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Quantitation of Hydrogen Ion and Potential Gradients in Gastric Plasma Membrane Vesicles†

E. Rabon, H. Chang, and G. Sachs*

ABSTRACT: The ATP-dependent uptake of H^+ by hog gastric parietal cell vesicles was quantitated by using the pH indicator dyes bromcresol green and malachite green, the weak bases, aminopyrine and 9-aminoacridine, and the pH electrode. A K^+-dependent H^+ uptake was found, with a significant difference between the quantity of H^+ disappearing from the medium (ΔH_o) and the quantity appearing inside the vesicle (ΔH_i). 9-Aminoacridine gave a lower value for the ΔH_i than any of the other probes. Probes of potential such as diethyloxadicarbocyanine or oxonol dyes showed that only secondary diffusion potentials occurred during H^+ uptake and that the cationic dyes in the presence of protonophores could also be used to quantitate H^+ uptake. The potential in the presence of protonophore indicated a ΔH_i greater than that found with the other probes. Binding sites for acridine orange were generated either by ATP or an artificial pH gradient and corresponded to the ΔH_i indicated by aminopyrine. SCN^- (30 mM) only partially inhibited the H^+ gradient, and this, coupled with the failure to detect the physiological ΔpH of 6.6, indicated that these vesicles may be an incomplete model of gastric acid secretion.

Purified hog gastric vesicles derived from the acid secreting (parietal) cell of gastric epithelium (Saccomani et al., 1978) contain as a major protein a K^+-activated ATPase (Forte et al., 1974; Sachs et al., 1976) distinct from the $Na^+ + K^+$ ATPase (Skou, 1965). These vesicles, due to the ATPase, are capable of simultaneously translocating H^+ and K^+ (Sachs et al., 1976) apparently by an electroneutral exchange (Schackmann et al., 1977; Lewin et al., 1977). These vesicles are oriented so that the cytoplasmic side faces the external medium (Saccomani et al., 1977). In the absence of ATP the vesicles have a low permeability to K^+ and Cl^- and a low, but measurable H^+ conductance (Chang et al., 1977).

Since these vesicles provide a unique opportunity to study the transport activity of a plasma membrane ATPase of a eukaryotic cell, a variety of probes were used in order to quantitate the pH gradient and any primary or secondary potentials generated by the transport process.

Osmotic gradient experiments have proved useful in distinguishing uptake and binding (Kinne et al., 1975) and are also helpful in discriminating between net uptake and exchange mechanism. 9-Aminoacridine has been used extensively in detection and quantitation of H^+ trapped in vesicles (Schuldiner et al., 1972; Fiolet et al., 1974). pH indicator dyes are useful in discriminating pH changes in the medium or in the vesicle space (Chance, 1975). Uptake of a weak base or weak acid has been employed for studies of pH gradients in gastric glands (Berglindh, 1977) and in vesicles (Ramos & Kaback, 1977). Carbocyanine dyes as probes of potential have been applied to intact tissue such as cornea (Graves et al., 1978), squid axon (Waggoner, 1976) or chromatophores (Pick & Avron, 1976), red cells (Sims et al., 1974), and mitochondria (Laris et al., 1973) and appear to be useful probes of potentials across membranes. Acridine orange appears to alter its binding characteristics in mitochondria (Dell'Antone et al., 1972) and gastric vesicles (Lee et al., 1976) as a function of what has been termed energization, or more probably H^+ gradients. All of these probes may act as ATPase inhibitors, or as uncouplers; thus optical measurements are combined with measurement of enzyme activity and measurement of ΔH_o using the pH electrode.

The application of these techniques to gastric vesicles, as described herein, indicates that a quantitative discrepancy may exist between the amount of H^+ disappearing from the medium and the quantity of H^+ appearing inside the vesicle with different probes. As concluded previously, the H^+ pump appears to have nonelectrogenic characteristics. SCN^- did not inhibit transport by these vesicles to the extent expected from its action in the intact tissue.

Materials and Methods

A. Vesicle Preparation

The purified gastric vesicles were obtained as detailed elsewhere (Chang et al., 1977). The fraction used was obtained at the sucrose–ficoll interface in a Beckman Z60 rotor.

For studies of transport parameters, the vesicles were routinely incubated in a suspension at a final concentration of 150 mM KCl, 2 mM $MgCl_2$ and 1–20 mM buffer (pH 6.1 or 7.4) for 2 h at room temperature. The suspension was then placed on ice and used within 3 h. Longer times of incubation at room temperature resulted in increased leak by the vesicles. On occasion vesicles were used which had not been KCl equilibrated. Ionophores were added as described and SCN^-, when used, was present at 30 mM final concentration. Vesicle protein was set to a final concentration of 0.25 to 0.3 mg/mL, corresponding to 0.50 to 0.66 μL of vesicle volume mL^{-1}. Protein was measured according to Lowry et al. (1951) and ATPase as previously described (Saccomani et al., 1977). The probes were added at concentrations identical with those used in the optical experiments.

B. Measurements

1. *Proton Uptake Measurements.* Proton uptake with a pH electrode was measured as described elsewhere (Chang et al., 1977), using a Radiometer pHM 64 meter. Proton uptake was measured in the presence of each probe used under conditions identical with those chosen for the optical assays, at pH 6.1.

For measurement of the effects of osmolarity on proton

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uptake, two conditions were chosen. In one set of experiments vesicles were added to 150 mM KCl in 5 mM glycylglycine buffer containing increasing concentrations of mannitol giving an osmolarity range of 310 mosM to 770 mosM. Since ΔH_o was measured in these experiments, a weak buffer was used in the medium. ATP (0.6 mM) was added 2 min later. Alternatively, vesicles were preequilibrated in 150 mM KCl at 4 °C for 24, 48, and 72 h before the addition of the mannitol and ATP. At 4 °C the vesicles were virtually equilibrated between 48- and 72-h incubation (Schackmann et al., 1977). The 4 °C temperature was chosen in order to maintain vesicle function over the prolonged period required to achieve equilibration of KCl. In each case the maximum transport was measured and plotted as a function of the reciprocal of the osmolarity.

Calibration of the pH signal was carried out in all experiments by measuring the deflection caused by the addition of 10 nmol of HCl in 10 μL to the final mixtures, or by returning the external pH to the initial value, by successive additions of 10 nmol of HCl in 10 μL volumes.

2. *Measurements Using Bromcresol Green.* Absorbance changes of this dye were measured in the Aminco DW2 spectrophotometer in the dual beam mode set at 617 and 680 nm. The reaction mixture consisted of 1.0 mg/mL vesicle suspension incubated for 2 h in 150 mM KCl, 2 mM $MgCl_2$, and 5 mM glycylglycine buffer (since external pH is being monitored), pH 6.1, also containing bromcresol green at 15 μM. ATP was added to a final concentration of 0.6 mM to initiate the reaction and ionophores were added in 4 μL of methanol. Calibration was carried out using additions of 10-μL aliquots of 10^{-3} M HCl and measuring the absorbance change, since this dye monitored extravesicular pH.

