J. Membrane Biol. 32, 361–381 (1977)

Cation Transport by Gastric $H^+ : K^+$ ATPase

R. Schackmann, A. Schwartz, G. Saccomani, and G. Sachs*

Laboratory of Membrane Biology, University of Alabama in Birmingham, Birmingham, Alabama 35294, and Department of Cell Biophysics, Baylor University, Houston, Texas

Received 13 May 1976; revised 2 September 1976

Summary. A vesicular microsomal fraction isolated from hog fundic mucosa demonstrates the capacity to take up equal amounts of RB^+ and Cl^-. The amount of the Rb^+ uptake is sensitive to the extravesicular osmolarity, and rate of uptake is sensitive to temperature. $^{86}Rb^+$ efflux is dependent upon the cation composition of the diluting solution. ATP, but not β-γ methylene ATP, induces a reversible efflux of $^{86}Rb^+$ from loaded vesicles, and this is dependent upon a functional K^+-ATPase. The ATP induced efflux is not affected by CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) or TCS (tetrachlorosalicylanilide) nor by lipid soluble ions or valinomycin. Nigericin inhibits the efflux by 40%. Uptake of the lipid soluble ion $^{14}C\text{-}SCN^-$ has been demonstrated and is enhanced by ATP only in the presence of valinomycin. The results are consistent with a neutral or isopotential exchange of H^+ for Rb^+ mediated by K^+-ATPase.

The availability of a vesicular preparation from hog gastric mucosa has allowed an experimental approach to the role of K^+ in gastric acid secretion due to the capacity of the vesicles to take up H^+ in the presence of ATP, in a K^+ dependent fashion.

Work on the intact amphibian mucosa has shown that K^+ is essential for acid secretion (Davis *et al.*, 1965). Rb^+ and Cs^+, but not Na^+ or Li^+, can partially substitute for K^+ (Forte, Forte & Saltman, 1967). In K^+-free solutions with complete inhibition of the acid rate, the intracellular K^+ content remains high, falling only by 10 to 20% (Davis *et al.*, 1965). This has been interpreted as evidence for a K^+ compartment necessary for H^+ secretion. In intact dog stomach, insulin inhibition is also reversed by injection of K^+ or Rb^+, and results of flux studies have also been interpreted as providing evidence for a K^+ compartment (Hirschowitz & Sachs, 1967). In contrast, high K^+ levels in the serosal solution of frog mucosa reduce active Cl^- secretion significantly (Hogben, 1968). Measurement of K^+ movement across gastric mucosa showed that the unidirectional K^+ flux is considerably less than the H^+ rate

* *To whom correspondence should be addressed.*

(Sachs *et al.*, 1969) and this K^+ flux was inhibitied by SCN^- as was the H^+ rate. In Cl^- free, SO_4^{2-} containing solutions, high mucosal [K^+] results in a significant increase in H^+ rate as well as in an inversion of the potential difference from mucosal positive to mucosal negative (Davis, Rutledge & Rehm, 1963). Passage of current to produce the same P.D. change as produced by K^+ in SO_4^{2-} solutions also stimulates H^+ secretion.

The discovery of a K^+ *p*-nitrophenyl phosphatase (Forte *et al.*, 1967) K^+-acetyl phosphatase and K^+-ATPase activity (Forte *et al.*, 1975; Sachs *et al.*, 1966; Saccomani *et al.*, 1975) in fundic mucosa of various species suggested that the role of K^+ might be as an activator of a proton transporting ATPase (Sachs & Hirschowitz, 1968). The demonstration of H^+ transport by dog microsomes (Lee, Simpson & Scholes 1974) or hog gastric membrane fractions (Sachs *et al.*, 1975, 1976) allowed a direct test of the transport functions of these preparations and the K^+-ATPase in particular. It was established that K^+ (or Rb^+ and Cs^+) was necessary for H^+ uptake (Sachs *et al.*, 1976). In this work we establish that the role of K^+ in the vesicle is not only as an activator of the K^+-ATPase but that K^+ (or Rb^+) is transported in exchange for H^+ by the K^+-ATPase.

Materials and Methods

Preparation of Vesicles

The method has been described in detail elsewhere (Saccomani *et al.*, 1977). Fundic mucosa is homogenized in 0.25 M sucrose and centrifuged for 40 min at $26000 \times g$ and the supernatant is centrifuged for 60 min at $100{,}000 \times g$ to provide the microsomal pellet. This fraction was further separated on a discontinuous gradient of 7% ficoll and 20% ficoll both in 250 mM sucrose in a Beckman Z60 zonal rotor at 60,000 rpm for 2 hr. The fraction at the 7% ficoll interface was free of mitochondrial markers such as succinic dehydrogenase, monamine oxidase or cytochrome oxidase and was 20-fold enriched in K^+-ATPase, K^+-pNPPase and 5′-AMPase activity. Further purification of this fraction was obtained by free flow electrophoresis using 8 mM tris-acetate buffer at pH 7.4, 0.1 mM MgATP in 0.25 M sucrose as curtain buffer and 100 mM tris-acetate pH 7.4 as electrode buffer. The separation was carried out at 7.5 °C with a voltage gradient of 120 v/cm and a flow rate of 180 ml/hr distributed through 90 tubes. This fractionation produced a fraction 35-fold enriched in K^+-ATPase (*F1*) and another fraction enriched in 5′-AMPase (*F11*). The *F1* fraction was essentially free of 5′-AMPase or Mg^{2+}-ATPase and contained essentially only 1 peptide region of 100,000 M_r on SDS gel electrophoresis. The zonal gradient or *F1* fractions were used for the studied outlined as specified in the text (Table 1), but the majority of transport studies were carried out on the gradient fraction.

ATPase Assay

ATPase assays were performed in the same incubation medium as the Rb^+ efflux studies. The incubation contained in 1 ml: ca. 170 μg protein, 75 mM RbCl, 50 mM tris-

Table 1. Purification of gastric membranes

	μmole Pi mg^{-1} hr^{-1}		
	Mg^{2+}-ATPase	K^+-ATPase[a]	5′-nucleotidase
Total homogenate	6.2±0.6	2.0±1.0	0.5±0.3
Microsomal fraction	15.9±1.5	7.1±4.4	1.5±0.6
Gradient fraction (*GI*)	6.4±1.3	32.5±3.2	4.6±0.1
Electrophoretic fraction *FI*	2.7±0.6	64.1±3.9	0.6±0.2
Electrophoretic fraction *FII*	16.6±2.0	17.3±1.2	14.7±1.2

(n=5)

[a] The K^+-ATPase activity is the difference between activity is presence and absence of K^+.

acetate or 5 mM glycylglycine buffer, pH 6.1, 125 mM sucrose, 2 mM $MgCl_2$ and 2 mM ATP. The reaction was carried out at room temperature for varying times between 10 sec and 15 min. The phosphate liberated was measured by butyl acetate extraction of the phosphomolybdrate complex (Yoda & Hokin, 1970) and protein by Lowry's method (Lowry *et al.*, 1951).

