Biochimica et Biophysica Acta, 464 (1977) 313—327

BBA 77580

PROTON TRANSPORT BY GASTRIC MEMBRANE VESICLES

H. CHANG, G. SACCOMANI, E. RABON, R. SCHACKMANN and G. SACHS *

Laboratory of Membrane Biology, University of Alabama in Birmingham, Birmingham, Ala. 35294 (U.S.A.)

(Received May 31st, 1976)

Summary

A highly purified membrane fraction was derived from hog gastric mucosa by a combination of differential and density gradient centrifugation and free flow electrophoresis. This final fraction was 35-fold enriched with respect to cation activated ouabain-insensitive ATPase. Antibody against this fraction was shown to be bound to the luminal surface of the gastric glands. The addition of ATP to this fraction or the density gradient fraction resulted in H^+ uptake into an osmotically sensitive space. The apparent K_m for ATP was $1.7 \cdot 10^{-4}$ M in the absence of a K^+ gradient similar to that found for ATPase activity. The reaction is specific for ATP and requires cation in the sequence $K^+ > Rb^+ > Cs^+ > Na^+ > Li^+$ and is inhibited by ATPase inhibitors such as *N,N'*-dicylclohexylcarbodiimide. Maximal H^+ uptake occurs with an outward K^+ gradient but the minimal apparent K_A is found in the absence of a K^+ gradient. The pH optimum for H^+ uptake is between 5.8 and 6.2 which corresponds to the pH range for phosphorylation of the enzyme, but is considerably less than the pH maximum of the K^+ dependent dephosphorylation. In the presence of an inward K^+ gradient, protonophores such as tetrachlorsalicylanilide only partially abolish the H^+ gradient but valinomycin dissipates 75% of the gradient, and nigericin abolishes the gradient. The vesicles therefore have a low K^+ conductance but a measurable H^+ conductance, hence a K^+ gradient can produce an H^+ gradient in the presence of valinomycin. The uptake and spontaneous leak of H^+ are temperature sensitive with a similar transition temperature. Ultraviolet irradiation inactivates ATPase and proton transport at the same rate, approximately at twice the rate of *p*-nitrophenylphosphatase inactivation. It is concluded that H^+ uptake by these vesicles is probably due to a dimeric $(H^+ + K^+)$-ATPase and is probably non-electrogenic.

* To whom correspondence should be addressed.
Abbreviation: DCCD, *N,N'*-dicyclohexylcarbodiimide.

Introduction

The generation and utilization of proton gradients by bacteria [1], bacterial vesicles [2], chloroplasts [3] and mitochondria [4] have now assumed primary importance in understanding coupling of ion gradients to chemical synthesis or solute transport. In all these systems one is dealing in general with a complex series of redox reactions, a multi-subunit ATPase and a large number of putative ion and organic solute carriers. The parietal cell of gastric fundic mucosa at rest contains a large number of smooth surfaced closed vesicular structures in the apical cytoplasm which fuse with the intracellular canalicular membrane during secretion forming a complex microtubular system [5]. In this state the cell is capable of generating a better than 10^6 : 1 H^+ gradient.

There are many indications that the purified membrane fractions of gastric mucosa are vesicular apart from the H^+ transport studies to be reported. Thus the K^+ ATPase activity [6] is enhanced in fresh preparations by the addition of ionphores such as valinomycin [7] and electron micrographs show the preparations to be vesicular, often with a double membrane appearance [8]. Using a lactoperoxidase method, it has also been shown that only certain peptides can be iodinated in fresh preparations from dog mucosa and that additional peptides iodinate in frozen thawed fractions [9]. Recently, it has been shown that a crude microsomal fraction from dog stomach is capable of transiently alkalinising the medium at pH 6.1 with the addition of ATP, the alkalinisation being reversed only by adding both valinomycin and protonophore [10]. The importance of this finding to proton secretion by the stomach and to studies relating proton gradients to ATP turnover makes it necessary to define the characteristics of this gradient, such as its magnitude, location, ion requirements, substrate specificity, electrogenicity and relationship to ATPase activity.

Materials and Methods

1. Vesicle preparation. Scrapings of hog gastric mucosa, after flooding with 3 M NaCl to remove mucus and large numbers of surface cells [11], were homogenized in unbuffered 0.25 M sucrose and the crude microsomal pellet prepared by centrifuging the post 20 000 $\times g$ supernatant at 78 000 $\times g$ (mean value) for 1 h. The pellet was resuspended at a concentration of 25 mg/ml protein again in 0.25 M sucrose, layered on top of a discontinuous gradient of 7% (w/v) ficoll and 30% sucrose, and centrifuged in a Z60 zonal rotor for 2 h at 59 000 rev./min. Three particulate fractions were obtained, one at the 0.25 M sucrose-7% ficoll interface, one at the interface between 7% ficoll-30% sucrose layers and one at the bottom of the gradient. Similar procedures applied to the hog antrum (with the exception that polytron (Brinkmann Instruments) homogenization was required) produced only two fractions, that at the sucrose ficoll interface, the other at the bottom of the tube.

The fraction at the sucrose ficoll interface (GI) was used in most of the experiments. The step gradient produced a sufficiently concentrated fraction to allow use without further centrifugation. Ficoll was found to be superior to sucrose in producing vesicles of low permeability.

In addition GI fraction was subjected to free flow electrophoresis on a Hanning FF5 free flow machine (Biomedical Instruments, New York) using 8 mM

Tris/acetate in 250 mM sucrose adjusted to pH 7.4 with 2 M NaOH with the addition of 0.1 mM MgATP in the curtain buffer with a voltage gradient of 100 V per cm and a flow rate of 180 ml/h at 7.5° C. This procedure separates the gradient fraction into an FI and FII fraction. Both of these were studied for H^+ uptake.

Gel electrophoresis was carried out in sodium dodecyl sulphate as previously described [9].

2. H^+ uptake. The experiments were carried out in a magnetically stirred vessel and the change of pH measured by a Radiometer pHM 64 pH meter with a servorecorder coupled to a REA 112 high sensitivity module. The amplification was so arranged that a deflection of 1 cm corresponded to a change of pH of 0.01 units.

