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A STUDY OF H^+ TRANSPORT IN GASTRIC MICROSOMAL VESICLES USING FLUORESCENT PROBES *

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Summary

Fluorescent amines, 9-aminoacridine, acridine orange and quinacrine, were used as probes for a pH gradient (ΔpH) across gastric microsomal vesicles. Analysis of probe uptake data indicates that 9-aminoacridine distributes across the membrane as a weak base in accordance with the ΔpH. On the other hand, acridine orange and quinacrine show characteristics of binding to membrane sites in addition to accumulation in response to ΔpH. A discussion of the advantages and limitations of the probes is presented.

Application of these probes to pig gastric microsomal vesicles indicates that that K^+-stimulated ATPase is responsible for the transport of H^+ into the vesicles and thus develops a ΔpH across the membrane. The ΔpH generated by the K^+-ATPase has a definite requirement for internal K^+. The proton gradient can be discharged slowly after ATP depletion or rapidly either by detergent disruption of the vesicles or by increasing their leakiness using both H^+ and K^+ ionophores. On the other hand, the sole use of the K^+ ionophore, valinomycin, stimulates the ATP-induced formation of ΔpH by increasing the availability of K^+ to internal sites. This stimulation by valinomycin requires the presence of permeable anions like Cl^-. Analysis of the Cl^- requirement indicates that in the presence of valinomycin the net effect is the accumulation of HCl inside the gastric vesicles. With an external pH of 7.0, the ATP-generated ΔpH was calculated to be from 4 to 4.5 pH units. The results are consistent with the hypothesis that the K^+-stimulated ATPase drives a K^+/H^+ exchange across the gastric vesicles. Since other lines of evidence suggest that these gastric microsomes are derived from the tubulovesicular system of the oxyntic cell, the participation of the ATP-driven transport processes in gastric HCl secretion is of interest.

* These studies were previously reported in preliminary form, (1977) Fed. Proc. 36, 274.
Abbreviations: Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); CCCP, carbonyl cyanide, *m*-chlorophenyl hydrazone.

Introduction

A preparation of microsomes isolated from fundic gastric mucosa of several species has been shown to possess K^+-stimulated, Mg^{2+}-dependent, ATPase activity [1]. Immunocytochemical and other studies have demonstrated that these membranes are largely derived from oxyntic cells [2,3]. Furthermore, the gastric microsomal membranes can be isolated largely in a vesicular form and can transport (accumulate) H^+ in the presence of K^+, Mg^{2+} and ATP [4,2]. The possible relationships of these processes to the mechanism of gastric HCl transport is of great interest.

In a previous report, we demonstrated that metachromatic dyes, like acridine orange and neutral red, are useful probes for monitoring the development of a pH gradient by gastric microsomal vesicles [5]. In this report, we further characterize the mechanism of the spectral changes observed with acridine orange and extend the study to two other dyes, 9-aminoacridine and quinacrine, which have been shown to measure pH gradient in chloroplasts [6], inside-out vesicles of *Escherichia coli* [7], submitochondrial particles [8] and liposomes [9].

Materials and Methods

Vesicle preparation

Stomachs of freshly slaughtered pigs were obtained from an abattoir and placed into ice-cold 0.15 M NaCl containing 5 mM Tris buffer, pH 7.4, for transport to the laboratory (60—90 min). All tissue manipulations and fractionation procedures were carried out at 0—4°C. The epithelium from the fundic region of the stomach was scraped with a stainless steel spatula and placed into homogenizing medium consisting of 113 mM mannitol/37 mM sucrose/0.2 mM EDTA (ethylenediaminetetraacetic acid)/5 mM Pipes buffer (piperazine *N,N'*-bis(2-ethanesulfonic acid), pH 6.68. The scrapings of gastric epithelium were triturated in a Potter-Elvejhem homogenizer by 1—8 passes of a rotating Teflon pestle (60 cycles per min). The homogenate was diluted to approximately 10 g of tissue per 100 ml of homogenizing medium and centrifuged at 14 600 × *g* for 15 min. The pellets were discarded and the supernatant was recentrifuged at 143 000 × *g* for 55 min to produce the crude microsomal pellet. The microsomal pellets were resuspended in homogenizing medium to about 5 mg of protein per ml, and subsequently layered over a discontinuous sucrose density gradient consisting of 4 ml of 20%, 10 ml of 27% and 14 ml of 33% sucrose. After centrifuging over night at 131 000 × *g*, two distinct membrane bands and a pellet were obtained. The lightest membrane band was collected and either immediately frozen for subsequent use or first washed by centrifugation in homogenizing medium before freezing. Gastric microsomes prepared this way can be stored frozen for at least two months without sign of decay.