Transport Studies

Uptake. For uptake studies vesicles obtained from the gradient at a concentration of 2–6 mg/ml protein were added to an equal volume of a medium containing 150 mM RbCl containing 10 μCi $^{86}Rb^+$, 80 mM tris-acetate or 10 mM glycylglycine buffer pH 6.12 and 4 mM $MgCl_2$, to give a final volume of 400 μl. The pH of 6.12 was chosen so as to be able to compare the data with those of H^+ uptake. Incubation was carried out at varying temperature and 20 μl samples were taken and added to 1 ml of ice-cold "stop" solution containing 150 mM choline chloride and 40 mM tris-acetate buffer (or 5 mM glycylglycine) buffer pH 6.12. After vortexing the sample was filtered on a Millipore HAWP filter (0.45 μ). The filter was washed twice with 4 ml of the stop solution, dried and the radioactivity measured in a LKB 8100 scintillation counter. As necessary osmolarity was varied by adding mannitol to the incubation medium.

Efflux. Efflux studies were performed on vesicles which had been equilibrated with radioisotope by incubating for 48 hr at 4 °C in the uptake conditions just described. This incubation time was necessary to achieve equilibrium at that temperature. High temperatures of preincubation even for times as short as 2 hr at 37 °C resulted in vesicular damage. Two types of efflux study were performed: efflux with a gradient of Rb^+ or $^{86}Rb^+$, and efflux induced by the addition of ATP with no initial gradient present.

For efflux with a gradient the preloaded vesicles were diluted into a medium containing different external cation such as choline, K^+, Rb^+, Cs^+, Na^+ or Li^+ as chloride salts at 150 mM with other constituents the same as in the equilibration solution. Sampling and washing were carried out as for the uptake studies. These efflux studies allowed measurement of the cation selectivity of the exchange efflux pathway. Ionophores such as valinomycin, 10^{-6} M, nigericin, 1 mg/ml, tetrachlorsalicylanilide (TCS), 10^{-5} or 10^{-6} M, or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 10^{-4} or 10^{-5} M, were added in 4 µl methanol.

Efflux in the equilibrium situation was assessed by adding ATP at pH 6.12 at varying concentrations directly to the vesicles in the equilibrated medium and sampling as usual. Ionophores as above or lipid permeable ions such as dimethyl dibenzyl ammonium (DDDA), dipicrylamine (DPA), or SNC^- were added as necessary at 1 or 10 mM concentrations.

Control experiments to determine binding of isotope to the filters were performed by solubilizing the vesicles with 1% triton X-100 in the stop solution, or by diluting the vesicles into H_2O, rather than stop solution before filtration. The former procedure reduced the radioactivity by 95% and the latter by 85% in terms of the activity usually trapped on the filter.

On occasion $^{22}Na^+$ or $^{137}Cs^+$ were used; and the experiments were performed as for $^{86}Rb^+$. In other experiments $^{36}Cl^-$ or ^{14}C-SCN^- were used, and with these 120 mM Li_2SO_4 was used as stop solution instead of choline chloride. For experiments in which SO_4^{2-} replaced Cl^-, 75 mM Rb_2SO_4 replaced the 150 mM RbCl solutions. Sucrose was used to adjust the SO_4^{2-} medium to maintain isotonicity.

H^+ Uptake Studies. Vesicles preincubated in 150 mM salt as above were added to a medium containing 150 mM salt and 5 mM glycylglycine pH 6.12 in a stirred vessel at room temperature. The change of pH was measured by a combination pH and calomel electrode connected to a pHM64 pH meter and a Radiometer Servorecorder, with a sensitivity of 1 cm per 0.01 pH units. 0.3 mM ATP was added to initiate the reaction and ionophores were added as above in methanol.

Results

Uptake of Cations

Kinetics. Uptake of Rb^+, Cs^+ or Na^+ was temperature and time dependent. Little difference was noted in uptake rate between the different

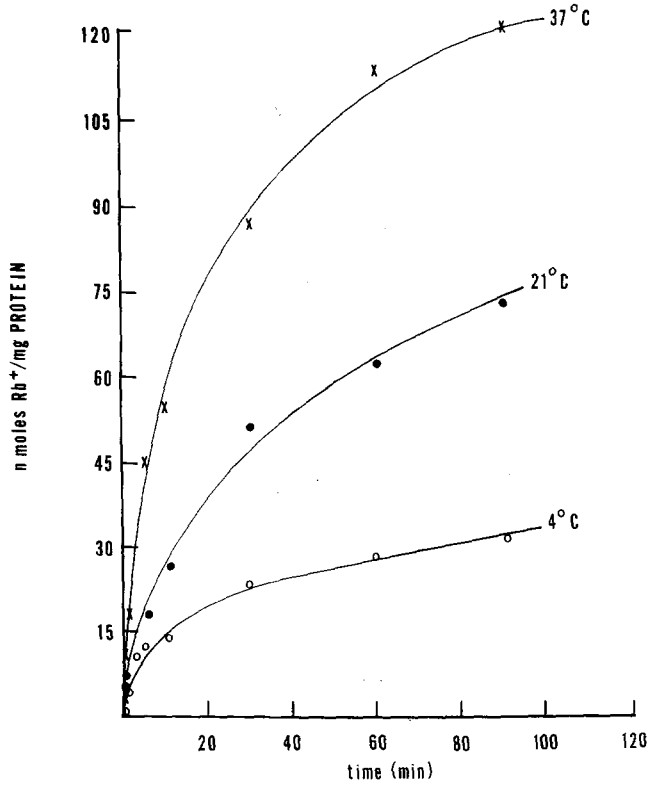

Fig. 1. Uptake of Rb^+ at three temperatures. *G1* vesicles in 250 mM sucrose, 200 µl, were mixed with 200 µl of a solution containing 150 mM RbCl, 10 mM glycylglycine, pH 6.12, 4 mM $MgCl_2$, ×—×, 37 °C; ●—●, 21 °C; o—o, 4 °C. Sampling was performed in triplicate at times longer than 5 min and in duplicate up to five min. The final protein concentration was 1.9 mg/ml and the final levels of uptake were equal after 48 hr further incubation

cations, and the final level reached was identical for the three cations studied.