The pH of all solutions was adjusted to the exact pH of the experiment prior to mixing any of the solutions and in general was adjusted to pH 6.11. The quantity of membranes added gave a final concentration of 0.17 mg/ml in 6 ml volume. The standard buffer was 5 mM glycyl glycine. KCl (or other salt) was added at 150 mM with 2 mM $MgCl_2$ to maintain osmolarity. Nucleotide or other substrate was added usually at $1.7 \cdot 10^{-5}$ M. For experiments in which osmolarity was varied, salt concentration was reduced to 90 mM and mannitol was added to the solution at various concentrations. Ionophores such as valinomycin (10^{-6} M) tetrachlorsalicylanilide (10^{-6} M) nigericin (1.7 μg/ml) were added in 10 μl methanol to the final volume of the solution of 6 ml. The internal medium of the vesicles was controlled by preincubating the vesicles for 48 h at 4° C in appropriate medium such as 150 mM KCl or other cation chloride. The time to reach equilibrium was determined in separate experiments using radioactive tracers such as $^{86}Rb^+$ [12], which technique also allows an estimate of the intravesicular volume.

Control experiments in the absence of vesicles showed that the addition of ATP did not change the pH. Rupture of the vesicles or the presence of nigericin abolished the pH changes showing that these changes were not due to proton released by breakdown of ATP. The pH of 6.11 was chosen to prevent any artefact due to this phenomenon [13].

In experiments to determine the H^+/ATP ratio, 150 mM K^+ equilibrated vesicles had 0.3 mM ATP added and the data obtained at 10 s were used. For inactivation comparison, $6 \cdot 10^{-5}$ M ATP and 111 $\mu g\ ml^{-1}$ protein was used for H^+ uptake and 2 mM ATP and 20 $\mu g\ ml^{-1}$ protein was used for hydrolysis measurements.

The change in pH was converted to nmol H^+ absorbed by the vesicles by back titration with 10^{-3} M HCl under each experimental condition.

3. Enzyme assays. ATPase activity was measured in a medium containing approximately 10 μg protein, 2 mM MgCl, 2 mM ATP in 40 mM Tris/acetate with or without 20 mM KCl at pH 7.4, following incubation at 37° C in a final volume of 1 ml. In some experiments pH 6.1 and room temperature was used. Phosphate released was measured as described elsewhere [14]. In others the concentration of MgATP was varied.

p-Nitrophenylphosphatase was measured in a medium containing 10 μg protein, 6 mM $MgCl_2$ and 6 mM *p*-nitrophenyl phosphate in 40 mM Tris/acetate buffer, pH 7.4 following incubation at 37° C [8].

Protein was measured by the method of Lowry et al. [15] and all chemicals used were the highest purity grade available.

4. Irradiation experiments. A vesicular suspension at a protein concentration of about 2 mg/ml was diluted in 0.25 M sucrose to give a final concentration of 400 μg/ml. The sample was shielded with aluminum foil and kept in an ice bath at a constant temperature of 4° C. The mercury lamp, used as the ultraviolet light source, was placed 2.0 cm above the sample suspension. At 30-s intervals the ultraviolet lamp was turned off and 1.2 ml aliquots were removed for H^+ uptake, ATPase and *p*-nitrophenyl phosphatase activity measurements.

The data were interpreted based on the equation

$$a = a_0 \, e^{-VD}$$

where a is activity at time t, a_0 is initial activity, V is volume of target and D is dose of irradiation which was taken as proportional to the time of irradiation.

5. Localization of the active fraction. Antibody against the gastric K^+-ATPase was produced in rabbits by injection of the frozen dried free flow FI fraction at concentration of 0.5 mg/ml, suspended in Freund's adjuvant. The cation activated ATPase was shown to be inhibited by this antibody in studies described in detail elsewhere (Saccomani, G., Sachs, G., Shaw, D. and Mihas, A., in preparation).

Immunofluorescence experiments were carried out using the method of indirect staining [16]. Small pieces (about 1 cm^2) of hog gastric mucosa from both fundus and antrum were fixed in 95% ethanol for 24 h at 4° C, dehydrated and embedded in paraffin. Cryostat section measuring 6—8 μm were cut after paraffin was removed by xylene, and incubated with the antibody for 1 h at 25° C. After washing in 0.1 M phosphate buffer containing 0.15 M NaCl at pH 7.8, the sections were treated with fluorescein-isothiocyanate conjugated goat anti-rabbit γ-globulin. Following three more washes in phosphate-saline buffer, the sections were covered under buffered glycerine, sealed with nail polish and examined in a Leitz fluorescence microscope. The specificity of the staining was determined by using "pre-immune" rabbit serum and anti-IgG, anti-IgA and anti-IgM nonspecific antibodies as controls.

Results

1. Purification of vesicles. H^+ uptake dependent upon the addition of ATP occurred in the microsomal fraction, the lighter density gradient fraction (GI) and in only the anodic peak of the free flow fractionation (FI).

In accord with the H^+ uptake activity, there was enrichment of cation activated ATPase in these fractions associated with the cation activated phosphatase (Table I). At the same time there is a progressive enrichment of the 100 000 M_r peptide on the acrylamide gels (Fig. 1) so that the final free flow fraction contains more than 75% of its peptide at the 100 000 M_r location. This peptide region is phosphorylated by [γ-^{32}P]ATP and dephosphorylated in the presence of K^+ [9]. The fractionation technique is described in detail elsewhere [8]. Antral membrane fractions contained neither the cation activated ATPase or H^+ uptake properties with ATP addition. Fundic vesicles were stable up to 5 days after preparation.

TABLE I

PURIFICATION OF VESICLES

Assay conditions as described in Materials and Methods. The K^+-ATPase activity is the difference between activity in presence and absence of 20 mM K^+ (mean of 10 fractionations ± S.E.).