Dye uptake by microsomes

We want to distinguish two cases of dye uptake from the bulk solution, a binding to the membrane vs. accumulation within the vesicular space. For the case of binding, if it is assumed that each site binds one dye molecule and there are n number of membrane sites (μmol/g protein), mass action law will

give:

$$K = \frac{[\text{dye}]_{\text{free}}(n - [\text{dye}]_{\text{bound}})}{[\text{dye}]_{\text{bound}}} = \text{dissociation of dye} \cdot \text{membrane complex}$$

$$\text{or } \frac{[\text{dye}]_{\text{bound}}}{[\text{dye}]_{\text{free}}} = \frac{n}{K} - \frac{[\text{dye}]_{\text{bound}}}{K} \quad (1)$$

where $[\text{dye}]_{\text{free}}$ is the dye concentration in μM, $[\text{dye}]_{\text{bound}}$ is the concentration of dye associated with membrane sites in μmol/g protein. Thus, the bound/free ratio of the dye will be a linear function of the concentration of dye bound.

If one assumes dye molecules are merely accumulated inside membrane vesicles as weak base according to the ΔpH existing across the membrane, a different relationship can be derived as follows [6] since:

$$[\text{dye}]_{\text{in}} = [R]_{\text{in}} + [RH^+]_{\text{in}} \quad (2)$$

$$[\text{dye}]_{\text{out}} = [R]_{\text{out}} + [RH^+]_{\text{out}} \quad (3)$$

$$\text{and } K_a = \frac{[R]_{\text{in}}[H^+]_{\text{in}}}{[RH^+]_{\text{in}}} = \frac{[R]_{\text{out}}[H^+]_{\text{out}}}{[RH^+]_{\text{out}}} \quad (4)$$

where the subcripts, "in" and "out", refer to intra- and extra-vesicular quantities; [dye] is the total dye concentration; [R] and $[RH^+]$ are the concentration of the uncharged and charged form of the dye, respectively; and K_a is the dissociation constant of the dye. Taking the ratio of Eqns. 2 and 3 and using Eqn. 4 to eliminate $[RH^+]$, we have

$$\frac{[\text{dye}]_{\text{in}}}{[\text{dye}]_{\text{out}}} = \frac{[R]_{\text{in}} + \frac{1}{K_a}[R]_{\text{in}}[H^+]_{\text{in}}}{[R]_{\text{out}} + \frac{1}{K_a}[R]_{\text{out}}[H^+]_{\text{out}}} \quad (5)$$

Assuming that only the uncharged form of the dye is always in equilibrium,

$$[R]_{\text{in}} = [R]_{\text{out}} = [R] \quad (6)$$

Thus, Eqn. 5 can be reduced to

$$\frac{[\text{dye}]_{\text{in}}}{[\text{dye}]_{\text{out}}} = \frac{K_a + [H^+]_{\text{in}}}{K_a + [H^+]_{\text{out}}} \quad (7)$$

for $K_a << [H^+]_{\text{in}}$ and $[H^+]_{\text{out}}$, Eqn. 7 reduces to

$$\frac{[\text{dye}]_{\text{in}}}{[\text{dye}]_{\text{out}}} \approx \frac{[H^+]_{\text{in}}}{[H^+]_{\text{out}}} \quad (8)$$

In our assay systems, $[\text{dye}]_{\text{out}}$ is the external dye concentration which would be equivalent to $[\text{dye}]_{\text{free}}$; $[\text{dye}]_{\text{in}}$ is the dye concentration within the vesicles ($[\text{dye}]_{\text{trapped}}$) and converted to $[\text{dye}]_{\text{bound}}$ of Eqn. 1 by multiplying it by the vesicular volume expressed in l/g protein. Therefore, Eqn. 2 can be rewritten in the same units as Eqn. 1 as follows:

$$\frac{[\text{dye}]_{\text{bound}}}{[\text{dye}]_{\text{free}}} = \text{a constant independent of } [\text{dye}]_{\text{bound}}$$

Thus, by plotting $[dye]_{bound}/[dye]_{free}$ vs. $[dye]_{bound}$ (i.e., Scatchard plot), we can distinguish between a binding and an accumulation mechanism.