The uptake of $^{86}Rb^+$ with time is shown in Fig. 1 for the same preparation at 3 different temperatures. It can be seen that the process is time and temperature dependent. At 37 °C, the uptake curve is fitted to a single exponential with a $t_{1/2}$ of 16.5 min ($r=0.991$). At room temperature, the equilibrium value is not reached except after several hours incubation, and this value is lower than that reached at 37 °C in 2 hr, suggesting that some impairment of vesicular impermeability has occurred. This is substantiated by the finding that H^+ uptake is reduced and a greater leak of the ATP induced H^+ gradient is observed. The $t_{1/2}$ using the actual equilibrium value obtained at room temperature had a range of 28 to 51 min with a mean of 40 ± 9 min ($n=5$). The

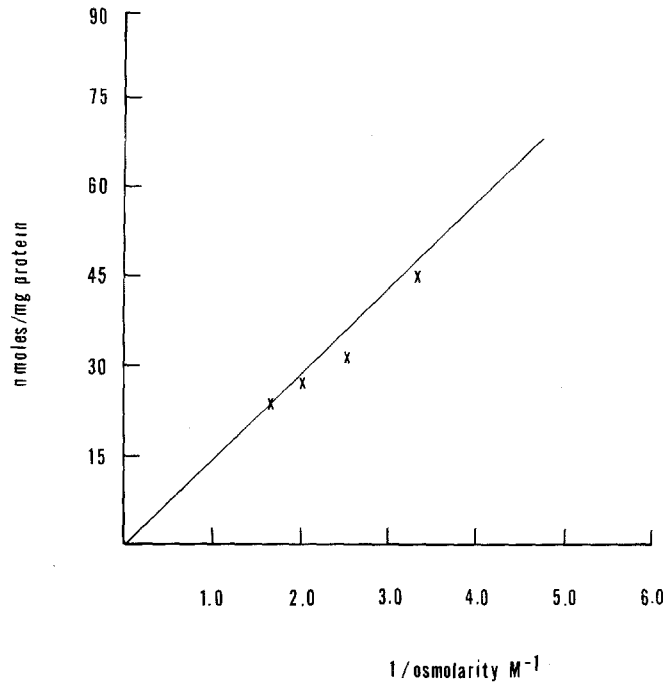

Fig. 2. Variation of osmolarity on $^{86}Rb^+$ uptake. *GI* vesicles were added in 250 mM sucrose to a medium containing 150 mM RbCl, 10 mM glycylglycine, pH 6.12, 4 mM $MgCl_2$ with varying amounts of mannitol to vary the medium osmolarity. The samples were taken in triplicate 10 min after mixing. The uptake was performed at 21 °C and the final protein concentration was 2.9 mg/ml

variation from preparation to preparation is presumably due to a varying degree of permeability of the vesicles as they are prepared. Maximal final uptake was observed by incubating the vesicles at 4 °C for 48 hr, which procedure had the least effect on H^+ uptake; hence, this procedure was used to load the vesicles with cations. The equilibrium level at this temperature allowed calculation of an intravesicular volume of 2.0 ± 0.3 µl per mg protein ($n = 23$).

Binding. Variation of medium osmolarity results in alteration of Rb^+ uptake as shown in Fig. 2. It can be seen that there is minimal binding of Rb^+, since there is no apparent uptake predicted at infinite osmolarity. The mannitol used as a nonpermeant species does, however, slowly penetrate the vesicles ($t_{1/2} > 2$ hr), hence only short exposures to the mannitol medium were used.

A Scatchard plot of the Rb^+ taken up at varying initial Rb^+ concentrations (Fig. 3) showed that above 10 mM a zero slope was observed as would be expected of uptake into a nonsaturating intravesicular

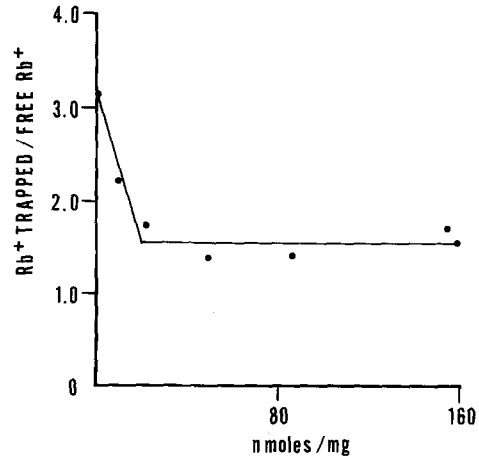

Fig. 3. Scatchard plot for equilibrium uptake of Rb^+. Uptake was performed at 4 °C for 72 hr with varying Rb^+ concentrations with 2 mM $MgCl_2$, 40 mM trisacetate, pH 6.1. Isotonicity was maintained by varying the sucrose concentration. *Ordinate:* nmoles Rb^+ trapped/nmoles Rb^+ in the aliquot. *Abscissa:* nmoles Rb^+ trapped mg^{-1}. The sampling was performed in quadruplicate at 21 °C. The final protein concentration was 0.82 mg/ml

volume. Below 10 mM the slope of the line suggests that some binding may occur. Subtraction of the uptake of Rb^+ allows calculation of the total number of binding sites to be approximately 5 nmoles/mg protein. This value may be neglected when dealing with the usual uptake of 150 nmoles or so.

Nature of Vesicle. Uptake of Rb^+ into the *F1* and *F11* fractions showed similar levels compared to the *G1* fraction (Table 2). The combined uptake of Rb^+ in *F1* and *F11* in this experiment was 191 nmoles/mg based on 72% of *G1* as *F1* and 28% *F11* (Saccomani *et al.*, 1977). This level compares within experimental error to the 181 nmoles/mg uptake by the *G1* fraction implying that during free flow electrophoresis little vesicular damage occurs.

Table 2. $^{86}Rb^+$ uptake and ATP-stimulated efflux from *G1* and *G1* free-flow electrophoresis

Fraction	Percentage of *G1* as protein	Equilibrium uptake nmole/mg protein	ATP (2 mM) induced efflux nmole/mg protein
G1	100	181	79
G1–F1	72	199	115
G1–F11	28	171	15

Efflux was measured 10 min after the addition of ATP

Effect of ATP. The addition of 2 mM ATP to the uptake medium reduces the uptake in the first 30 min by 50%.

Uptake of Anion

Chloride. The uptake of Cl^- at room temperature is similar to that of Rb^+ and reaches the same equilibrium level as Rb^+. The mean $t_{1/2}$ was 36 min. The ratio of the $t_{1/2}$ shows that the permeability ratio of Rb^+ to Cl^- is approximately equal. However, addition of nucleotide is without effect on Cl^- uptake or distribution under any of the conditions of study.

Thiocyanate. SCN^- shows a much more rapid uptake ($t_{1/2}=2$ min). This is probably due to the presence of an additional uptake path for SCN^-, i.e., a lipophilic route (Scarborough, 1976). In the presence of valinomycin the uptake is further increased. This enhancement of uptake is independent of the initial K^+ gradient.

The presence of valinomycin and a K^+ gradient would be expected to induce a potential which would increase SCN^- uptake and result in a potential-dependent SCN^- concentration gradient. This gradient would then dissipate at the same rate as the K^+ gradient. This expectation is contrary to the experimental findings. Thus the enhanced uptake in the presence of valinomycin is present even in vesicles equilibrated in KCl prior to $S^{14}CN^-$ addition. The interpretation of the increase uptake of SCN^- in the presence of valinomycin is problematic. It could be due to binding of SCN^- to the membrane that is dependent on the presence of the lipophilic valinomycin-K^+ complex. ANS shows a similar enhanced binding (Lewin *et al.*, 1977). This binding could then obscure a transient due to the K^+ gradient dependent potential. Alternatively, the combination of a lipophilic anion and the lipophilic valinomycin K^+ complex could result in rapid equilibration of K^+.