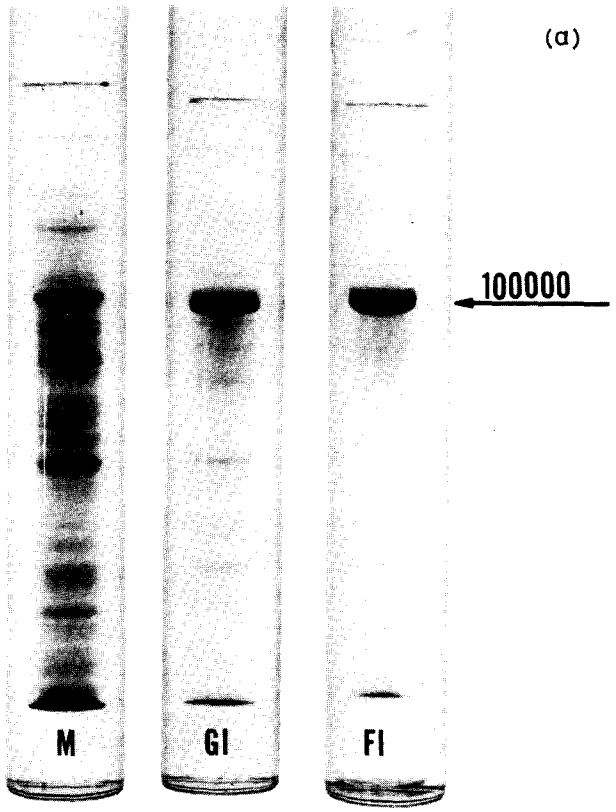
	Protein in mg	5'-AMPase	Mg^{2+}-ATPase (μmol · P_i mg^{-1} · h^{-1})	K^+-ATPase	K^+-pNNPase (μmol pNP mg^{-1} · h^{-1})
Total homogenate	4800	0.5 ± 0.3	6.2 ± 0.6	2.0 ± 1.0	3.1 ± 0.5
Microsomal fraction	360	1.5 ± 0.6	15.9 ± 1.5	7.1 ± 4.3	22.7 ± 3.9
Light membrane fraction (GI)	31	4.6 ± 0.1	6.4 ± 1.3	32.5 ± 3.2	51.6 ± 3.6
Electrophoretic fraction (FI)	16	0.6 ± 0.2	2.7 ± 0.6	64.1 ± 3.9	54.0 ± 4.0
Electrophoretic fraction (FII)	7	14.7 ± 1.2	16.6 ± 2.0	17.3 ±1.2	18.8 ± 2.4

2. Localization of active fraction. Using the immunofluorescence technique, it was possible to show fluorescence present in hog fundic sections, but not antral sections. The fluorescence appears to have mainly a supranuclei and apical surface localization which corresponds to the region of the tubulovesicles or microvilli of the parietal cell [5] (Fig. 2). Fluorescence was not obtained when non-immunized rabbit γ-globulin was used. Longitudinal sections showed that the majority of the fluorescence was in the middle third of the gastric glands, which is the region of the majority of parietal cells.

3. Uptake of H^+. No change of pH was observed with the addition of ATP to the medium alone. Moreover, freeze drying the vesicles abolished more than 90% of the pH change normally observed. Sonication of the vesicles progressively reduced H^+ uptake with a much more rapid decline of H^+ uptake than of ATPase activity (Fig. 3).

Ionophores also reversed the H^+ gradient and all these data suggest that the disappearance of H^+ was due to uptake rather than binding of H^+. To quantitate the contribution of these two processes, the medium osmolarity was varied and the effect of this on H^+ uptake measured. Plotting H^+ uptake as a function of the reciprocal of osmolarity showed a good fit to a straight line ($r = 0.992$) and extrapolation of the line to infinite osmolarity gave a zero intercept on the H^+ uptake axis (Fig. 4). These data show that, under the conditions of study, binding of H^+ does not contribute significantly to the H^+ disappearance.

4. Characteristics of ATP effect. The addition of vesicles to the KCl medium resulted in no change of pH until the addition of ATP (Fig. 5). With addition of ATP there is a rapid uptake of H^+, followed by release of H^+ when the ATP is consumed. The addition of tetrachlorsalicylanilide results in a rapid but small release of H^+. Valinomycin alone results in a larger release of H^+ under the conditions of an inward K^+ gradient. This suggests that the K^+ conductance of the vesicle is lower than that of H^+. If valinomycin is added before ATP to the vesicles, then there is initially a slight acidification due to the K^+ gradient exchanging K^+ for H^+. If ATP is added under these conditions there is a more rapid uptake and more rapid release of H^+. From the above, it seems that ion gradients as well as ATP can produce H^+ gradients in these vesicles. This is confirmed by incubating the vesicles in 1 M KCl and diluting into equimolar choline chloride thus creating an outward K^+ gradient. There is no change of pH as



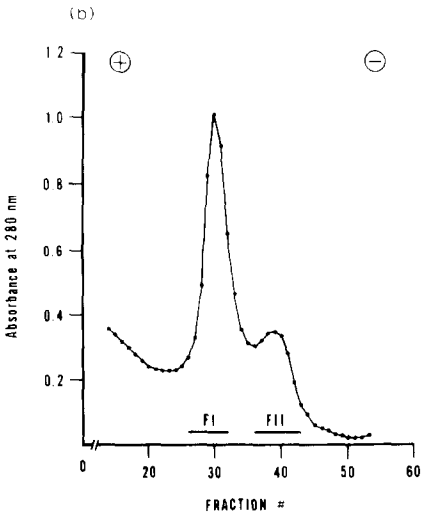


Fig. 1. (a) Sodium dodecyl sulphate gel patterns of the microsomal fraction, the gradient fraction and the electrophoretic fraction showing the increasing purification of the 100 000 M_r band. (b) The free flow profile of the gradient fraction.