3. *Aminoacridine.* Fluorescent quenching of 9-aminoacridine was measured in a Perkin-Elmer MPF 44 scanning fluorometer with 398-nm excitation and 430-nm emission wavelengths. The standard experimental sample consisted of a 500-μL aliquot of vesicles (0.25–0.3 mg mL^{-1}) equilibrated in 150 mM KCl, 2 mM $MgCl_2$, and 2 mM Pipes[1]-Tris (pH 6.1 or 7.4); then 3.5 μL of aqueous 1 mM 9-aminoacridine was added and 7 μL of 30 mM Mg ATP at identical pH was added to initiate the reaction. Ionophores such as nigericin, valinomycin, or tetrachlorosalicylanilide (TCS) were added in 3.5 μL of methanol. Control experiments showed that this quantity of methanol was without effect. In experiments designed to shrink the preequilibrated vesicles, the volume of the initial suspension, with concentrations as above, except for twice the protein, was 200 μL to which was added 200 μL of a solution containing 150 mM KCl, 2 mM $MgCl_2$, 2 mM Pipes–Tris (pH 6.1 or 7.4) and 67–333 mM mannitol, the mannitol being added to vary the osmolarity. Subsequent additions were as above. In nonequilibrated experiments, only 15 min exposure to the KCl solution was allowed before ATP addition.

For direct calibration of the ATP-induced signal since this was due to a change in intravesicular pH, the vesicles were incubated in 0.25 M sucrose containing 5 mM sodium succinate (pH 5.5) for 30 min at room temperature. The succinate was used to increase the proton capacity of the intravesicular space. At this point 100 μL of 100 mM Pipes–Tris buffer adjusted to pH 6, 6.5, or 7 and 100 mM Tris buffer adjusted to pH 8, 8.5, 9, 9.5, or 10 was added to 350 μL of this suspension giving a final protein concentration of 0.3 mg mL^{-1}. The pulse-generated change in fluorescence was recorded. Varying sucrose concentrations were used with each pulse to maintain constant osmotic pressure and a plot made of the log of fluorescence quenching ratio against the change of pH. Parallel experiments with appropriate volume increments were used to measure the exact pH change induced by buffer addition, using the pH meter. The principles of this method have been worked out for other subcellular particles (Schuldiner et al. 1972).

4. *Measurements Using [^{14}C]Aminopyrine.* Twenty micrograms of the vesicles was suspended to a final volume of 450 μL in 150 mM KCl, 2 mM $MgCl_2$, 5 mM glycylglycine (pH 6.1) or 5 mM Pipes–Tris (pH 7.4) for 24 h at 4 °C. This time was chosen since an equivalent KCl equilibration occurred as compared with the 2 h at room temperature used with the other probes. After warm up to room temperature, the reaction was initiated by addition of 50 μL of ATP and [^{14}C]aminopyrine giving a final concentration of 2 mM ATP and 5 μM aminopyrine with 100 000 cpm mL^{-1}. At timed intervals, the suspension was placed directly on a Millipore filter (HAWP, 0.45 μm) and immediately washed with 10 mL of 150 mM KCl, 2 mM $MgCl_2$, and 5 mM glycylglycine (pH 6.1) or 5 mM Pipes–Tris (pH 7.4) at 0 °C. Each time point was repeated in triplicate; the filters were dried and suspended in 10 mL of Aquasol. An LKB 81000 scintillation counter was used to measure radioactivity remaining on the filters. The ΔH_i was calculated from the equation, ratio in/out = $1 + 10^{pK-pH_i}/1 + 10^{pK-pH_o}$. This distribution ratio method has been used, for example, in bacterial vesicles (Ramos & Kaback, 1977).

5. *Measurements Using Malachite Green.* The experiments were carried out, also with the dual beam spectrophotometer set at 623 and 680 nm. Vesicles at 0.3 mg mL^{-1} were incubated with 15 μM malachite green, 150 mM KCl, 2 mM $MgCl_2$, and 2 mM Pipes–Tris buffer at pH 6.1 and 7.4 for 2 h at room temperature. ATP was added to a final concentration of 0.6 mM to initiate the reaction. Ionophores were added as usual in 3.5 μL of methanol.

6. *Potential Measurements.* Absorbance of 3,3′-diethyloxadicarbocyanine iodide (DOCC) and di-S-C_3-(5) was monitored in the Aminco DW 2 scanning spectrophotometer. Absorbance changes were measured at 598–636 nm for DOCC and 661–714 nm for di-S-C_3-(5). The reaction suspension consisted of vesicles (0.3 mg mL^{-1}; incubated for 2 h) in 150 mM KCl, 2 mM $MgCl_2$, and 2 mM Pipes–Tris at pH 6.1 and 7.4. Dye additions (4 μM) were made from a 400 μM solution in MeOH. ATP, 0.6 mM (adjusted to pH), was added at zero time to a 350-μL aliquot of the reaction suspension with or without 6 μM tetrachlorosalicylanilide (TCS).

For osmotic experiments, 386 mM mannitol was added immediately before the ATP. Calibration of the cyanine dye signal due to a change in intravesicular pH was carried out by suspending the vesicles (0.3 mg mL^{-1}) in a solution of 5 mM sodium succinate (pH 5.5) in 238 mM sucrose with 4 μM dye and adding 100 μL of 100 mM Pipes–Tris buffer adjusted to pH 6, 6.5, or 7 and at 100 mM Tris buffer adjusted to pH 8, 8.5, 9, 9.5 to 350 μL of the suspension. Absorbance was recorded for 20 s and then 6 μM TCS was added and absorbance recorded for a further 40 s. The actual change of pH was measured on a pH meter with the volume of reaction mixture scaled up to 2.0 mL. The standard curve was generated from a plot of the total absorbance change at each pH increment. The principle of these experiments was the same as used elsewhere, except that valinomycin-K^+ gradients have been more generally used for calibration (Waggoner, 1976).

7. *Measurements Using Acridine Orange.* Absorbance changes of acridine orange were measured in the DW2 spectrophotometer in the dual beam mode set at 446 and 496 nm.

[1] Abbreviations used: TCS, tetrachlorosalicylanilide; DOCC, 3,3′-dimethyloxadicarbocyanine iodide: di-S-C_3-(5), 3,3′-dipropyl-2,2′-thiodicarbocyanide iodide; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Ans, 8-anilino-1-naphthalenesulfonate.

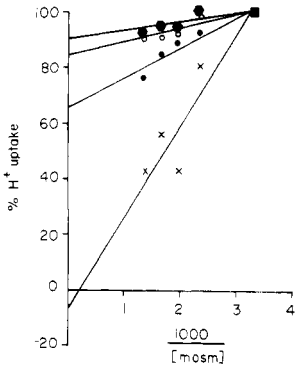

FIGURE 1: Vesicles were incubated with 150 mM KCl, 5 mM glycylglycine, 2 mM $MgCl_2$ at 4 °C for varying lengths of time [(X—X) 2 min; (●—●) 24 h; (○—○) 48 h; (●—●) 72 h], and 0.6 mM ATP was added with varying concentrations of mannitol. The maximal H^+ uptake measured by the pH electrode (calculated as % of control) was plotted as a function of the reciprocal of total medium osmolarity.