The addition of ATP has no effect on the equilibrium level of SCN^- in the absence of valinomycin, as for $^{36}Cl^-$. In contrast to Cl^- however, in the presence of a valinomycin there is an enhanced SCN^- uptake (Fig. 4). From the data presented below, there is a K^+ gradient induced by ATP, which in the presence of valinomycin would induce a positive potential inside the vesicles. This is the simplest interpretation of the ATP induced redistribution. The ATP dependent SCN^- uptake is osmotically sensitive, hence is not due to binding. Since the fraction of transporting vesicles is not known, calculation of the potential difference gives a minimal value, assuming all vesicles are transporting equally (i.e., have an identical K^+ gradient), of approximately 18 mV.

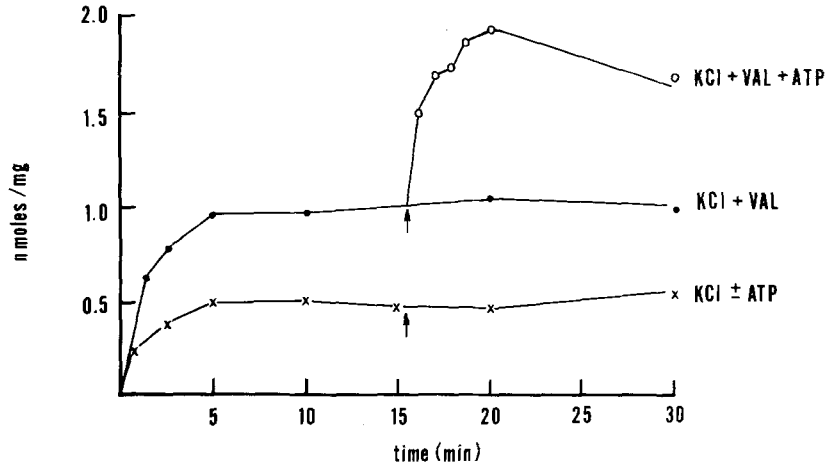

Fig. 4. Uptake of $S^{14}CN^-$. *GI* vesicles in 250 mM sucrose, 200 ml were mixed with 200 µl of a solution containing 150 mM KCl, 80 mM tris-acetate, 4 mM $MgCl_2$, and 1.8 mM NaSCN (4 µCi ^{14}C). Valinomycin was added in 4 µl methanol to give 10^{-6} M. ATP was added (arrows) to give a final concentration of 2 mM. ×—×, $-S^{14}CN$ uptake with or without ATP; ●—● $-S^{14}CN$ uptake with valinomycin; ○—○ $-S^{14}CN$ uptake with valinomycin and ATP added after 15 min

Efflux of Cation

Dilution Efflux

This was performed as detailed in *Materials and Methods*. The rate of efflux of $^{86}Rb^+$ was influenced by the cation composition of the external medium. The sequence of effectiveness of various cations (Table 3) in terms of the inverse of the $t_{1/2}$ of efflux was, with the rate in the presence of K^+ set arbitrarily at 100, K^+ 100; Rb^+ 82; Cs^+ 64; Na^+ 20; Li^+ 11; choline 2. This efflux seems to be due, therefore,

Table 3. Exchange efflux of $^{86}Rb^+$

External cation	$t_{1/2}$ (min)
K^+	1.8
Rb^+	2.2
Cs^+	2.8
Na^+	9
Li^+	16
Choline	80

The mean of three experiments.

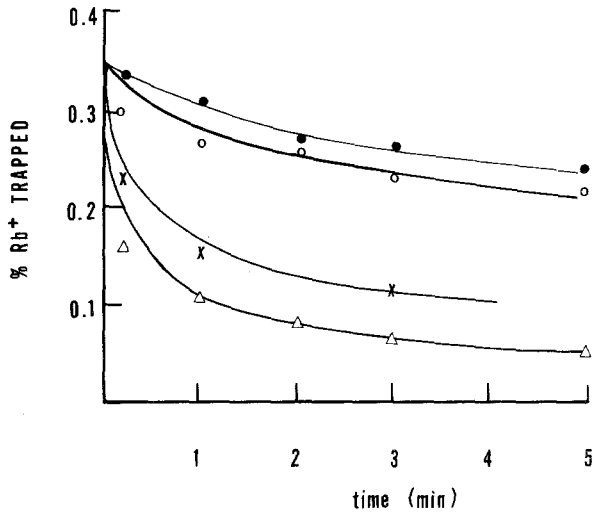

Fig. 5. Exchange efflux of $^{86}Rb^+$ in the presence of ionophores. Vesicles were equilibrated with $^{86}Rb^+$ in 75 mM RbCl, 125 mM sucrose, 2 mM $MgCl_2$, 5 mM glycylglycine, pH 6.12 for 72 hr at 4 °C. The efflux of $^{86}Rb^+$ was initiated by diluting 200 µl of the vesicles into 800 µl of solutions containing 150 mM MCl, 2 mM $MgCl_2$, 5 mM glycylglycine, pH 6.12. ●—●, M=Na^+; ○—○, M=Na^+ with 10^{-6} M valinomycin; ×—×, M=Na^+ with 1 µg/ml nigericin; △—△, M=Rb^+ with 10^{-6} M valinomycin. The experiment was performed at 21 °C and the final protein concentration was 2 mg/ml

to a cation/cation exchange process, the selectivity of this exchange corresponding to the sequence of activation of the ATPase or H^+ uptake (Sachs *et al.*, 1976). It seems possible, therefore, that the pathway is due to pump sites.

Addition of ionophores affects the dilution efflux according to their known selectivities. Thus valinomycin (Fig. 5) enhances efflux of Rb^+ with Rb^+ or K^+ as external cation, but does not affect efflux with Na^+ as external cation. Nigericin also stimulates efflux in the presence of Na^+. The permeability in uptake studies was calculated to be 10^{-9} cm sec^{-1} and for efflux with external K^+, 2×10^{-8} cm sec^{-1} at RT.

Equilibrium Efflux

Effect of ATP. Most of the studies were performed on efflux from an equilibrium situation. This transport may be primary active, but could be secondary active due to coupling to a potential of an electrogenic pump or due to contraction of the vesicles. The filtration technique can detect a transient efflux due to various possibilities. First, the treatment with ATP could result in a sudden increase in Rb^+ permeability.