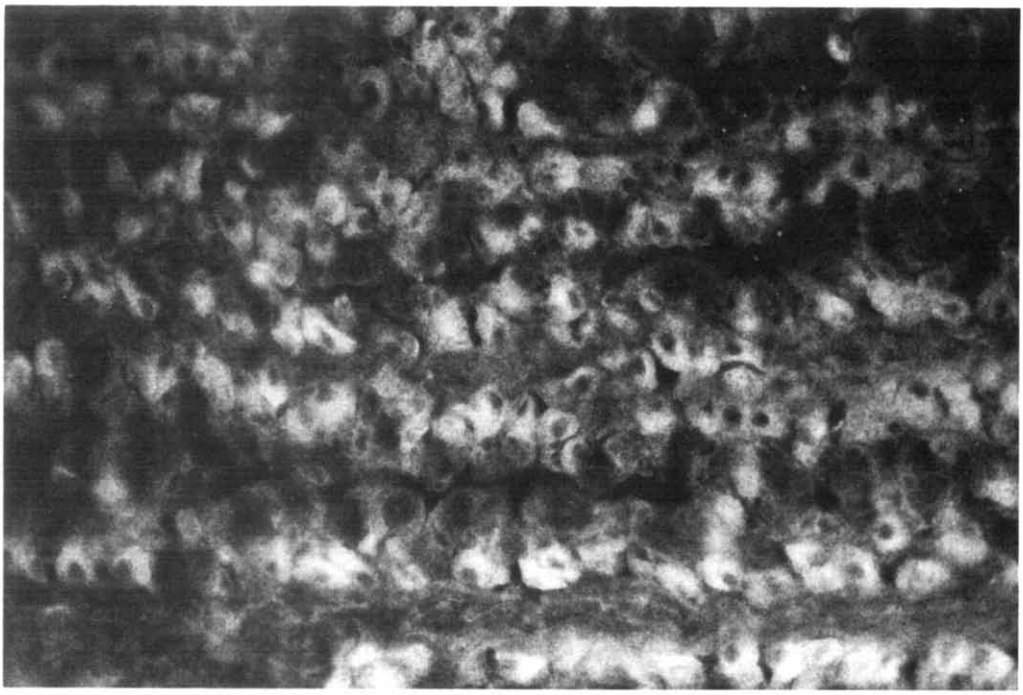


Fig. 2. The appearance of a section of gastric gland from hog fundus following treatment with rabbit antiserum to FI fraction of the free flow electrophoresis and the staining with fluorescein conjugated goat anti-rabbit gamma globulin (× 176).

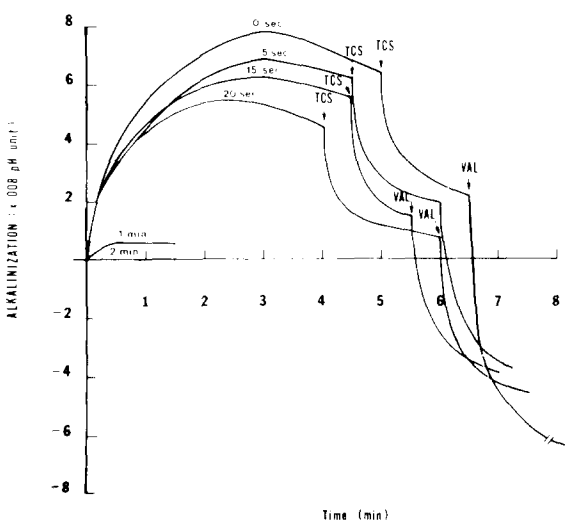


Fig. 3. The effect of various times of sonication of the vesicles prior to addition to the standard uptake medium using a Labsonic microtip sonifier. This demonstrates progressive loss of H^+ uptake and at 1 min where 75% residual ATPase activity is found actually no H^+ uptake is seen. Again, tetrachlorsalicylanilide (TCS) only partially dissipates the gradient, and the subsequent addition of valinomycin (VAL) results in an acid overshoot since these experiments were performed in the presence of an inward KCl gradient.

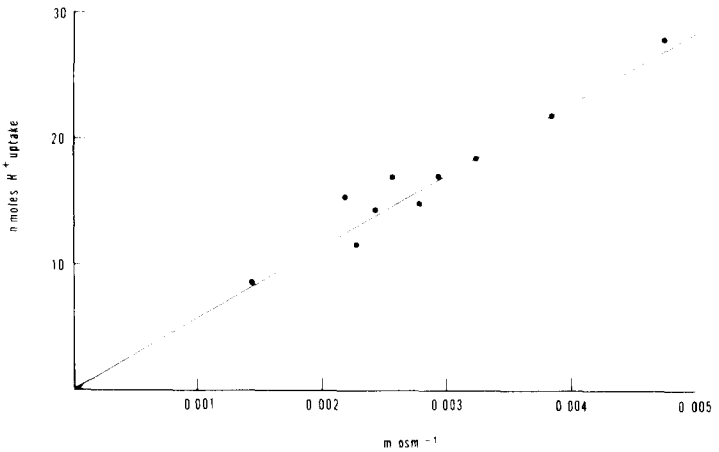


Fig. 4. The effect of vesicular volume on H^+ uptake. The vesicles were added to a solution containing 90 mM KCl with appropriate addition of mannitol to vary the osmolarity and other additions as in text. ATP $1.7 \cdot 10^{-5}$ M was added, and the H^+ uptake measured.

would be predicted from the low K^+ conductance inferred above. The addition of a protonophore, *m*-chlorocyano carbonyl phenylhydrazone produces only a slight alkalinisation showing that H^+ or Cl^- conductance are not limiting. The addition of valinomycin produces a transient alkalinisation (Fig. 6).