We varied $[dye]_{bound}$ in two ways: first, by keeping protein constant and varying the dye concentration; second, by keeping dye concentration constant and varying the protein. Similar results were obtained both ways. To measure $[dye]_{bound}$, two methods were employed:

(a) Centrifugation. Membranes vesicles (2—20 μg protein/ml) were incubated at room temperature (approx. 22°C) in appropriate medium (see individual experiments) with dye and 1 mM ATP. After 15 min, centrifugation was initiated and continued for 20 min at 220 000 × *g* at 22°C. Supernatant was taken out and the concentration of acridine orange was determined by the fluorescence intensity at 530 nm; this gives $[dye]_{free}$. The difference between total [dye] and $[dye]_{free}$ divided by protein concentration gives $[dye]_{bound}$.

(b) Spectrophotometric determination. This method is based on the assumption that the change in absorbance or fluorescence intensity is proportional to the amount of dye taken up by the microsomes. This assumption was found to be valid by direct comparison between optical changes and flow dialysis results under identical conditions and also from the fact that it gives similar results to those obtained by the centrifugation method.

Flow dialysis

To measure the uptake of dye by microsomes direclty and continuously, the flow dialysis method of Colowick and Womack was used [10]. The apparatus consisted of two chambers separated by a dialysis membrane (Union Carbide Company). The disc-shaped upper and lower chambers (19 mm diameter × 10 mm) both had a capacity of 2.8 ml, the contents of which were mixed continuously by two small magnetic stirring bars. The lower chamber was completely filled with buffer solution which was pumped through at a constant rate. The concentration of dye in the lower chamber is given by the equation:

$$C_{lower}(t) = C_{upper} \cdot \frac{D}{f}(1 - e^{-(ft/V)}) + C_{lower}(t = 0)\, e^{-(ft/V)}$$

where C_{upper} is the concentration of free dye in the upper chamber; C_{lower} (t) is the concentration of dye in lower chamber at time t; D is a constant depending on the nature of the diffusing molecule as well as the proporties of the apparatus; f is the flow rate through the lower chamber (ml/min); and V is the volume of the lower chamber (ml). If we start with C_{lower} $(t = 0) = 0$, then after a period of time t such that ft/V is large (>4), then $e^{-(ft/V)}$ approaches zero and the dye concentration in the upper chamber is directly proportional to the dye concentration in the lower chamber.

Fluorescence and absorbance measurement

All fluorescence measurements were performed with the Perkin-Elmer spectrofluorometer MPF-44A with the corrected spectra attachment. Unless specified otherwise, fluorescence measurements were performed in a medium containing 2 mM $MgCl_2$, 150 mM KCl, 10 mM Pipes (pH 7.0) and 0.1 mM EDTA (referred to as K^+-Mg^{2+}-medium, pH 7.0) at room temperature (21—23°C). Wavelengths used were 493 → 530 nm (excitation → emission wave length) for

acridine orange, 422 → 455 nm for 9-aminoacridine and 425 → 505 nm for quinacrine.

Absorbance measurements were carried out with an Aminco-Bowman dual wavelength spectrophotometer. For measurements on acridine orange, A was measured at 493 nm using 550 nm as the reference.