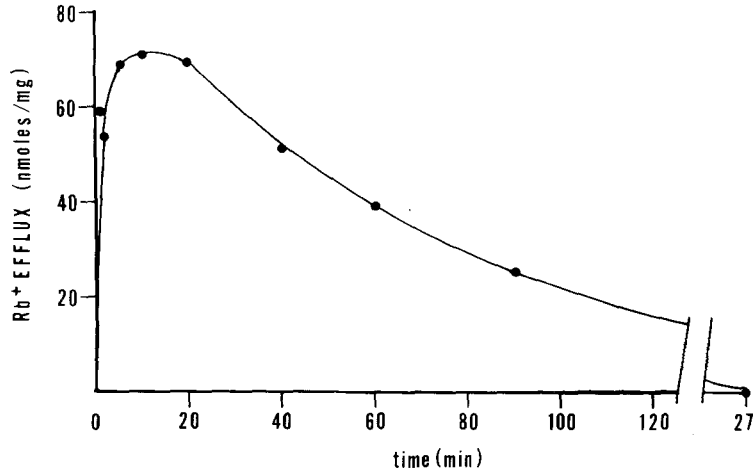

Fig. 6. Typical ATP stimulated efflux of Rb^+ from equilibrated vesicles. *G1* vesicles were incubated in 75 mM RbCl, 125 mM sucrose, 2 mM $MgCl_2$, 5 mM glycylglycine, pH 6.12 for 48 hr at 4 °C. ATP (2 mM) was added in 6.5 μl to initiate the efflux at 21 °C. Sampling was performed in triplicate at times over five min. and in duplicate up to five min

Such vesicles placed in the stop solution would lose Rb^+ more than untreated controls. However, there are several findings which exclude this possibility. Proton uptake studies show that the vesicles do not become extremely permeable to either H^+ or K^+. With the addition of ATP Rb^+ uptake is decreased by ATP. The effect of valinomycin on SCN^- uptake and ANS fluorescence engendered by ATP can be most easily explained by the development of a K^+ gradient (Lewin *et al.*, 1977). $^{36}Cl^-$ efflux or $S^{14}CN^-$ efflux would be expected with an increased leakiness and is not observed. Contraction of the vesicles would likewise enhance Cl^- or SCN^- efflux. A reduction of ATP induced efflux in the presence of nigericin occurs and this would not be expected of an increased wash out phenomenon.

Alternatively, an actual concentration gradient develops in the solution to which the ATP is added, and with loss of ATP due to hydrolysis, the previous equilibrium is reestablished. Fig. 6 shows the typical time course of the Rb^+ efflux and reequilibration with ATP addition to the *G1* fraction.

Kinetics. The efflux produced by ATP is rapid and the maximum efflux obtained is a function of the ATP concentration used. The K_M calculated from the Eadie-Hofstee plot was 2.5×10^{-4} M. This value is similar to the K_M for the ATPase and H^+ uptake activities of the vesicles (Sachs *et al.*, 1976).

Table 4. $^{86}Rb^+$ Efflux as a function of high energy phosphates

Nucleoside phosphate (2 mM)	nmole/mg uptake[a]	nmole/mg efflux[b]	Percentage of ATP induced efflux[c]
ATP	187	91	100
CTP	168	56	87
GTP	194	10	17
ITP	182	16	28
UTP	198	0	0
β-γ methylene ATP	134	0	0
Acetyl phosphate	118	8	15
pNitrophenyl phosphate	117	1	2

[a] Differences in uptake levels are due to the different preparations used.
[b] Maximal efflux taken 10 min after the addition of the nucleotide.
[c] Percentages were based on the average ATP induced effluxes for the preparations used.

Varying the ATP concentration also changes the initial rate of Rb^+ efflux, but with the filtration technique used, the initial 15 sec values are close together and could not be adequately analyzed.

The maximal efflux observed is a function of passive influx and passive efflux as well as of active efflux. The finding of a correspondence between the K_M of the efflux process with respect to ATP and the H^+ uptake and ATPase activity suggests that the active efflux is predominant. The long $t_{1/2}$ values for both efflux and influx as compared to the ATP effect substantiates this suggestion.

With increasing ATP the gradient is maintained for longer periods of time, as would be expected. The H^+ gradient is similarly affected (Sachs *et al.*, 1976).

The efflux of Rb^+ was relatively specific for ATP. Of the other nucleoside triphosphates tested (GIP, UTP, TTP, CTP) only CTP was capable of inducing Rb^+ efflux, but at a slower rate (Table 4). β-γ methylene ATP shows no transport activity though known to compete with ATP for the hydrolysis site (Saccomani *et al.*, 1977). pNPP also shows no transport though hydrolyzed by the enzyme in the presence of K^+. The average maximum efflux obtained for the *G1* fraction was $44 \pm 7\%$ of the initial level of Rb^+. The pH curve from 6.0 to 7.4 of the maximum transported Rb^+ activity corresponded to the K^+-ATPase curve. Inhibitors of K^+-ATPase activity such as Zn^{++}, F^- (Forte *et al.*, 1975) and DCCD at 1 mM (Sachs *et al.*, 1976) completely inhibited Rb^+ efflux.

Following efflux there is reuptake of Rb^+ to the same level as found before ATP addition to the equilibrated vesicles. The reuptake can be

described by a single exponential with a $t_{1/2}$ of 53 min which is to be compared to the $t_{1/2}$ of 40 min under gradient conditions. This reuptake therefore presumably occurs by a single pathway into one type of vesicle.

Stoichiometry. The stoichiometry of the Rb^+ and H^+ transported and ATP hydrolyzed, as well as a direct comparison of the moles H^+ taken up as compared to Rb^+ extruded have implications as to the electrogenicity of the pump mechanism. Since the only added ions are Rb^+ (or K^+) and Cl^- maintenance of electroneutrality of the H^+ uptake process requires cotransport of Cl^- or countertransport of Rb^+ (or K^+). Since no Cl^- movement could be shown, the ratio of H^+ and Rb^+ transported is likely to be unity. Measurement of Rb^+ efflux, H^+ uptake, and ATP hydrolysis on the same preparations gave a value for Rb^+ efflux of 6.5 μmole per mg per hr and an ATPase activity of 3.6 μmole Pi per mg per hr in the presence of Rb^+ and 1.8 μmole Pi per mg per hr in the absence of Rb^+. The calculated ratio of Rb^+ transported to ATP hydrolyzed is therefore 1.6 ± 0.2 using total ATPase activity and 3.5 ± 0.4 ($n=4$) using only the cation activated component. These studies were done at room temperature at pH 6.1, and this accounts for the relatively large contribution of "basal" ATPase activity. The same conditions, using the cation activated ATPase activity for the calculation, the H^+ transported to ATP hydrolyzed ratio was 4.1 ± 0.2 ($n=10$) (Sachs *et al.*, 1976). It should be noted however, that the H^+ measurement is electrometric, as compared to the filtration method for Rb^+. The relative ratio obtained is reasonably close to one, as was predicted. This does not prove that the pump is nonelectrogenic. As discussed in more detail elsewhere, however (Sachs, *et al.*, 1976; Lewin *et al.*, 1977), the results with ionophores and probes of vesicle potential strongly suggest that the transport mechanism is different from the H^+ ATPase of mitochondria or neurospora, for example (Scarborough, 1976).

Effects of Ionophores. Ionophores have variable effects on ATP induced Rb^+ efflux from preloaded vesicles (Table 5). Valinomycin and TCS did not affect Rb^+ efflux but valinomycin accelerated the reuptake phase of Rb^+ movement. Nigericin reduced Rb^+ efflux by 40% at 1 μg/ml. Lipid permeable ions such as DDA, DPA or SCN^- had no effect on the level of Rb^+ efflux at concentrations up to 10 mM, alone or in combination.

Effect of Rb^+ Concentration. Variation of Rb^+ concentration does not alter the steady state ratio of Rb^+ between vesicles and medium achieved by the addition of ATP. Moreover, it appears that the quantity of Rb^+ transported is a linear function of the Rb^+ concentration below

75 mM (Fig. 7). In contrast, ATPase activity is a saturable function of cation concentration, the apparent K_A for Rb^+ being 15 mM at pH 6.1 at room temperature.