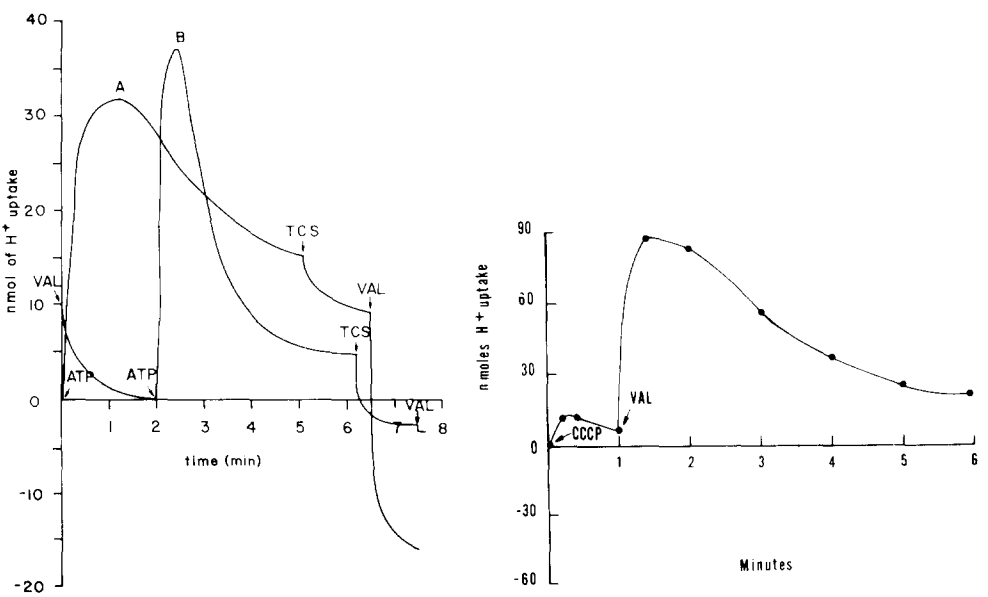


Fig. 5. H^+ uptake by the vesicles: In the curve A vesicles were added to a solution of 150 mM KCl with additions as in text, followed by $1.7 \cdot 10^{-5}$ M ATP. At times indicated tetrachlorsalicylanilide (TCS) and valinomycinin (VAL) were added. In curve B, valinomycin was added immediately following the addition of the vesicles from the same preparation, then ATP at $1.7 \cdot 10^{-5}$ M.

Fig. 6. The effect of preincubating the vesicles in 1 M KCl and then diluting into equimolar choline solution (●——●) with the addition of 10^{-5} M CCCP and then 10^{-6} M valinomycin showing the development of an H^+ gradient in the absence of ATP.

5. Effect of cations. Since the cation conductance of the vesicles is low, the effect of varying the nature and distribution of the cations was determined. Both the rate and maximal uptake of H^+ was a function of the nature of the cation. The sequence was $K^+ > Rb^+ > Cs^+ > Na^+ > Li^+$, using initial rate values. This is the same sequence of activation as for the ATPase or *p*-nitrophenyl phosphatase [17].

The rate of uptake of H^+ was increased by preincubating the vesicles in KCl and the apparent K_A for K^+ was reduced from 62 to 32 mM. However, the K_A for the ATPase was about 7 mM under identical conditions. The maximum uptake and fastest rate was obtained with an outward K^+ gradient i.e. when the vesicles were diluted into choline chloride. This suggests that when ATP is added, the outward K^+ gradient can contribute to the uptake of H^+. This can be explained by the development of a K^+ limitation due to efflux of K^+ with the addition of ATP [12], and development of a K^+ gradient even with preequilibrated vesicles. The outward K^+ gradient would reduce the gradient limitation (Table II). These data suggest that H^+ uptake is accompanied by cation efflux as detailed elsewhere [12].

6. Effect of anions. The conclusion that cation and cation movement is obligatory for H^+ uptake suggests that anion is not required for the process. The removal of Cl^- and substitution by other anions gives a selectivity sequence for anions of $Cl^- > Br^- > I^- > F^-$. The latter anion is inhibitory in the presence of Cl^- since the ATPase is also inhibited [6].

It has been demonstrated that H^+ secretion by gastric mucosa is electrogenic in the presence of SO_4^{2-} as a substitute for Cl^-. Under SO_4^{2-} conditions in the vesicles H^+ uptake was reduced by 40% even after preincubation. SO_4^{2-} also reduced cation uptake [12] and hence the inhibition might be due to reduction of cation in the intravesicular medium. No evidence was found for development of a potential in the presence of SO_4^{2-}.

SCN^- has been much used as an inhibitor of acid secretion [19]. The addition of SCN^- partially inhibited H^+ uptake but the results were variable. The rate of H^+ leak from the vesicles was increased by SCN^- which is a lipid permeable anion [20,22]. No effect of SCN^- was found on ATPase activity.

7. Effect of lipid permeable ions. The above data strongly suggest a non-elec-

TABLE II

EFFECT OF K^+ GRADIENT AND CONCENTRATION ON H^+ UPTAKE

The preincubation was carried out at 4°C for 48 h and the ionic concentrations were varied from 1 mM to 150 mM, mannitol being used to maintain isotonicity. The initial gradient was kept constant under two conditions (i.e. outward and zero) and only the ionic concentrations were varied allowing calculation of apparent K_A from a best fit Lineweaver Burk plot.

$[K^+]_{in}/[K^+]_{out}$	Initial velocity (nmol $H^+ \cdot mg^{-1} \cdot min^{-1}$)	V (nmol $H^+ \cdot mg^{-1} \cdot min^{-1}$)	K_A (mM)
0.006 *	130	216	62
1.0	163	218	32
6.0	563	1370	182

* With the inward gradient this varied due to the conditions used. The value of 0.006 was the gradient under standard conditions.

trogenic $H^+ : K^+$ exchange. If electrical coupling does play a role in this phenomenon, then modification of membrane conductance by the addition of lipid permeable cation or anion should increase H^+ uptake. Dimethyldibenzylammonium and SCN^- at 1 mM concentration had no effect on H^+ uptake. These data suggest that development of a potential does not limit H^+ uptake under these conditions, contrary to what would be predicted for electrogenic H^+ uptake.

8. Relationship to ATPase activity. The uptake of H^+ was a saturable function of the ATP concentration and was specific for ATP. The apparent K_m in the absence of a K^+ gradient was $1.7 \cdot 10^{-4}$ M with a V of 154 μmol $H^+ \cdot mg^{-1}$ protein per h^{-1}.

The molar ratio of H^+ uptake to ATP hydrolyzed measured in the first 10 s, under standard conditions was 3.1 ± 0.4 ($n = 10$) if the total ATPase activity was used and 4.1 ± 0.2 ($n = 10$) if only the cation activated component of the ATPase was used in the calculation. Those ratios are to be compared to the value of 4 obtained for H^+ transport per high energy bond for chloroplasts [3] or mitochondria [4].