Fluorescence depolarization measurements

Measurements of polarization of fluorescence were carried out with the polarization accessory for the MPF-44A by obtaining $I_{\parallel}$ and $I_{\perp}$ and calculating the polarization, P, by the equation

$$P = \frac{(I_{\parallel} - I_{\perp})}{(I_{\parallel} + I_{\perp})}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities detected through a polarizer oriented parallel and perpendicular, respectively, to the polarization of the beam. To correct for scattering contribution [11] and the unequal transmission of differently polarized light [12] a modified equation was used:

$$P = \frac{(I_{\parallel} - I_{\parallel}^{s}) - C(I_{\perp} - I_{\perp}^{s})}{(I_{\parallel} - I_{\parallel}^{s}) + C(I_{\perp} - I_{\perp}^{s})}$$

where $I_{\parallel}^{s}$ and $I_{\perp}^{s}$ were measured with dye-free suspension and $C = I'_{\parallel}/I'_{\perp}$, the primes indicating excitation polarized in a perpendicular direction. This latter factor was used to correct for the unequal transmission of differently polarized light [12].

Artificially imposed pH gradient

pH Gradients across the vesicular membranes were achieved according to the method by Jagendorf and Uribe [13]. Microsomes were incubated in 150 mM KCl/10 mM succinate at pH 4.0 for 1—4 min to bring the internal pH down to 4.0. The extra-vesicular pH was then rapidly elevated to specified alkaline values by adding different amounts of Tris-base. Thus, a known pH gradient can be imposed across the microsomal membranes.

Uptake of ^{36}Cl by microsomes

Gastric microsomes were harvested by centrifugation, then resuspended in homogenizing medium to a concentration of 4—6 mg protein/ml. The membrane suspension (0.5 ml) was mixed with 0.5 ml radioactive reaction medium, which consisted of 190 mM KCl/4 mM $MgCl_2$/20 mM Pipes (pH 7.0)/0.2 mM EDTA/20 μCi ^{36}Cl. 50-μl aliquots were taken out at various times and added to 1 ml ice-cold stop solution consisting of 120 mM K_2SO_4/2 mM $MgSO_4$/10 mM Pipes (pH 7.0)/0.1 mM EDTA. The suspension was filtered through Gelman GN-6 filters (0.45 μm pore size) under suction. The filters were washed immediately with 8 ml ice-cold stop solution, dried under suction for 30 s, then put into 10 ml of Aquasol (New England Nuclear) for scintillation counting.

Miscellaneous

ATPase was measured by the liberation of P_i according to the method of Sanui [14]. Assays were run on all preparations to insure their purity and

high activity of K^+-stimulated ATPase as described in our earlier work [1,3]. Protein concentration was determined according to the method of Lowry and co-workers [15]. Acridine orange was obtained from Eastman. Quinacrine dihydrochloride and the proton ionophore, CCCP (carbonyl cyanide, *m*-chlorophenyl hydrazone), were obtained from Aldrich Biochemicals. ^{36}Cl was obtained from ICN. Valinomycin was from Cal Biochem. All ionophores were dissolved in methanol. Nigericin was a gift from Dr. W. Scott of Hoffman-LaRoche, Inc.

Results

(a) Characterization of dye-microsomal interactions

The addition of ATP to gastric microsomal vesicles produced a characteristic quenching of fluorescence for the three dyes tested, acridine orange, quinacrine and 9-aminoacridine, as shown in Fig. 1. The fluorescence quench also required that Mg^{2+} and K^+ were present and, as shown in Fig. 1, the ATP-induced effect was reversed by adding both CCCP and valinomycin (addition of the K^+/H^+-exchange ionophore, nigericin, was also effective in rapidly dissipating the ATP effect). The qualitative similarity of response for the three dyes suggested that that might be measuring the same transport process in the gastric vesicles; thus, it was of interest to further investigate and compare the mechanisms of this ATP-induced fluorescence quenching for each dye.