Two interpretations may be suggested for these data. If the Rb^+ movement were in response to electrogenic H^+ uptake, then the final ratio would be independent of Rb^+ concentration, and also the quantity

Table 5. $^{86}Rb^+$ Efflux in the presence of ATPase inhibitors and ionophores[a]

Efflux stimulus and concentration	Total uptake nmole/mg	efflux nmole	% ATP stimulated efflux[b]
ATP+TCS (0.9×10^{-6} M)	154	72	98%
ATP+valinomycin (10^{-6} M)	152	70	92%
ATP+SCN (10 mM)	155	66	106%
ATP+nigericin (1 g/ml)	151	39	63%
ATP+F^- (1 mM)	162	3	5%
ATP+Zn^{2+} (1 mM)	161	1	1%
ATP+DCCD (1 mM)	159	0	0%
ATP+10 mM DDA+1 mM NaSCN	152	75	90%
ATP+10 mM DDA+10 mM NaSCN	192	117	127%
ATP+10^{-5} M DPA	146	75	77%
ATP+1 mM NaSCN+1 mM DDA	174	85	88%

[a] Efflux measured 10 min after the addition of ATP.
[b] Based on ATP stimulated effluxes for same preparation.

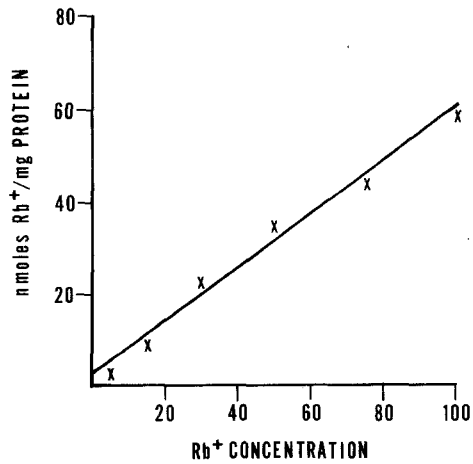

Fig. 7. ATP stimulated Rb^+ efflux as a function of Rb^+ concentration *G1* vesicles were incubated at 4 °C for 48 hr in X mM RbCl, 125 mM sucrose, 2 mM $MgCl_2$ 5 mM glycylglycine, pH 6.12 and (75-X) mM choline chloride. Efflux was initiated at room temperature with 2 mM ATP and measured ten min after ATP addition. Samples were taken in triplicate

transported would be a linear function of Rb^+ concentration. The distribution ratio would then suggest a value of 18 mV for the vesicular potential.

However, there are many suggestive lines of evidence that argue against the idea that Rb^+ is acting as a passive counterion for an electrogenic H^+ ATPase. Thus increasing the conductance of the vesicular membrane to Rb^+, by the addition of valinomycin would be predicted to increase Rb^+ efflux rate, which does not happen. The provision of alternate conductance paths, such as with lipid permeable cations and anions such as DDA^+ and SCN^- should reduce Rb^+ efflux, which again does not occur (Table 5). If a potential does develop during ATP hydrolysis, then ATP hydrolysis should be stimulated by increasing membrane conductance. In general this does not occur, unless K^+ selective ionophores are used (Ganser & Forte, 1973*b*). The activation of K^+ stimulated ATPase by these ionophores is due to increased levels of intravesicular K^+, since vesicular disruption abolishes the ionophore effect and NH_4^+ activation is not enhanced by valinomycin (NH_3 being highly permeable) (Saccomani *et al.*, 1977).

An alternative interpretation of the final steady state ratio is that only a fraction of the vesicles transport as a function of the addition of ATP. This fraction then achieves a similar internal level of Rb^+ independent of the initial level of ATP. Separation of the density gradient fraction by free flow electrophoresis produced two vesicular fractions which trap approximately the same quantity of Rb^+ (Table 1). However, only the anodic fraction demonstrates Rb^+ efflux with ATP addition, and a higher level of efflux (58%) is obtained as compared to the original zonal fraction (44%). If there is a random orientation of the ATPase in the fraction, only half of this fraction will actually transport. Hence the approximate 3:1 steady state ratio achieved may be misleading. When ANS was used as a probe of intravesicular potential, the initial rate of change of fluorescence with the addition of ATP in the presence of valinomycin was a linear function of the log of the added K^+ concentration (Sachs *et al.*, 1976; Lewin *et al.*, 1977). This implies that the internal cation reaches a fixed value. Hence the Rb^+ ratio of the fraction of the vesicles transporting is likely to be greater than 3:1.

H^+ Uptake: Fig. 8 shows the H^+ uptake induced with the addition of 0.3 mM ATP in vesicles equilibrated with RbCl as for the Rb^+ transport studies. The addition of a protonophore such as tetrachlorsalicylanilide (TCS) only partially dissipates the H^+ gradient and the further addition of valinomycin is required. This suggests the apparent absence of

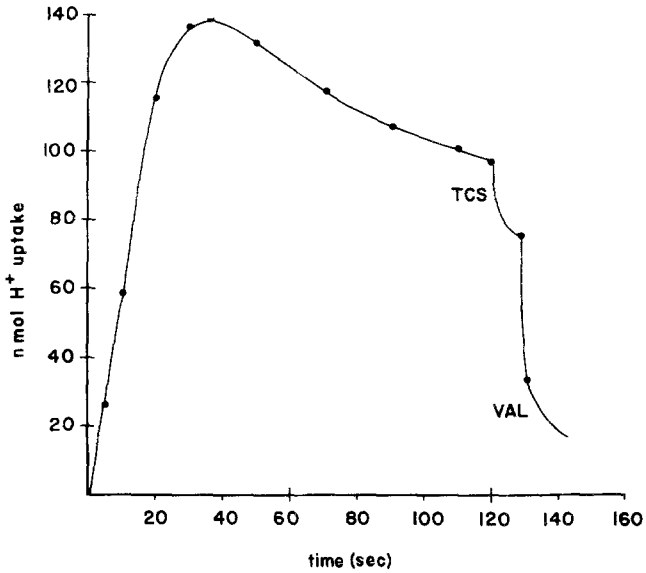

Fig. 8. The uptake of H^+ when 0.3 mM ATP was added to vesicles equilibrated with RbCl in 5 mM glycylglycine buffer pH 6.1 and the effect of addition of tetrachlorsalicylanilide (TCS) and valinomycin (val)

sufficient K^+ or Cl^- conductance to adequately dissipate the gradient even with an added H^+ conductance. This is further evidence against potential dependent redistribution of Rb^+. In this particular experiment the initial 10 sec uptake rate of H^+ was equivalent to 21.2 μmole per mg per hr, and the initial hydrolysis rate corresponded to 8.16 μmole Pi per mg per hr giving a ratio of H^+ transported to ATP hydrolyzed of 2.9. Correcting for basal ATPase activity in this particular experiment gave a ratio of 4.2 μmole H^+ transported per mole ATP hydrolyzed. The sensitivity of H^+ uptake to ATPase inhibitors is identical to that of the Rb^+ efflux.