The pH of the luminal solution of intact mucosa at maximal secretion is less than 1. From this, it can be calculated that transport of 1 mol of H^+ would require about 9 kcal. From values for the energy available from ATP this would suggest a ratio of 1 mol H^+ transported per mol ATP hydrolyzed. In the intact mucosa this discrepancy with the vesicle data may be explained by a variable coupling ratio of the enzyme as a function of the gradient.

From the quantity of H^+ taken up, and the vesicular volume 2 μl mg^{-1} protein), an internal pH of 1.7 can be calculated giving a ΔpH of 4 or so. The actual value is probably less due to buffering inside the vesicles.

The optimum pH of H^+ uptake was found to be between 5.8 and 6.2, correcting for the change in H^+ released by the ATPase reaction. This corresponds generally to the pH optimum of phosphorylation of the enzyme by [γ-^{32}P]ATP not to the pH optimum of dephosphorylation. The correction at a pH of 7.0 and above due to H^+ release by the ATPase is larger than the observed uptake. To establish that an H^+ gradient is indeed present, the uptake of [^{14}C]-imidazole was measured, at pH 7.4. This depends on the assumption that the protonated form of imidazole (pK 6.8) is charged and hence reactively impermeable, and thus the neutral form equilibrates across the vesicle membrane. The increment of imidazole inside the vesicles is shown in Fig. 7. This allowed calculation of a pH difference of 1.5 at this pH. The molar ratio of H^+ taken up to ATP hydrolyzed would be much less at pH 7.4 than at 6.1. However, the imidazole technique is much slower in response than the pH electrode, and is also less sensitive and quantitation depends on the impermeability of the charged form.

Thus a pH gradient is developed across a broad range of pH but seems more closely related to the phosphorylation stage of the overall ATPase reaction namely:

$$ATP + H^+ + ENZ \rightarrow P\text{-}ENZ\text{-}H^+ + ADP$$

$$P\text{-}ENZ\text{-}H^+ + K^+ \rightarrow P\text{-}ENZ\text{-}K^+ + H^+$$

9. Effect of temperature. The availability of a plasma membrane derived

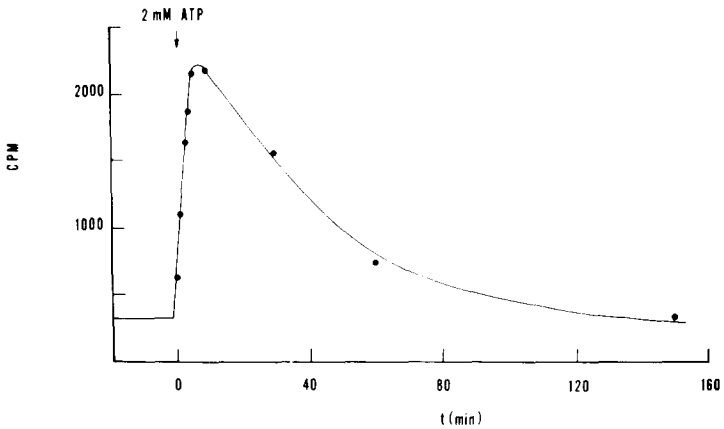


Fig. 7. The uptake of ^{14}C-labelled imidazole as a function of addition of 2 mM ATP. The vesicles were equilibrated using 0.1 mM imidazole as determined by radioactivity trapped on a Millipore HAWP filter and ATP added at zero time on the graph. There was a transient uptake as shown, which decayed over a period of two and a half hours due to the large quantity of ATP used.

vesicle with low permeability and active transport properties made the effect of temperature on transport and ionophore action of interest. Fig. 8 shows the effect of increasing temperature on H^+ uptake as a function of initial rate of maximal uptake under zero K^+ gradient conditions. Both parameters are temperature sensitive, but the rate of uptake is more sensitive with a temperature optimum of 37°C, as for the ATPase activity. When the data for initial rate of uptake and leak of H^+ were plotted as an Arrhenius plot a transition temperature of 22°C was found, with an activation energy of 3.0 kcal/mol above the

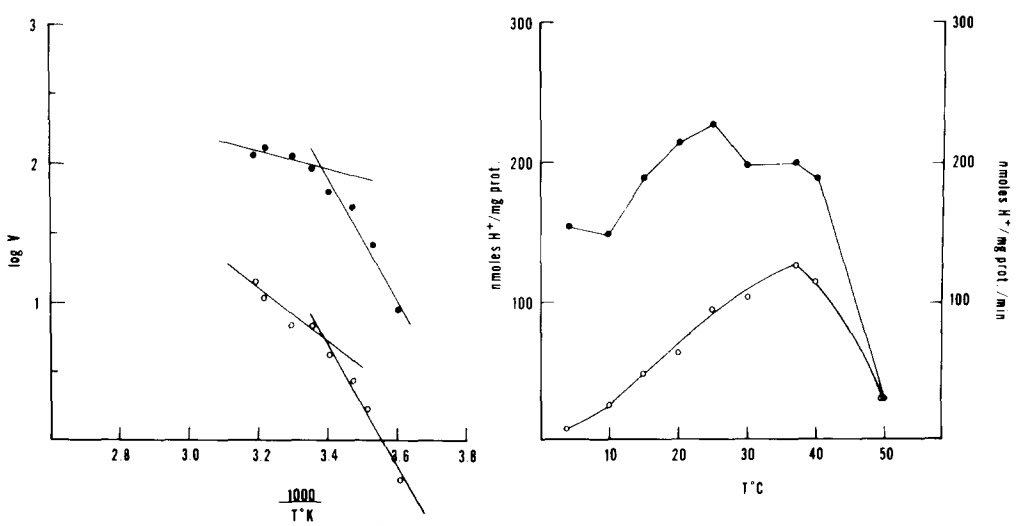


Fig. 8. The effect of temperature on H^+ uptake by gastric vesicles with the addition of $1 \cdot 10^{-4}$ M ATP in the absence of a K^+ gradient with 150 mM KCl. The left hand side of the figure shows the Arrhenius plot of the initial rate (nmol $H^+ min^{-1}$) with ATP addition (●——●) and the leak (nmol $H^+ min^{-1}$) (○——○). The right hand side shows the maximal H^+ uptake observed (●——●) and the initial rate (○——○) as a function of temperature.