To 350 μL of a vesicle suspension (0.3 mg mL^{-1}) incubated for 2 h with 150 mM KCl, 2 mM $MgCl_2$, and 2 mM Pipes-Tris buffer (pH 6.1 or 7.4), ATP at identical pH to the incubation buffer was added to give a final concentration of 0.6 mM. Acridine orange was present at 5 μM final concentration. Ionophores were added in 3.5 μL of methanol before or after ATP addition.

Two methods were used to calibrate the absorbance change. Again, since the change was due to an increase in H^+_i, vesicles were incubated in 250 mM sucrose containing 5 mM sodium succinate at pH 5.5, 6, 6.5, 7, 7.5, 8, 8.5, and 9 and the pH was increased abruptly by addition of 100 μL of 100 mM Pipes-Tris buffer adjusted to 1 pH unit greater than the 350 μL of the vesicle suspension containing 5 μM dye. Alternatively, 350-μL vesicles (0.3 mg mL^{-1}) equilibrated with 238 mM sucrose containing 5 mM sodium succinate at pH 5.5 were pulsed with 100 μL of Pipes-Tris buffer at pH 6.0 and 6.5 or 100 mM Tris buffer adjusted to pH 7, 7.5, 8, 8.5, 9, and 9.5. Again, varying sucrose concentrations were used to maintain isoosmotic conditions. The exact pH gradient was calibrated in a volume of 2.0 mL using a pH meter as before. The methods used were similar to those previously applied (Lee et al., 1976; Dell'Antone et al., 1972).

8. *ATPase.* ATPase was measured as previously described (Saccomani et al., 1977). The conditions in the measurement of P_i release were 40 mM Tris-Cl (pH 7.4), 2 mM $MgCl_2$, 2 mM ATP, and 10 μg of enzyme per tube. The material was incubated for 15 min at 37 °C in 1 mL total volume. Probe additions were added at concentrations identical with those used in the optical experiments. In the presence of SCN^- the conditions were 50 mM Tris-acetate (pH 7.4), 2 mM $MgCl_2$, 2 mM ATP, and 20 g of enzyme per tube and incubation time was increased to 30 min at 37 °C.

C. Chemicals

ATP was purchased from Sigma, 3,3′-diethyloxadicarbocyanine iodide from Eastman, and 9-aminoacridine from Aldrich. Di-S-C_3-(5) was a gift from Dr. A. Waggoner. Acridine orange was a gift from Dr. J. M. Menter. Malachite green was a gift from Dr. T. Christian. Ionophores were purchased from Sigma Chemical Co. or obtained as gifts from Dr. H. A. Lardy. Tributyltin was purchased from K & K Lab, Inc. All other chemicals were the highest purity grade available. Hog stomachs were donated by R. L. Zeigler Meats, Tuscaloosa, Ala.

TABLE I: A Comparison of the ΔpH Obtained Using Various Probes of pH Inside or Outside the Vesicles at pH 6.1 or 7.4.

transport measurement	pH 6.1	pH 7.4
pH electrode	4.92	*c*
fluorescent quench 9-aminoacridine	2.2[a], 1.9[b]	2.8[a], 2.8[b]
[^{14}C]aminopyrine accumulation	3.37	4.41
absorption of (DOCC + TCS)	3.7	5.9
absorption of acridine orange	3.2	4.6
absorption of bromcresol green	4.89	*c*

[a] This value was calculated from the equation in text. [b] This value was obtained from the standard curve. The values given here were obtained on the same vesicle population for which the calibration curves were generated but reflect the average signal obtained in several experiments. [c] Not measurable at this pH. The values of ΔpH were obtained directly from the calibration curves as shown in the figures and are typical of the different probes used.

Results

A. Probes of pH

(a) *External.* Here two methods, the pH electrode and bromcresol green, were used.

1. pH Electrode. Under the standard conditions (2-h incubation, room temperature) the addition of 0.6 mM ATP resulted in a change of pH of the external medium equivalent to the uptake of 69 nmol of H^+ μL^{-1} vesicle space (Table I). These data have been detailed elsewhere (Chang et al., 1977).

The osmotic sensitivity of vesicular transport may be used to distinguish various aspects of uptake. It was previously shown that uptake of ^{86}Rb was time and temperature dependent, and 48 h or more at 4 °C was required for equilibration in the presence of Cl^- (Schackmann et al., 1977) but considerably longer was necessary in the presence of SO_4^-. As shown in Figure 1, the time of equilibration with KCl has marked effects on osmotic sensitivity. With only 2-min incubation, at infinite osmolarity, zero H^+ uptake is predicted. As the vesicles approach equilibrium, osmotic sensitivity is progressively reduced, and essentially disappears after 72-h incubation. If K_2SO_4 was used, even after 72 h, partial osmotic sensitivity was retained. The addition of lipid permeable ions did not reduce the osmotic sensitivity in SO_4^{2-} solutions (data not shown), suggesting the presence of a KCl symport pathway, rather than a coupled K^+ and Cl^- conductance.

2. Bromcresol Green. The addition of ATP to the preincubated vesicles resulted in a rapid shift of the dye absorbance (Figure 2). Calibration of this dye signal showed that the change of pH indicated by this dye corresponded almost exactly to the change measured by the pH electrode, that is, a change of 4.89 at pH 6.1 (Table I). Partial inhibition of ATPase activity occurred with this dye, which probably accounts for the small inhibition of H^+ uptake seen using the pH electrode in the presence of this dye. The OD change shown by bromcresol green was reversed by nigericin or a combination of valinomycin and TCS. Addition of a stronger buffer to the medium (10 mM Pipes-Tris) abolished the OD response.

(b) *Internal.* 1. 9-Aminoacridine. ATP addition to incubated vesicles in the presence of 9-aminoacridine resulted in quenching of the fluorescence as would be predicted from the development of intravesicular acidity (Schuldiner et al., 1972) and hence trapping of the dye. Nigericin reversed the change of fluorescence (Figure 3) and, after exhaustion of ATP, the addition of valinomycin also reversed the signal. In contrast to the enhancement obtained with the pH electrode (Sachs et al., 1977) the addition of valinomycin in the presence of ATP

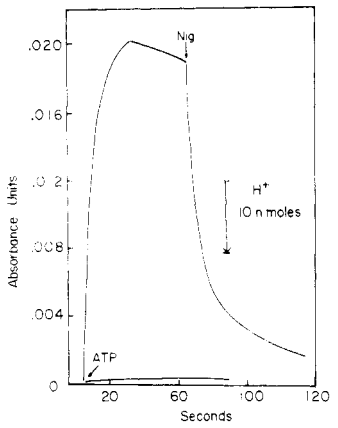

FIGURE 2: Change in absorbance of 15 μM bromcresol green resulting from addition of 0.6 mM Mg–ATP to 350 μL of incubation medium. As in text, incubation medium consisted of 150 mM KCl, 2 mM $MgCl_2$, 5 mM glycyglycine (pH 6.1) incubated for 2 h at room temperature with GI at a final protein concentration of 1 mg mL^{-1}. The OD difference between 680 and 617 nm was monitored. The calibration signal corresponds to the addition of 10 nmol of HCl. The lower curve shows the optical change in the presence of 10 mM Pipes–Tris buffer (pH 6.1).