It should be noted that the maximal H^+ gradient is developed at about 30 sec under these equilibrated conditions as compared to about 5 min for the Rb^+ efflux. At 1 min, directly comparing H^+ uptake and Rb^+ efflux, approximately a 2:1 ratio is obtained whereas the ratio approaches 1:1 at longer time intervals. The interpretation of this result is made difficult since the measurement of H^+ uptake electrometrically is more sensitive than the filtration technique used for Rb^+ efflux. Cation electrodes are insufficiently sensitive for cation efflux studies with these preparations. However, these data as compared to the calculated stoichiometry based on ATP hydrolysis suggest that the latter must be viewed with caution.

Discussion

From the data presented above, it has been possible to prepare, from hog gastric mucosa, a vesicular fraction which contains a cation activated ATPase (Ganser & Forte, 1973*a*). This preparation contains vesicles of low ion permeability and this low permeability is maintained during the fractionation procedures including free flow electrophoresis. If these vesicles derive from the tubulo-vesicular system of the parietal cell, this implies that they are preformed in the cell and are not actually vesiculated by homogenization as would be necessary for plasma membranes of other cell types. The tubulovesicles fuse with the apical plasma membrane of the acid secreting cell with formation of microvilli (Sedar, 1965) and presumably contain the acid secreting system. It is therefore striking that these vesicles contain few peptides revealed on SDS gel electrophoresis, more than 75% being accounted for in the 100,000 M_r region (Sachs *et al.*, 1976).

The vesicles, either the zonal gradient or the free flow fraction, take up H^+ with addition of ATP as previously established for dog microsomes (Lee *et al.*, 1974). Since both a proton and a cation conductance were required to dissipate the H^+ gradient (Lee *et al.*, 1974; Sachs *et al.*, 1975, 1976) it was suggested that the uptake was nonelectrogenic. Since changes in membrane conductance such as those induced by lipid permeable ions or protonophores did not affect ATPase activity (Sachs *et al.*, 1976) and since no potential was detected during transport (Fig. 4 and Lewin *et al.*, 1977) also using lipid permeable ions it was likely that the mechanism of H^+ uptake was not by a mechanism similar to the electrogenic H^+-ATPase (Mitchell, 1966). Since cation and chloride ion were the only additions to the H^+ uptake medium which could satisfy the ionic requirements for the uptake process, pathways for these ions were presumed to be present in this vesicular preparation.

Both cation and chloride were taken up by this preparation into an osmotically sensitive space at about the same rate. The low interactionic selectivity of uptake is typical of channels such as gramicidin (Goodall, 1973) or of noninteractive sites such as large diameter waterfilled pores. Both of these structures have a high H^+ permeability, and if present in these vesicles must be few in number.

Efflux of Rb^+ into choline solutions was very slow as would be expected from the slow uptake observed, but external cation stimulated efflux considerably and selectively although the discrimination between K^+ and Na^+ was less than that for H^+ uptake or ATPase activity (Sachs

et al., 1976). Carriers of the valinomycin type are more selective and conductive whereas nigericin is less selective and essentially neutral (Henderson, McGiven & Chappell, 1969). The selectivity of this exchange process suggests that it is distinct from the nonselective uptake pathway revealed by the influx studies. It is also likely to be neutral since cation gradients do not result in H^+ movement across the vesicle membrane even in the presence of a proton conductance (i.e., in the presence of TCS).

From both these types of studies the cation permeability and conductance of the unenergized membrane preparation is low as is the anion permeability.

The addition of ATP significantly affects cation but not anion movements in the untreated membranes. Thus influx is apparently reduced and efflux stimulated. Studies of Rb^+ movement following equilibration showed that Rb^+ was transported uphill following ATP addition. The cation requirement for H^+ uptake and the identical effects of inhibitors on ATPase, H^+ uptake, and Rb^+ movement suggested that these processes were interlinked. Indeed, the absence of Cl^- redistribution under identical conditions implied that countertransport of cation was probably obligatory for H^+ uptake and that the ratio of cation efflux to H^+ uptake would therefore have to be 1:1. Studies on H^+ uptake showed that intravesicular cation was required (Sachs *et al.,* 1976) and the evidence against electrogenicity of H^+ uptake or ATPase activity and against a cation conductance path made it likely that cation transport was due to direct coupling to the pump rather than due to indirect coupling by a potential due to electrogenic H^+ transport. This was substantiated in these studies by the finding that shunt conductances (e.g., lipid permeable ions) added to the vesicles did not affect Rb^+ efflux; and that SCN^- did not detect a positive interior potential with the addition of ATP.

However, the final steady state ratio achieved by the vesicles for the gradient fraction ($\bar{c}$2:1 and 3:1 for the electrophoretic fraction) could be interpreted as evidence for an 18–25 mV potential gradient. The data derived for the stoichiometry of the pump showed close to a 1:1 ratio if the ratio relative to ATP hydrolysis was used. A discrepancy did exist comparing pH measurements directly to Rb^+ movement at short time intervals, which might be due to the lower sensitivity and longer response time of the filtration technique.

Thus the ATPase of these vesicles simultaneously translocates H^+ and cation in a directly coupled reaction.

From other studies cations dephosphorylate the ATPase as they do the $Na^+ + K^+$ ATPase (Tanisawa & Forte, 1971). This ATPase therefore can be classified as an $H^+:K^+$ ATPase in analogy to the $Na^+ + K^+$ or Ca^{++} ATPases (Skou & Hilberg, 1969; Martonosi, 1969). A major difference between the preparation described here, and similar preparations of the other enzymes is the ion tightness of the vesicle. Ca^{++}-ATPase containing vesicles have a high anion permeability (Duggan & Martonosi, 1970) making it very difficult to determine the electrical characteristics of the pump. With this enzyme analogs such as carbamyl phosphate energize transport in contrast to the $H^+:K^+$ ATPase. Most preparations of $Na^+ + K^+$ ATPase are too leaky for transport studies, and this enzyme is usually present in membranes of a more complex peptide composition than the $H^+:K^+$ ATPase (Walter, 1975). Reconstituted vesicles containing this enzyme do exhibit Na^+ and K^+ transport (Hilden & Hokin, 1975).

Although from these studies it would appear that the $H^+:K^+$ ATPase catalyzes a transmembranal neutral $K^+:H^+$ exchange, results with frog gastric mucosa in SO_4^{2-} solutions are compatible only with an electrogenic process in the gastric mucosa (Davis *et al.*, 1963). Metabolic data in dog gastric mucosa (Sarau *et al.*, 1975) and spectroscopic observations (Hersey, 1974) are also not incompatible with a redox energy source for secretion. On the other hand, based on antibody studies (Saccomani, *unpublished observations*) it is clear that this $H^+:K^+$-ATPase is a specific component of the parietal cell and is a transport enzyme requiring K^+, which cation is essential for acid secretion (Davis *et al.*, 1965). One might suggest that the electrogenic properties of the intact tissue reside in the Cl^- pump (Hogben, 1951) and perhaps in another membrane associated component removed by our isolation procedures or absent from the plasma membrane in nonsecreting tissue.