Acridine orange. Fig. 2 shows the results of a flow-dialysis experiment. Since

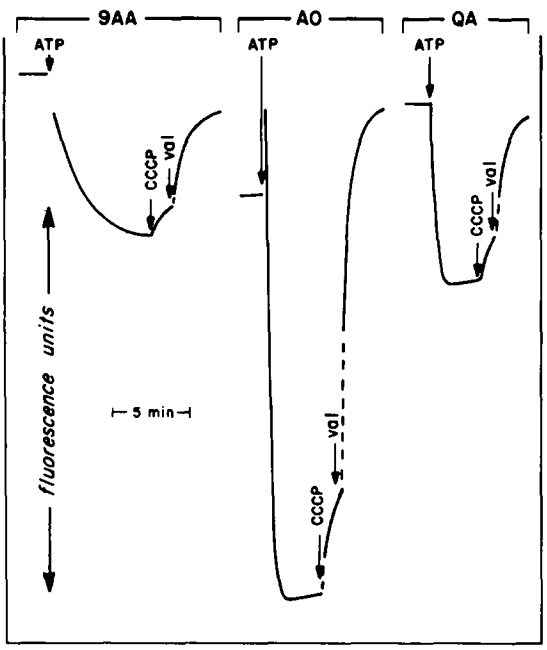

Fig. 1. Gastric microsomes (0.25 mg protein/ml) were incubated in K^+-Mg^{2+}-medium, pH 7.0, for 4 days at 0—5°C. 10 μM of various dyes shown were added and the reaction was started by the addition of 1 mM ATP. Ionophores, CCCP (1 μM) and valinomycin (Val) (1 μM), were added to dissipate the gradient. 9AA, 9-aminoacridine; AO, acridine orange; QA, quinacrine.

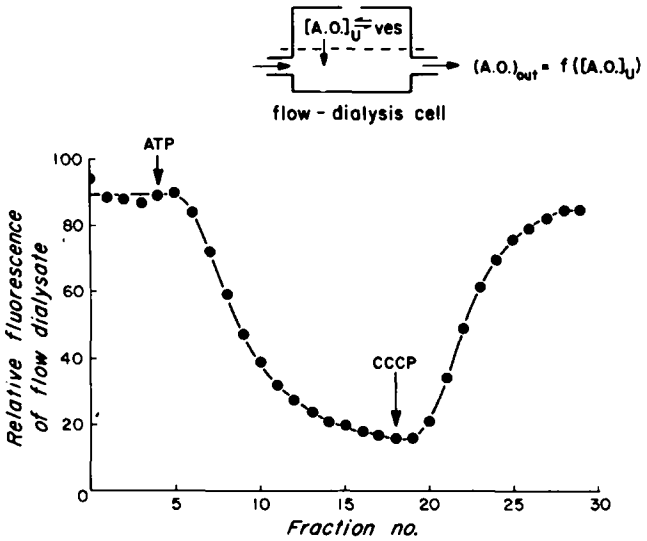

Fig. 2. Flow dialysis measurement of acridine orange (A.O.) uptake. The upper chamber contained 2 ml of K^+-Mg^{2+}-medium, 25 μM valinomycin, 25 μM acridine orange and gastric microsomal vesicles at a concentration of 0.4 mg protein/ml. K^+-Mg^{2+}-medium was pumped through the lower chamber at a rate of 3.7 ml/min and was collected as 1-min fractions. 1 mM ATP was added to the upper chamber as indicated by the arrow. CCCP was added where indicated to give a final concentration of 25 μM.

the amount of dye diffusing into the flow cell is a function of its concentration in the upper chamber (see Materials and Methods), it is clear that ATP produces a dramatic dye uptake by these gastric microsomes. This removal from bulk solution can either be the consequence of binding to membrane sites or accumulation within intravesicular space. To distinguish between these two possibilities, kinetic analyses were performed on the dye uptake data as shown in Fig. 3. In the Scatchard plot, two regions can be distinguished, at low and at high dye to protein ratios. In the region where dye to protein ratio is low, the form of the plot indicates that either the apparent number of binding sites or

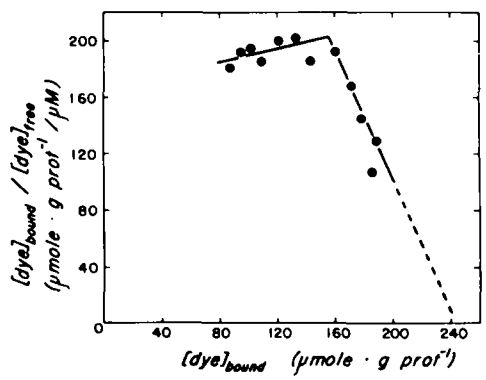

Fig. 3. Scatchard plot of acridine orange binding data. Acridine orange binding was measured by the centrifugation method with 2 μM acridine orange, 10 μM valinomycin and 1 mM ATP. Amount of gastric microsomes was varied from 1.5 to 17.5 μg protein/ml. Extrapolation of the linear negative slope provides an estimate of 244 μmol dye bound/g protein with a dissociation constant of 0.43 μM.