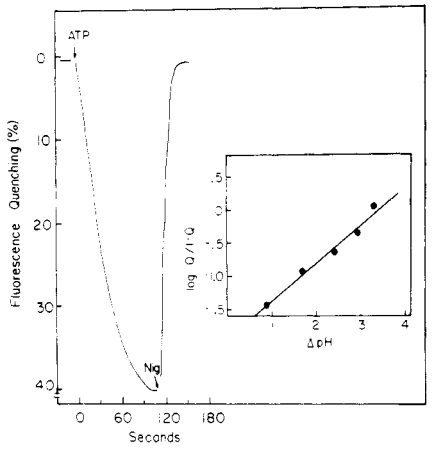

FIGURE 3: The vesicles were incubated with 150 mM KCl, 2 mM $MgCl_2$, and 2 mM Pipes–Tris buffer (pH 7.4) for 2 h at room temperature. Following 9-aminoacridine addition to a 250-μL aliquot of the incubated material, 0.6 mM Mg–ATP was added and the reaction followed in a fluorimeter set at excitation 398 nm, emission 430 nm. Nigericin (1 μg mL^{-1}) was added at the point of maximal fluorescent quenching. The insert shows the plot of log of quench ratio as a function of an applied pH gradient by addition of external base, the initial pH being 5.5. When the ratio is unity, the vesicular volume is calculated as 1.9 μL mg^{-1}.

resulted in a slight reduction of the signal. This dye has a slightly larger effect on the quantity of H^+ transport as compared with the inhibition of ATPase activity (Table II).

The response to 9-aminoacridine was maximal in 90 s, in contrast to the 30-s maximum given by the pH electrode, bromcresol green, and acridine orange. However, the rate of reversal by nigericin was as fast as with the other methods. Imidazole at 0.3 mM reduced the signal observed, without an effect on ATPase activity, but with a reduction in H^+ uptake as also measured by the pH electrode (Table II).

SCN^- (30 mM) partially inhibited the dye signal (Table III) in contrast to the complete inhibition of H^+ transport observed in the intact mucosa (Rehm, 1972) and isolated glands (Berglindh, 1976).

The magnitude of the signal was linearly dependent on protein concentration in the range of 0.125–0.5 mg of protein at 10 μM aminoacridine. Above that concentration, the degree of quench was reduced.

TABLE II: The Effect of Different Probes and Other Additions on ATPase Activity and H^+_o of Gastric Vesicles at pH 6.1.[a]

additions to controls	% of control activity: proton transport	% of control activity: K^+-ATPase
6 μM TCS	70.3	77 5
50 μM $TPMP^+$	68.4	77.5
4 μM DOCC	80.2	83.0
4 μM acridine orange	73.0	97.6
0.3 mM imidazole	76.0	95.3
3 mM imidazole	19.0	97.0
10 μM 9-aminoacridine	87.0	94.0
15 μM malachite green	87.3	85.0
15 μM bromcresol green	90.0	84.5
0.5 μM tributyltin	10.0	46.0
5 μM aminopyrine	94.5	101.4

[a] The concentrations of material used were the same as used in the experiments in the text. Proton transport was measured using the pH electrode with vesicles preincubated with KCl for 2 h at room temperature, and the compounds of interest were added before ATP. ATPase activity was measured at 37 °C and reflects the effect of the compounds on the K^+ increment of activity.

TABLE III: The Effect of SCN^- (30 mM) on the Transport Parameters of Gastric Vesicles at pH 6.1 and 7.4.

transport measurement	% inhibition with 30 mM SCN^-: pH 6.1	% inhibition with 30 mM SCN^-: pH 7.4
10 μM 9-aminoacridine	39.7	40.0
4 μM DOCC (+TCS)	77.5	73.3
4 μM acridine orange	7.9	29.0
5 μM aminopyrine	36.8	30.7
pH electrode	48.8	*a*

[a] Could not be measured at this pH. These experiments were done on a single preparation of vesicles but are generally representative of the effects found, following addition of SCN^- before the addition of ATP. The inhibitory action of SCN^- on ATPase under these conditions was minimal.

Osmotic sensitivity of the dye signal, using 10 M dye, was measured under conditions where ΔH_o was osmotically sensitive. The signal was partially osmotically sensitive, the intercept at zero vesicular volume corresponding to the bound component, constituting about 20% of the total signal. With preincubated vesicles, no effect on the signal was observed due to an osmotic gradient showing that the decrease in trapping volume was exactly balanced by the increase in $[H^+_i]$.

From the equation

$$\frac{A_i}{A_o} = \frac{[H^+_i]}{[H^+_o]} = \frac{Q}{100 - Q}\frac{V}{v}$$

(where A_i and A_o are internal and external aminoacridine concentrations, $[H^+_i]$ and $[H^+_o]$ are internal and external H^+ concentrations, Q is the % quench, V is medium volume, and v is vesicle volume), the ΔH_i can be calculated (Table I). Equivalently, the ΔH_i can be obtained directly from the calibration curve of aminoacridine (Table I). It can be seen that, although the maximum pH gradient was observed at pH 7.4, more than two orders of magnitude difference was obtained with this technique comparing ΔH_i and ΔH_o at pH 6.1. In part, this may be due to uncoupling by the dye (Table II) or due to internal buffering capacity of the vesicles or other H^+ binding sites.

2. Aminopyrine Uptake. Since the data using aminoacridine did not agree quantitatively with the pH electrode observations,

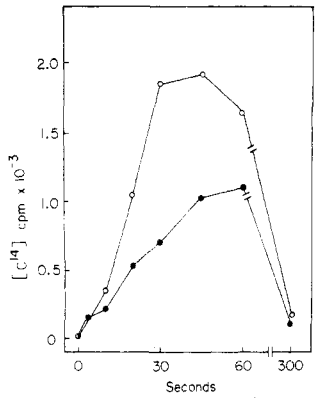

FIGURE 4: Vesicles were incubated 24 h at 4 °C in 150 mM KCl, 2 mM $MgCl_2$, and either 5 mM glycylglycine (pH 6.2) or 2 mM Pipes–Tris (pH 7.4); protein concentration was 44 μg mL^{-1}. For each point, a 450-μL aliquot was removed and equilibrated to 23 °C. At zero time, 50 μL of solution containing 20 mM Mg–ATP (pH 6.1) and 50 μM aminopyrine (with 100 000 dpm) was added to the incubated vesicles. At the indicated time intervals, the reaction mixture at pH 6.1 (O—O) or pH 7.4 (●—●) was placed directly on a HAWP (0.45 μm) Millipore filter and washed, dried, and counted as detailed in the text.

uptake of [^{14}C]aminopyrine was measured under identical conditions. As shown in Figure 4, this base was rapidly accumulated by the vesicles at pH 6.1 or 7.4 with an accumulation ratio of 209 and 160, respectively, upon the addition of ATP. Aminopyrine did not affect ATPase activity and slightly inhibits H^+ uptake at these concentrations (Table II). Again, as shown in Table I, the ΔH_i calculated from the distribution ratio was considerably less than that found using the pH electrode; this corresponds to a ΔpH of 3.37 units, larger than that calculated with 9-aminoacridine.