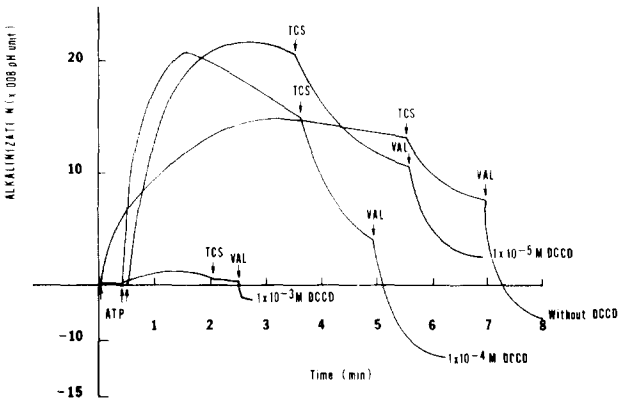


Fig. 9. The effect of various concentrations of the carboxyl reagent DCCD as compared to control on the H^+ uptake induced by ATP showing stimulation of rate and magnitude of H^+ uptake at 10^{-5} and 10^{-4} M and inhibition of 10^{-3} M. TCS, tetrachlorsalicylanilide; VAL, valinomycin.

transition temperature and 18.1 kcal/mol below that temperature for H^+ uptake. The leak rate had a higher activation energy of 8.7 kcal/mol above the transition temperature and a similar 19.4 kcal below.

The action of valinomycin disappeared below 20° C but nigericin was still effective in dissipating the gradient at 4° C.

10. Effect of ATPase inhibitors. Inhibitors of ATPase activity, such as Zn^{2+}, F^- and *p*-chloromercuribenzoate, inhibited H^+ uptake. The action of *N,N'*-dicyclohexylcarbodiimide (DCCD) was investigated in more detail since this reagent also inhibits proton translocation in intact mitochondria [22]. Fig. 9 shows that low concentrations of DCCD apparently increase the rate and maximum of H^+ uptake, whereas at concentrations which inhibit the ATPase, DCCD inhibits H^+ transport.

The action of this inhibitor is therefore complex, but confirms the role of the ATPase in H^+ transport by these vesicles.

11. Effect of irradiation. When the effect of irradiation on ATPase and *p*-

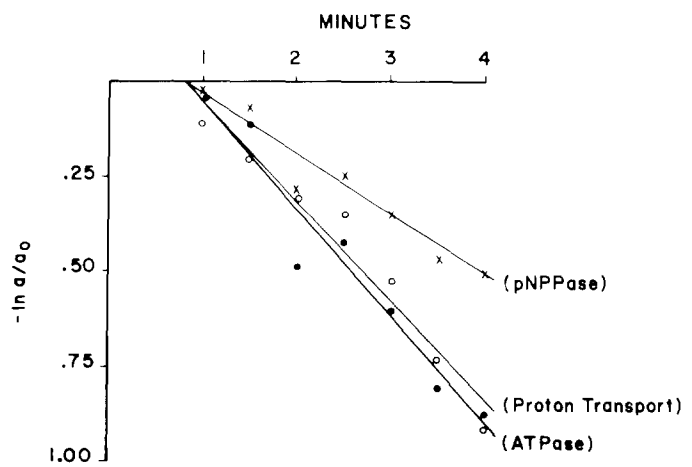


Fig. 10. The effect of irradiation of the vesicles on H^+ uptake (○———○) ATPase (●———●), and *p*-nitrophenyl phosphate (X———X) activities plotted as ln a/a_0 against time of irradiation where a is activity at time t and a_0 is initial activity.

nitrophenyl phosphatase was compared, as shown in Fig. 10, the rate of inactivation of the ATPase was twice that of phosphatase. This suggested that the molecular weight of the ATPase was twice that of the phosphatase, hence that a dimeric form of the enzyme was required for ATPase activity, but either half of the dimer was effective in hydrolysing *p*-nitrophenyl phosphate. H^+ transport was inactivated at the same rate as the ATPase. Similar inactivation data have been found for $(Na^+ + K^+)$-ATPase [23].

Discussion

The above data appears to establish that a vesicular preparation derived from the luminal surface of the parietal cell is capable of taking up H^+ with the addition of ATP.

The uptake of H^+ is K^+ dependent and appears to involve a $K^+ : H^+$ exchange as established above and by direct measurement of cation and anion movement [12].

Measurement of metabolite levels in resting and secreting mucosa of frog [24] and dog mucosa [25] showed a decline in phosphorylation potential. In addition the intramitochondrial pyridine nucleotide became relatively oxidized with onset of acid secretion [26]. These data are compatible with an ATP based mechanism of H^+ secretion, as are data using inhibitors [27].

It has also been known for some time that K^+ is required for H^+ secretion [28] and that this K^+ is only a minor component of the total cellular K^+ [29].

These data derived from the intact mucosa are therefore compatible with a $K^+ : H^+$ exchange ATPase being responsible for H^+ secretion.

This H^+ transport mechanism, occurring in the plasma membrane of a eukaryotic cell appears quite distinct from the H^+ transport process of lower life forms [1]. These latter are clearly electrogenic, do not require cation, involve cooperation of an ATPase and several other subunits [30] and no protein bound phosphate appears to be involved. In mechanism therefore this ATPase seems more related to the $(Na^+ + K^+)$- or Ca^{2+}-ATPase of higher organisms, where the hydrolytic and transport functions have been fused into a single protein. Critical to their transport is the formation of a protein bound phosphate, as for this ATPase.

If this enzyme is the only component of the H^+ pump of gastric mucosa, then the HCl secretion of the tissue must be accounted for by an additional mechanism. A possibility is the diffusional movement of KCl across the mucosal membrane with reabsorption of K^+ in exchange for H^+ with therefore net HCl production.

The electrogenicity of H^+ secretion by the intact mucosa also raises problems for this ATPase mechanism. Various lines of evidence, such as measurement of potentials using SCN^- [12] and 1 anilino-8-naphtosulfonate [31] and effects of ionophores on ATPase activity and H^+ uptake argue against electrogenicity of the vesicle transport system. It is possible that this ATPase is only a partial mechanism and another component, such as a redox component is lost during purification. Another possibility is that the conductance of the pump changes drastically at pH 7.4.