The authors are grateful for the assistance and discussion given by Dr. E. Rabon, Ms. H. Hung Chang and Mr. H.B. Stewart.

This work was supported by grants NIH AM 15878; NSF Gb31075; CA 16877 and CA 16722 (G.S.) and HL07906 and contract HV52998 (A.S.).

References

Davis, F.L., Rutledge, J.R., Keesee, D.C., Bajandas, F.J., Rehm, W.S. 1965. Acid secretion, potential and resistance of frog stomach in K^+-free solutions. *Am. J. Physiol.* **209**:146

Davis, F.L., Rutledge, J.R., Rehm, W.S. 1963. Effect of potassium on secretion and potential of frog's gastric mucosa in Cl^- free solutions. *Am. J. Physiol.* **205**:873

Duggan, P.F., Martonosi, A. 1970. Sarcoplasmic reticulum. IX. The permeability of sarcoplasmic reticulum membranes. *J. Gen. Physiol.* **56**:147

Forte, J.G., Forte, G.M., Saltman, P. 1967. K^+-stimulated phosphatase of microsomes from gastric mucosa. *J. Cell Physiol.* **69**:293

Forte, J.G., Ganser, A., Beesley, R., Forte, T.M. 1975. Unique enzymes of purified microsomes from pig fundic mucosa. K^+-stimulated adenosine triphosphatase and K^+-stimulated pNPPase. *Gastroenterology* **69**:175

Ganser, A., Forte, J.G. 1973*a*. K^+-stimulated adenosine triphosphatase in purified microsomes of frog oxyntic cells. *Biochim. Biophys. Acta* **307**:169

Ganser, A.L., Forte, J.G. 1973*b*. Ionophoretic stimulation of K^+-ATPase of oxyntic cell microsomes. *Biochem. Biophys. Res. Commun.* **54**:690

Goodall, M.C. 1973. Action of two classes of channel forming synthetic peptides in lipid bilayers. *Arch. Biochem. Biophys.* **157**:514

Henderson, P.J.F., McGivan, J.D., Chappell, J.B. 1969. The action of certain antibiotics on mitochondrial, erythrocyte, and artificial phospholipid membranes. The role of induced proton permeability. *Biochem. J.* **111**:521

Hersey, S.J. 1974. Interaction between oxidative metabolism and acid secretion in gastric mucosa. *Biochim. Biophys. Acta* **344**:157

Hilden, S., Hokin, L.E. 1975. Active potassium transport coupled to active sodium transport in vesicles reconstituted from purified sodium and potassium ion-activated adenosine triphosphatase from the rectal gland of *squalus acanthias*. *J. Biol. Chem.* **250**:6296

Hirschowitz, B.I., Sachs, G. 1967. Insulin inhibition of gastric secretion and reversal by rubidium. *Am. J. Physiol.* **213**:1401

Hogben, C.A.M. 1951. The chloride transport system of the gastric mucosa. *Proc. Nat. Acad. Sci. USA* **37**:393

Hogben, C.A.M. 1968. Observations on ionic movement through the gastric mucosa. *J. Gen. Physiol.* **51**:2405

Lee, J., Simpson, G., Scholes, L. 1974. An ATPase from dog gastric mucosa: Changes of outer pH in suspensions of membrane vesicles accompanying ATP hydrolysis. *Biochem. Biophys. Res. Commun.* **60**:825

Lewin, M., Saccomani, G., Schackmann, R., Sachs, G. 1977. The use of ANS as a probe of gastric vesicle transport *J. Membrane Biol.* **32**:301

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**:265

Martonosi, A. 1969. Sarcoplasmic reticulum VII. Properties of a phosphoprotein intermediated implicated in calcium transport. *J. Biol. Chem.* **244**:613

Mitchell, P. 1966. Chemiosmotic coupling in oxidative and photosynthetic phosphorolation. *Biol. Rev.* **44**:445

Saccomani, G., Shah, G., Spenney, J.G., Sachs, G. 1975. Characterization of gastric mucosal membranes. VIII. The localization of peptides by iodination and phosphorylation. *J. Biol. Chem.* **250**:4802

Saccomani, G., Stewart, H.B., Shaw, D., Lewin, M., Sachs, G. 1977. Characterization of gastric mucosal membranes. X. Fractionation and purification of K^+-ATPase containing vesicles by zonal centrifugation and free-flow electrophoresis technique. *Biochim. Biophys. Acta (in press)*

Sachs, G., Collier, R.H., Pacifico, A., Shoemaker, R.l., Zweig, R.A., Hirschowitz, B.I. 1969. Action of thiocyanate on gastric mucosa in vitro. *Biochim. Biophys. Acta* **173**:509

Sachs, G., Hirschowitz, B.I. 1968. Secretion by in vitro amphibian gastric mucosa. *In:* The physiology of Gastric Secretion. L.S. Semb and J. Myren, editors. Vol. 186, p. 202. Universitetsforlaget, Oslo

Sachs, G., Chang, H., Rabon, E., Schackmann, R., Lewin, M., Saccomani, G. 1976. A non-electrogenic H^+ pump in plasma membranes of hog stomach. *J. Biol. Chem.* **251**:7690

Sachs, G., Rabon, E., Saccomani, G., Sarau, H.M. 1975. Redox and ATP in acid secretion. *Ann. N.Y. Acad. Sci.* **264**:456

Sachs, G., Rose, J.A., Shoemaker, R.L., Hirschowitz, B.I. 1966. Phosphatase reactions of transport ATPases. *Physiologist* **9**:281

Sarau, H.M., Foley, J., Moonsamy, G., Wiebelhaus, V.D., Sachs, G. 1975. Metabolism of dog gastric mucosa. I. Nucleotide levels in parietal cells. *J. Biol. Chem.* **250**:8321

Scarborough, G.A. 1976. The neurospora plasma membrane ATPase in an electrogenic pump. *Proc. Nat. Acad. Sci. USA* **73**:1485

Sedar, A.W. 1965. Fine structure of the stimulated oxyntic cell. *Fed. Proc.* **24**:1360

Skou, J.C., Hilberg, C. 1969. The effects of cations, *g*-strophanthin and oligomycin on the labeling from [^{32}P] ATP of the ($Na^+ + K^+$)-activated enzyme system and the effect of cations and *g*-strophanthine on the labeling from [^{32}P] ITP and ^{32}Pi. *Biochim. Biophys. Acta* **185**:198

Tanisawa, A.S., Forte, J.G. 1971. Phosphorylated intermediate of microsomal ATPase from rabbit mucosa. *Arch. Biochem. Biophys.* **147**:165

Walter, H. 1975. Tightness and orientation of vesicles from guinea pig kidney estimated from reactions of adenosine triphosphatase dependent on sodium and potassium ions. *Eur. J. Biochem.* **58**:595

Yoda, A., Hokin, L.E. 1970. On the reversibility of binding of cardiotonic steroids to a partially purified ($Na^+ + K^+$) activated adenosine triphosphatase from beef brain. *Biochem. Biophys. Res. Commun.* **40**:880