3. Malachite Green. This dye was chosen because of its low pK (0.8–1.2) and because it was shown to rapidly bind to the vesicles (95% within 10 min, 22 °C). As shown in Figure 5, the addition of ATP produced an acid shift of the dye absorbance, which was reversed by nigericin. At the concentration necessary for an observable optical signal, the dye showed partial inhibition of ATPase and H^+ transport activity (Table II). The response time of the dye, either at pH 6.1 or 7.4, was considerably slower than the pH electrode response. While the signal was reversed by nigericin, the rate of reversal was slow compared with the effects of nigericin using other probes. This slow effect was also produced by acid or base pulses or valinomycin addition at the point of maximum absorbance shift. This could be due to a slow displacement of the dye from membrane binding sites into intravesicular space or slow conformational changes in the vesicle membrane. Slow responses of malachite green have also been observed in squid axon (Cohen et al., 1974), explained as due to scattering changes.

B. Probes of Potential

It was shown previously that uptake of lipid permeable anions such as [^{14}C]SCN (Schackmann et al., 1977) or changes in fluorescence of 8-anilino-1-naphthalenesulfonate (Ans) were dependent on the presence of both ATP and valinomycin (Lewin et al., 1977). Moreover, in the case of Ans a sharp pH optimum was found at 6.1 in contrast to the transport of H^+ or Rb^+. Because Ans responses are highly pH dependent and certain ambiguities exist in the interpretation of the Ans signal (Aiuchi et al., 1977; Williams et al., 1977), carbocyanine dyes (Waggoner, 1976) were used in this study. Essentially identical data were obtained using either diethyl oxadicarbocyanine (DOCC) or Di S-C_3-(5); hence we will only describe the results using DOCC.

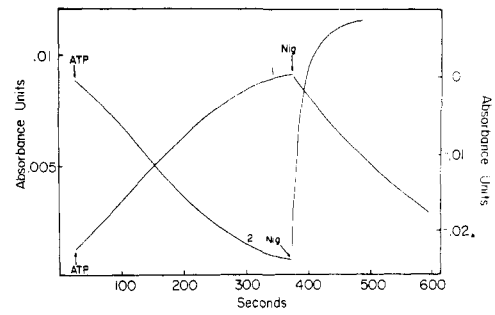

FIGURE 5: The vesicles (0.3 mg mL^{-1}) were incubated in 150 mM KCl, 2 mM $MgCl_2$, and 2 mM Pipes–Tris (pH 7.4) for 2 h at room temperature. Following a 10-min incubation of 350 μL of this material with either 15 μM malachite green or 4 μM DOCC, 0.6 mM Mg–ATP was added. Absorbance was monitored at 623–680 nm for malachite green (left-hand axis) and 588–636 nm for DOCC (right-hand axis). Curve 1 shows the change of OD of malachite green upon addition of Mg–ATP. The shift corresponds to an acid shift of the dye. Curve 2 shows the slow DOCC signal resulting from Mg–ATP addition. Nigericin (1 μg mL^{-1}) addition in both curves was at the point of the maximum absorbance shift.

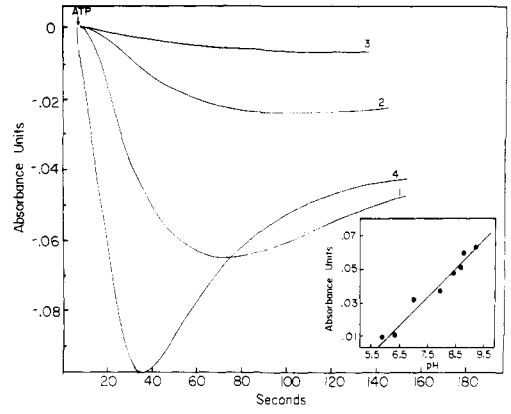

FIGURE 6: GI vesicles (0.3 mg mL^{-1}) were incubated in 150 mM KCl, 2 mM $MgCl_2$, and 2 mM Pipes–Tris (pH 7.4) for 2 h at room temperature. For each experiment, 4 μM DOCC and 6 μM TCS were added to 350-μL aliquots of the incubated material. Following addition of 0.6 mM Mg–ATP, the absorbance change at 588–636 nm was monitored. (Curve 1) Control signal for DOCC and TCS; (curve 2) 10-min incubation with 0.3 mM imidazole before addition of Mg–ATP; (curve 3) 10-min incubation with 50 μM $TPMP^+$ before addition of Mg–ATP; (curve 4) addition of Mg–ATP immediately after addition of 286 nM mannitol. According to details in the text, the insert shows a calibration curve obtained with the vesicles by adding a pulse of base to vesicles preincubated at pH 5.5 in sodium succinate.

(1) *Slow Changes.* Whether a ΔpH is applied chemically in the absence of a K^+ gradient, or ATP added to preincubated vesicles, a slow change in the absorbance of DOCC occurred which was rapidly dissipated by nigericin. The time course of the slow change, as shown in Figure 5, was slower than that observed under the same conditions with 9-aminoacridine, bromcresol green, or the pH electrode, but similar to that of malachite green.

No transient increase of absorbance was seen, and the development of this slow change, antagonized by nigericin, argues against an electrogenic H^+ pump. Moreover, oxonol dyes were altogether unresponsive to ATP addition arguing against development of a positive potential (data not shown) due to pump activity.