It is interesting also that the ATPase functions in its transport mode as a

dimer. The monomeric *p*-nitrophenylphosphatase reaction does not catalyze transport. On this basis, as for the ($Na^+ + K^+$)-ATPase [32] it can be argued that the enzyme exists as a dimer of unlike conformation E_1E_2 rather than E_1E_1 or E_2E_2. Either E_1 or E_2 can react with *p*-nitrophenylphosphate.

If only E_1 can bind and react with ATP and as a result bind H^+ and E_2P react with K^+ and release H^+ and then transport K^+, the transport reaction may be written as

$$H^+E_1P \cdot E_2PK^+ \rightarrow H^+E_2P \cdot E_1PK^+$$

with release of H^+ in the interior of the vesicle and K^+ and P_i on the exterior with reformation of E_1. In this model therefore the translocation of H^+ and K^+ are strictly coupled. The electrogenicity is thus a function of the pump stoichiometry as for the ($Na^+ + K^+$)-ATPase [33].

Further evidence for the role of this ATPase will depend on the development of specific inhibitors such as ouabain. The enzyme is not a component of other cell membranes which catalyze the development of pH gradients such as the pancreas or the distal tubule of the kidney [34] and thus represents a gastric specialization for H^+ secretion, if indeed it is the H^+ pump of the stomach.

Acknowledgements

Thanks are due to the excellent technical assistance of B. Stewart, D Shaw and D. Dailey and to Dr. A. Mihas and S. Goodson for collaboration in the antibody localization studies. This work was supported by NIH grant AM 15878; CVRTC HL 11310 and NSF grant GB31075.

References

1 Maloney, P.D., Kashket, E.R. and Wilson, T.H. (1974) Proc. Natl. Acad. Sci. 71, 3896—3900
2 Kaback, H.R. (1974) Science 186, 882—892
3 Witt, T.H. (1971) Ann. N.Y. Acad. Sci. 227, 203—206
4 Brand, M.D., Reynafarje, B. and Lehninger, A.L. (1976) Proc. Natl. Acad. Sci. U.S. 73, 437—441
5 Sedar, A.W. (1965) Fed. Proc. 24, 1360—1367
6 Ganser, A.L. and Forte, J.G. (1973) Biochim. Biophys. Acta 387, 169—180
7 Ganser, A.L. and Forte, J.G. (1973) Biochem. Biophys. Res. Commun. 54, 690—696
8 Saccomani, G., Shaw, D., Stewart, H.B., Lewin, M. and Sachs, G. (1976) Biochim. Biophys. Acta, submitted
9 Saccomani, G., Shah, G., Spenney, J.G., Sachs, G. (1975) J. Biol. Chem. 250, 4802—4809
10 Lee, J., Simpson, G. and Scholes, P. (1974) Biochem. Biophys. Res. Commun. 60, 825—832
11 Forte, J.G. and Ray, T.K. (1972) J. Appl. Physiol. 32, 714—717
12 Schackmann, R., Schwartz, A., Saccomani, G. and Sachs, G. (1976) J. Membrane Biol., in the press
13 Alberty, R.P. (1969) J. Biol. Chem. 244, 3290—3302
14 Yoda, A. and Hokin, L.E. (1970) Biochem. Biophys. Res. Commun. 40, 880—884
15 Lowry, O.H., Rosebrough, M.J., Farr, A.L. and Randall, R.I. (1951) J. Biol. Chem. 193, 265—275
16 Coons, A.H., Leduc, E.H. and Connolly, J. (1955) J. Exp. Med. 102, 49—60
17 Forte, J.G., Tanisawa, A. and Ganser, A.L. (1974) Ann. N.Y. Acad. Sci. 242, 255—267
18 Rehm, W.S. (1965) Fed. Proc. 24, 1387—1395
19 Davenport, H.W. (1940) Am. J. Physiol. 129, 505—514
20 Scarborough, G.A. (1976) Proc. Natl. Acad. Sci. 73, 1485—1489
21 Sachs, G., Chang, H.H., Rabon, E., Schackmann, R., Lewin, M. and Saccomani, G. (1976) J. Biol. Chem., in the press
22 Beechey, R.B., Robertson, O.N., Holloway, C.L. and Knight, I.G. (1967) Biochemistry 6, 3867—3879
23 Kepner, G.R. and Macey, R.I. (1968) Biochim. Biophys. Acta 163, 188—203
24 Durbin, R.P., Michelangeli, F. and Michel, A. (1974) Biochim. Biophys. Acta 367, 177—189

25 Sarau, H.M., Foley, J., Moonsamy, G., Wiebelhaus, V.D. and Sachs, G. (1975) J. Biol. Chem. 250, 8321—8329
26 Sachs, G., Rabon, E., Saccomani, G. and Sarau, H.M. (1976) Ann. N.Y. Acad. Sci. 264, 456—475
27 Sachs, G., Collier, R.H., Shoemaker, R.L. and Hirschowitz, B.I. (1968) Biochim. Biophys. Acta 162, 210—219
28 Harris, J.B. and Edelman, I.S. (1964) Am. J. Physiol. 206, 769—782
29 Rehm, W.S., Sanders, J.S., Rutledge, J.R., Davies, T.L., Kurfees, J.F., Keesee, D.C. and Bajandas, F.J. (1966) Am. J. Physiol. 210, 689—693
30 Catterall, W.A. and Pedersen, P.L. (1974) Biochem. Soc. Spec. Publ. 4, 63—90
31 Lewin, M., Saccomani, G., Schackmann, R. and Sachs, G. (1976) J. Membrane Biol., in the press
32 Glynn, I.M. and Karlish, J.D. (1975) Energy Transformations in Biological Systems, pp. 205—224, Ciba Press
33 Sen, A.K. and Post, R.L. (1964) J. Biol. Chem. 239, 345—352
34 Schwartz, I.L., Shaltz, L.J., Kinne-Saffran, E. and Kinne, R. (1974) Proc. Natl. Acad. Sci. U.S. 71, 2595—2649