ATPase upon stimulation is specific; there is very little change in the subcellular distribution of other markers (Table 1 and Wolosin & Forte, 1981*a*).

Accompanying the redistribution of the $(H^+ + K^+)$-ATPase is a dramatic change in the K^+ permeability. S.a. vesicles show maximal $(H^+ + K^+)$-ATPase activity and proton accumulation, even in low K^+ concentrations, with no further increases upon the addition of valinomycin (Table 4 and Fig. 1). Thus, in addition to the incorporated $(H^+ + K^+)$-ATPase, the s.a. vesicles also have pathways for K^+ and Cl^-, and there is evidence that these pathways are conductive (Wolosin & Forte, 1984). Therefore, the s.a. vesicles contain all the apparatus required for net transport of HCl by the secretory membrane of the parietal cell: the $(H^+ + K^+)$-ATPase in parallel with K^+ and Cl^- conductances. The origin and activation of the K^+ and Cl^- conductances are a central issue for parietal cell physiology, but remain outside the scope of the current study.

To aid in further characterization of s.a. vesicles, we purified the crude mitochondrial fraction (P_2) on a Ficoll density gradient (Table 2). For comparison, we similarly purified gastric microsomes from P_3 (Table 3). The fractions respectively designated as $P_2{}^S 16\%$ and $P_3{}^R 5\%$ had the highest specific activities of $(H^+ + K^+)$-ATPase and the associated K^+-pNPPase under the two physiological states, and showed reduced levels of contaminating organelles (Tables 2, 3, and 4). The specific activity of the $(H^+ + K^+)$-ATPase in s.a. vesicles ($P_2{}^S 16\%$) was about one-quarter of that of microsomes ($P_3{}^R 5\%$, 25.1 and 108.9 μmol/h per mg, respectively (Tables 2 and 3). If the maximum $(H^+ + K^+)$-ATPase activity in the s.a. vesicles after expression of latent activity with octyl glucoside is compared (68.4 μmol/h per mg; Fig. 2), then the difference between the two fractions is reduced, but still large. Octyl glucoside treatment may have partially inhibited the $(H^+ + K^+)$-ATPase, as is obvious with the higher concentrations (Fig. 2), accounting for some of the difference in specific activity. However, much of the difference in specific activity may be the result of the more complex nature of s.a. vesicles. Polyacrylamide-gel electrophoresis reveals that, compared with microsomes, which show one predominant band corresponding to M_r 100,000 and which is thought to comprise the polypeptides of the $(H^+ + K^+)$-ATPase (Saccomani *et al.*, 1977), there are several other bands in s.a. vesicles, most prominent of which is actin (Wolosin & Forte, 1981*a*). In addition, the K^+ and Cl^- conductances present in the s.a. vesicles are another possible source of protein which would result in a reduction in the specific activity of the ATPase in the s.a. vesicles.

The total $(H^+ + K^+)$-ATPase and K^+-pNPPase recovered from stimulated gastric mucosa was less than from the resting tissue (Table 1). In addition, the presence of actin and actin-like microfilaments within the s.a. vesicles (Wolosin *et al.*, 1983) is reminiscent of renal and intestinal brush border membranes. Both these observations suggested a proportion of the s.a. vesicles had a rso orientation. This was tested for by investigating for latent activity by permeabilizing the s.a. vesicles with octyl glucoside, which increased $(H^+ + K^+)$-ATPase activity by greater than 2-fold (Fig. 2). We estimate that approx. 55–65% of the $(H^+ + K^+)$-ATPase activity in the $P_2 16\%$ of s.a. vesicles is contained in rso vesicles.

There was less latent K^+-pNPPase activity in the s.a. vesicles. Octyl glucoside treatment increased K^+-pNPPase activity by 55% compared with the 124% increase in $(H^+ + K^+)$-ATPase activity (Fig. 2). A similar latent K^+-pNPPase was demonstrated by hypo-osmotic shock treatment. The difference in latency of ATPase and pNPPase activities may be explained if one assumes the vesicles show a greater permeability to *p*-nitrophenylphosphate as compared with ATP.

The latent $(H^+ + K^+)$-ATPase and K^+-pNPPase activities are both resistant to highly tryptic conditions (Fig. 3). In addition, about 20% of the non-latent $(H^+ + K^+)$-ATPase activity is also resistant to trypsin digestion. The resistance of the latent activities to trypsin digestion is consistent with these activities being contained in rso vesicles. We interpret the resistance of a proportion of the non-latent activities to the tryptic digestion, as a proportion of the enzyme being in rso vesicles which are leaky with respect to ATP and pNPP, but still impermeable to trypsin. With these assumptions, it can be estimated that approx. 55% of the $(H^+ + K^+)$-ATPase activity in s.a. vesicles is in rso tight vesicles, 10% in rso leaky vesicles, and 35% in iso vesicles. These latter vesicles are entirely responsible for the proton accumulation (Fig. 1).

Future studies will be involved in the separation of rso and iso orientated vesicles. Such separation of the two populations of s.a. vesicles will be of use in answering important questions concerning the surface properties of

the vesicles in relation to transport and protective functions. The resistance of the rso vesicles (latent activity) to highly degradative tryptic conditions is of particular interest to the latter. In addition, preliminary experiments demonstrate a degree of resistance to H^+. Incubation of s.a. vesicles at pH 1.9 for 4 min resulted in a 77% loss of non-latent activity, but only 22% loss of latent activity. The role of the apical coat of glycosubstances (Forte & Forte, 1970; Beesley & Forte, 1973) as a putative protective mechanism is of particular interest, and the rso vesicles are an attractive model for the study of such mechanisms.

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