(2) *Fast Changes.* The addition of a proton conductance, in the form of TCS, drastically altered the time course of the reduction of DOCC absorbance and increased its magnitude (Figure 6). Under these conditions, the addition of ATP, or a pulse of base, produced a rapid change of absorbance, with a

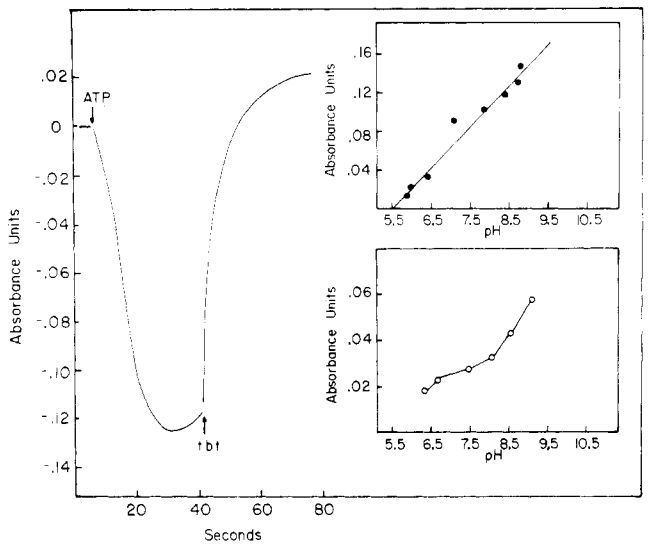

FIGURE 7: The change in acridine orange absorbance following the addition of 0.6 mM Mg-ATP to vesicles preincubated as in the text at pH 6.1 for 2 h at room temperature. At the maximum absorbance change dissipation was induced by addition of 5 μM tributyltin. The upper insert shows the calibration curve obtained by applying an increasing pH gradient to vesicles initially at pH 5.5 in sodium succinate. The lower insert shows a curve obtained by applying a constant pH pulse of 1 unit to vesicles preincubated at varying pH.

time course consistent with the development of the H^+ gradient. Imidazole reduced the DOCC signal indicating that the source of the signal is indeed an H^+ gradient (Figure 6). By comparison of the ATP-induced absorbance shift to the calibration curve determined by alkaline pulses on the same material, the pH gradient developed with ATP addition at pH 6.1 was 4.1 pH units and at pH 7.4 was 5.9 pH units, a higher value than observed with other probes.

When the vesicles are equilibrated with KCl, approximately doubling the osmotic gradient would transiently double the H^+ ion concentration inside the vesicles given an electroneutral H^+:K^+ exchange. This would result in an increase of the diffusion potential by a factor of 58 log 2, i.e., 17 mV, and a corresponding doubling of the dye concentration inside the vesicle, which would balance the decline in volume if dye uptake were the sole mechanism for potential sensitivity of this probe. Accordingly, as for 9-aminoacridine, no net change in signal would be expected. But, as shown in Figure 6, there is a more rapid and a larger change in absorbance.

$TPMP^+$ abolished the signal when added before ATP, as expected of a lipid permeable cation dissipating the potential in preference to the dye. This cation did not, under these conditions, abolish the H^+ gradient (Table II). Valinomycin (10^{-6} M) in the presence of TCS prevented or abolished the pH electrode response (Chang et al., 1977) and the dye signal. Imidazole also reduced the signal as with the pH electrode (Figure 6 and Table II). The dye reduced ATPase activity by 17% at 4 μM (Table II). SCN^- significantly reduced the signal, both at pH 6.1 and 7.4. Apparently, the action of SCN^- was additive to the action of TCS on H^+ uptake (48% + 30%) (Table III). Hence, the direct action of SCN^- on the H^+ gradient sensed by DOCC was similar to that found with other probes.

Substitution of K^+ with Na^+ abolished the response, and of Cl^- by SO_4^{2-} reduced the response, as observed previously with the pH electrode and all the other probes. In aged vesicles, the substitution of Cl^- by SO_4^{2-} increased the response slightly and reduced the leak rate of the gradient.

C. Probes of Energization

Acridine orange has been used in several vesicular systems as a probe of energization (Dell'Antone et al., 1972) and also in gastric vesicles (Lee et al., 1976). In the latter system, the interaction of the dye with the vesicles has been interpreted as being due to binding of the positively charged dye to negative sites generated by the energization process (Lee et al., 1976).

The application of a ΔpH resulted in a linear change of the signal as a function of the magnitude of the pH gradient, allowing calibration of the signal. In addition, the acridine orange signal was sensitive to the pH of the experiment. As shown in the insert in Figure 7, there was an increase of the signal with increase of pH, at a constant H^+ gradient. The two types of signal were reversible with nigericin, but not with TCS indicating the absence of an endogenous conductance other than that for H^+ in nonenergized vesicles.

The addition of ATP produced a rapid metachromatic shift of acridine orange as shown in Figure 7. Calibration of this response by comparison of the shifts produced by standard pH pulses of the same vesicle material indicated a ΔH_i of 3.2 units at pH 6.1. As with pH electrodes and other probes of pH, K^+ was required in the vesicle interior for the ATP dependent signal. Valinomycin with TCS, or nigericin alone, dissipated the signal. The effect of valinomycin was to increase the absorption change if added prior to the addition of ATP or in the presence of ATP. This effect was not given by $TPMP^+$ and, as before, the action of valinomycin (Sachs et al., 1976; Rabon et al., 1978) was interpreted as due to increased access of K^+ to the exchange sites. The signal under conditions where ΔH_o was osmotically sensitive was also osmotically sensitive.

SCN^- had a more variable effect on this dye than on any other signal studied. At pH 7.4, depending on the preparation, inhibition with 30 mM SCN^- ranged from 13 to 78%. Addition of SCN^- before ATP addition was required for the inhibition. Added after ATP, no effect was observed. It was also noted that in some preparations the relative size of the signal did not correlate with the pH electrode measured transport capacity of the vesicles, i.e., ΔH_o. Signals of similar magnitude could be obtained with vesicles transporting 10 or 50 nmol μL^{-1}, as indicated by the pH electrode.

D. Probes of OH^-:Cl^- Exchange

There are few anion selective ionophores known. Tributyltin, however, has been shown to be an OH^-:Cl^- exchange ionophore in mitochondria (Dawson et al., 1972). In the gastric vesicle, this compound had a dual action. It was a potent inhibitor of the ATPase apparently binding irreversibly (Kasbekar et al., in preparation) (Table II), but, in addition to inhibiting the ATPase, it was also capable of dissipating the H^+ (or OH^-) gradient when added after exhaustion of the ATP. Its action was also more rapid in abolishing the ΔpH than expected of an ATPase inhibitor (Figure 7), the rate being comparable to that of nigericin. Tributyltin also rapidly reversed the signals obtained with DOCC or acridine orange, as well as with the pH electrode. As expected of an OH^-:Cl^- antiport, replacement of Cl^- with SO_4^{2-} prevented the action of tributyltin.

E. Summary

Figure 8 summarizes our current concepts of the modes of action of the ATPase, ionophores, and the probes used in this work.

Discussion

Various techniques have been applied to attempt to further describe the mechanism and quantitative aspects of H^+ uptake by gastric vesicles. In most cases, the approaches have been used previously in other vesicular systems, but the gastric vesicles have properties which result in modifications of the use of, for example, osmotic shrinkage or potential probe dyes.

Based on an H^+:K^+ exchange mechanism for H^+ uptake, and a slow efflux of trapped K^+ (Schackmann et al., 1977), then as the vesicular K^+ content increases, shrinking the intravesicular space by application of an osmotic gradient will result in a progressively higher K^+ content being available for exchange. This will lead to a progressive loss in osmotic sensitivity, as was found. The finding of complete osmotic sensitivity in nonpreincubated (2 min) vesicles may therefore not be interpreted as showing absence of binding or nonpermeant buffers, but rather as evidence of K^+ entry being necessary for H^+ uptake. The absence of osmotic sensitivity at KCl equilibration (ca. 72 h at 4 °C) supports an exchange mechanism, which would be osmotically insensitive, rather than net uptake of HCl. The sensitivity of the technique does not allow distinction between equal or unequal exchange stoichiometrics.

These osmotic data also allow predictions to be made about dye responses to changes in vesicular volume. For a dye that is trapped by partition between vesicle water space and the medium due to a pH gradient, osmotic sensitivity of dye uptake should be observed in nonequilibrated vesicles, where osmotic sensitivity of H^+ uptake persists. Partial osmotic sensitivity of the 9-aminoacridine signal was found indicating some binding (ca. 20%) of the dye. In equilibrated vesicles no osmotic sensitivity of this signal would be predicted and was not found.

As the K^+ content of the vesicles increases, although the number of protons taken up will increase, this is into a progressively smaller intravesicular volume. The pH gradient should increase in exact proportion to the change of volume; hence no change in signal would be found with a dye such as 9-aminoacridine in preincubated vesicles. This is not the case however, for a potential sensitive probe such as DOCC. Although some of the signal generated by this dye may be due to diffusion of the dye from bulk aqueous phase into intravesicular water, driven by the H^+ diffusion potential in the presence of TCS, a considerable fraction of the signal is due to dye moving from the hydrophobic phase to the membrane surface in response to a potential difference (Waggoner, 1976). This latter component will be volume independent, and hence a larger pH gradient which will generate a larger potential will give an increased response. Since this was found (Figure 6), this further substantiates the concept of an H^+:K^+ exchange in these vesicles, and also shows that the pH gradient can be elevated above control levels by the osmotic gradient. The leak rate is also increased, showing that the steady-state gradient capacity of the vesicles is in part leak-rate limited.

The use of any probes in transport studies requires a careful analysis of their mechanism in a given system, before mechanistic or quantitative conclusions can be drawn.

The pH electrode has a relatively slow response, due to the mechanical stirring system used and only measures changes in medium pH. The actual H^+ gradient formed is unknown. Often, an optical probe of changes of external pH is convenient, especially for rapid kinetics. In the case of gastric vesicles, bromcresol green was shown to monitor exclusively the external pH. Inhibition of transport activity by this dye correlated with inhibition of ATPase activity.

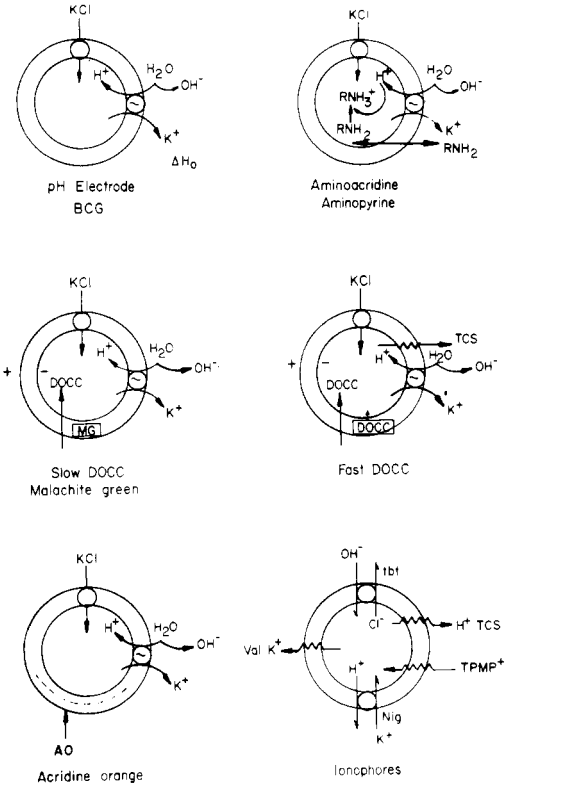

FIGURE 8: Models illustrating the mechanism of the different probes used to monitor pH changes and the different ionophores used to modify the signals. Abbreviations not used elsewhere are: MG, malachite green; BCG, bromcresol green; tbt, tributyltin; nig, nigericin; ⊖, pump; O, carrier; (〰) conductance; R, NH_2, 9-aminoacridine or aminopyrine.

For measurement of changes of internal pH, several probe types are available: weak bases which accumulate in the vesicle space, potential probe dyes used in the presence of protonophores and dyes which bind to the membrane as a function of the presence of a pH gradient.

The weak base, 9-aminoacridine, gives a rate of response significantly slower than the pH electrode. The rate of response is a function of the pump rate, the efflux of H^+, and the rate of penetration of the neutral form of the dye. The latter is excluded as rate limiting by the rapid dissipation of the signal by nigericin. Thus, given the greater reduction of the proton transport signal as compared with ATPase activity, this probe appears to act as an uncoupler of H^+ transport due to significant permeability of the protonated form. This is also shown by the reduction of the signal by valinomycin in the presence of ATP. Imidazole antagonizes the signal, due in part to the buffering effect of the accumulated imidazole, and at higher concentrations due again to the permeability of the protonated imidazole. Although we had previously used uptake of [^{14}C]imidazole as our index of intravesicular pH, these data showed that it was unsuitable (Rabon et al., 1978).

[^{14}C]Aminopyrine, on the other hand, accumulated in the vesicles with the addition of ATP and at the concentrations used showed slight uncoupling activity. As such, it was therefore more suitable than imidazole as a probe of internal pH.

Malachite green gave a slow acid shift with the addition of ATP, which was slowly dissipated by ionophores. Given the almost complete binding of the dye, and its low pK, it may be that this dye responds to a region of intramembranal acidity. However, it did not appear possible to calibrate this signal with pH pulses.

The probes of potential, DOCC or di-S-C_3-(5), which are positively charged, or oxonol dyes which are negatively

charged, gave no indication without added protonophore, of the development of a potential with a time course consistent with an electrogenic pump either in the H^+ or K^+ direction. In the absence of ionophore, a slow change in signal was observed. The inhibition by nigericin, at 1 $\mu g\ mL^{-1}$, at which concentration the ionophore acts as a neutral K^+:H^+ exchange carrier, indicates that this signal is due to a diffusion potential, rather than an electrogenic pump. Since a ΔpH artificially imposed also gave this slow response, and the H^+ conductance of the vesicles exceeds the K^+ conductance (Sachs et al., 1976), it is likely that this response is due to a H^+ diffusion potential, with development of interior negativity, calculated equal to -85 mV. A change in pump stoichiometry with an increase in internal $[H^+]$ could also account for this potential.

The response is increased, and accelerated by the protonophore TCS, and this response has the time course of the ΔpH sensed by the pH electrode. It is therefore reasonable to assume that the potential is due to the H^+ conductance induced by TCS, and the resulting H^+ diffusion potential. It is possible, however, that TCS-modified membrane structure and uncouplers have been shown to modify membrane structure (Zimmer, 1977) and TCS in particular partially inhibits ATPase activity (Table II). The use of this type of dye, in association with TCS, allowed calibration of the pH gradient by a means less dependent on vesicular volume, and independent of an uncoupling effect of the dye since it cannot act as a proton carrier. However, the dye on its own had significant effects on ATPase activity and H^+ transport and, hence, as in other systems (Waggoner, 1976) affects the membrane to which it binds.

Acridine orange has been shown to bind to gastric vesicles with development of an H^+ gradient (Lee et al., 1976). With a change of external pH, at a fixed pH, the increase of signal shows that deprotonation of a membrane group increases binding of this dye. Since both ATP and a ΔpH induce these binding sites, it suggests that these binding sites for acridine orange are in some way related to the transport activity of the membrane. In a given vesicle preparation, the size of the signal is quantitatively related to the magnitude of the H^+ gradient. It should be pointed out, however, that if the DOCC + TCS signal is compared with the acridine orange signal, different results may be obtained since the former signal is more sensitive to the presence of, for example, a Cl^- conductance. Also, since the latter signal depends on the presence of binding sites, such as phosphatidylserine, variation in content of this phospholipid from species to species, or preparation to preparation will alter the ratio of the acridine orange signal as compared with other probes.

An anion-exchange ionophore, such as tributyltin, has two apparently independent actions on the gastric vesicles, inhibition of ATPase activity, and a true ionophore effect that is Cl^- dependent. Hence, this compound can be used in this system to detect an active Cl^-:OH^- antiport which may coexist with the H^+:K^+ antiport.

Previous conclusions about the neutrality of the pump contained reservations since, if the addition of ATP activated a K^+ conductance, the vesicle potential would be given by, neglecting all other conductances:

$$PD = \frac{E_H g_H}{g_H + g_K}$$

where E_H is the EMF of the H^+ limb of the ATPase, and g_H and g_K are the specific conductances for H^+ and K^+, respectively. Thus, if $g_K > g_H$, a low potential would be observed as the g_K increased. Thus, perhaps only a transient would be observed. The absence of such a transient with either type of charged cyanine dye argues against this possibility. The removal of Cl^-, and substitution by SO_4^{2-}, an impermeant anion, also does not elicit a cyanine dye measurable potential. The experiments shown here are therefore not consistent with an electrogenic mechanism.

The electrochemical gradient obtained from an H^+ pump can be defined as (Mitchell, 1966)

$$\Delta\bar{\mu}_{H^+} = \Delta\Psi - 2.3\frac{RT}{F}\Delta pH$$

The intact mammalian stomach generates a pH of 0.8 and a potential difference (correcting for diffusion potentials) of about 60 mV lumen negative, due to Na^+K^+ ATPase dependent Cl^- transport (Rabon et al., 1978). Thus, the $\Delta\bar{\mu}_{H^+}$ anticipated from the isolated gastric H^+ pump is 333 mV, or a ΔpH of 5.74 units.

At pH 6.1, since no H^+ is released from the ATPase reaction, it is possible to compare directly the change of H^+ in the medium and in the vesicles. The loss of H^+ from the medium was calibrated with the pH electrode or the absorbance change of bromcresol green. The data indicate a possible pH change of 5 units in the intravesicular space, on the assumption that all vesicles transport H^+ equally and that there is no significant buffering capacity. However, when probes of internal pH are used, such as weak bases (9-aminoacridine and aminopyrine) or probes of H^+ gradient dependent sites (acridine orange), the change in internal pH is in the range of 2.2 to 3.7 units giving a final internal pH of about 2 to 4. This discrepancy between ΔH_i and ΔH_o may be explained by intravesicular buffer. If the gastric ATPase were capable of generating a ΔpH of 5 units, intravesicular buffering would result in an increase in ΔH_o but a final $[H^+_i]$ still equivalent to the gradient capacity of the pump. Thus, it must be concluded that the maximal gradient capacity of the vesicles is 3.7 units, almost two orders of magnitude less than expected. Another explanation is the presence of membranal bindings sites for H^+. Since the majority (ca. 90%) of membrane protein (Saccomani et al., 1977) has a subunit M_r of 105 000 containing 25% carbohydrate (Saccomani et al., in preparation), this would correspond to 10 mol of H^+ bound mol^{-1} protein. Alternatively, a leak pathway may be responsible but this would not account for the discrepancy between ΔH_o and ΔH_i.

At pH 7.4, although ΔH^+_o cannot be calibrated, the ΔpH found with the probes ranged from 3 to 4.5 (with the exception of DOCC + TCS), giving the same internal pH as at 6.1. This constancy of $[H^+_i]$ with varying $[H^+_o]$ could be interpreted as due to a buffering component at that pH or a pH dependent leak pathway fixing the internal pH. The application of an osmotic gradient in equilibrated vesicles increased the ΔpH showing that the gradient capacity of the vesicles was not limited by the leak path for H^+. Since the vesicles were preincubated with 150 mM KCl in these experiments, the initial uptake was not K^+ limited.

Of all the probe methodologies, only DOCC in the presence of TCS generated a large enough signal to approach the ΔH_o and even the predicted gastric H^+ pump gradient at pH 7.4. Although a probe of potential, it was converted to a probe of pH by the addition of a protonophore, TCS. The protonophore may provide a conductance across the whole vesicle membrane and perhaps also between a compartment of H^+ and the vesicle exterior. In this model, however, since the vesicle can be represented as two EMF's in parallel, no additional potential will be found, other than that expected from the pH gradient probed by the weak bases. This probe may also be pH sensitive at pH <2.

Considering the various probes, it seems that, at the least,

the expected ΔpH of 5.3 at pH 6.1 is not achieved and a discrepancy of about two orders of magnitude is found, when measuring intravesicular pH (Table I). In general the same is true at pH 7.4, although the ΔpH increases by one unit. This may indicate that the vesicles may not contain the complete system.

Another line of evidence which leads to caution in accepting the $H^+ + K^+$ ATPase as a complete model of the parietal cell proton pump is the lower effectiveness of SCN^- in blocking H^+ transport in these vesicles. Thus, although 30 mM SCN^- has a significant inhibitory action when added before, but not after ATP, the inhibition is not complete in contrast to the action of this compound in the intact tissue (Rehm & Sanders, 1977) or gastric gland (Berglindh, 1976). Moreover, imidazole can reverse the action of SCN^- on the intact tissue (Rehm & Sanders, 1977) which does not occur in the vesicles.

In conclusion, probes of pH and potential show that, in contrast to data obtained for intact frog mucosa (Rehm, 1972), no evidence can be obtained for an electrogenic H^+ pump in these vesicles and that the gradient achieved by the ATPase is less than expected based on the gradient capacity of the intact organ. This H^+ pump mechanism therefore is in contrast to the better known electrogenic H^+ pumps of mitochondria and chloroplasts (Mitchell, 1968).

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