the affinity of the binding for the ligand increases with the increase in the amount of dye bound to the membrane. Similar binding kinetics have been observed by others [16] for acridine orange and other dyes, and these have been interpreted in terms of some perturbation of the membrane giving this apparent cooperative binding effect. On the other hand, the relationships at high dye to protein ratio are characteristic of ligand binding to a fixed number of sites and with fixed affinity. Extrapolation of the data for the high ratio region gives a value for the apparent number of sites (n of Eqn. 1) of 244 μmol acridine orange/g protein, with a dissociation constant of 0.43 μM.

We and others have shown that the fluorescence of acridine orange is quenched by binding to model polyanions such as polystyrene sulfonate, agar and chondrotin sulfate [5,16]. These observations are consistent with the interpretation that the observed ATP-induced fluorescence quenching with gastric vesicles is due to a binding with negative sites. Another consequence of binding to membrane sites is the immobilization of the dye molecules which can be measured by the degree of polarization of the fluorescence. Table I shows that the major increase in the degree of polarization (P) of acridine orange occurs after addition of both ATP and valinomycin. Thus, a strong immobilization of the dye molecules, consistent with binding, is indicated under the conditions of maximal dye uptake (see later discussion).

Quinacrine. Table I shows that, similar to acridine orange, the degree of polarization for quinacrine indicates a strong immobilization of the dye induced by addition of ATP to the vesicle preparation. This, together with the fact that polystyrene sulfonate is also able to bind quinacrine, causing the characteristic spectral shift and fluorescence quenching [17], leads us to conclude that the ATP-induced fluorescence quenching of quinacrine is also due to an enhanced binding to negative sites on the gastric membranes. Since both quinacrine and acridine orange appear to bind to membrane sites, it was of interest to see if there were any interactions for their access to the same site. The fluorescence spectra of quinacrine (425 → 505) and acridine orange (493 →

TABLE I

IMMOBILIZATION OF ACRIDINE ORANGE AND QUINACRINE DURING STATES OF ENERGIZATION OF GASTRIC MICROSOMES AS MEASURED BY POLARIZATION OF FLUORESENCE

Sequential additions *	P values **,***	
	Acridine orange (25 μM)	Quinacrine (25 μM)
K^+—Mg^{2+}-medium	0.03	0.02
Gastric microsomes (125 μg protein/ml)	0.07	0.05
0.5 mM ATP	0.08	0.08
2.5 μM Valinomycin	0.15	0.13
2.5 μM Nigericin	0.06	0.06

* Indicates the order of sequential additions to the same cuvette.

** P values are the degree of polarization as defined in the text; and increase in P indicates an immobilization of the fluorophore and is especially prominent after adding valinomycin.

*** Due to its long fluorescence lifetime (15.5 ns) the P value of 9-aminoacridine is not sensitive to such immobilization studies.

525) are sufficiently separate to permit measurement of acridine orange fluorescence in the presence of quinacrine without much interference from the latter. Addition of quinacrine does indeed diminish the ATP-induced fluorescence quenching of acridine orange, thus there does seem to be some interaction for the same sites.

9-Aminoacridine. Fig. 4 compares the results for ATP-induced 9-aminoacridine uptake by gastric microsomes using the spectrofluorometric method (Fig. 4A) and the flow dialysis method (Fig. 4B). Note that the percentage of dye uptake measured by flow dialysis is very similar to the percentage of fluorescence quenched in the cuvette measured in the fluorometer. This validates using the latter as a direct measure of dye uptake which we have employed in the Scatchard analysis below. If, instead of using ATP-generated ion gradients, we use artificially imposed pH gradients across the vesicular membrane, a similar uptake of the dye is effected, as demonstrated in Fig. 5A and 5B. Once again, the amount of 9-aminoacridine uptake as measured by flow dialysis is

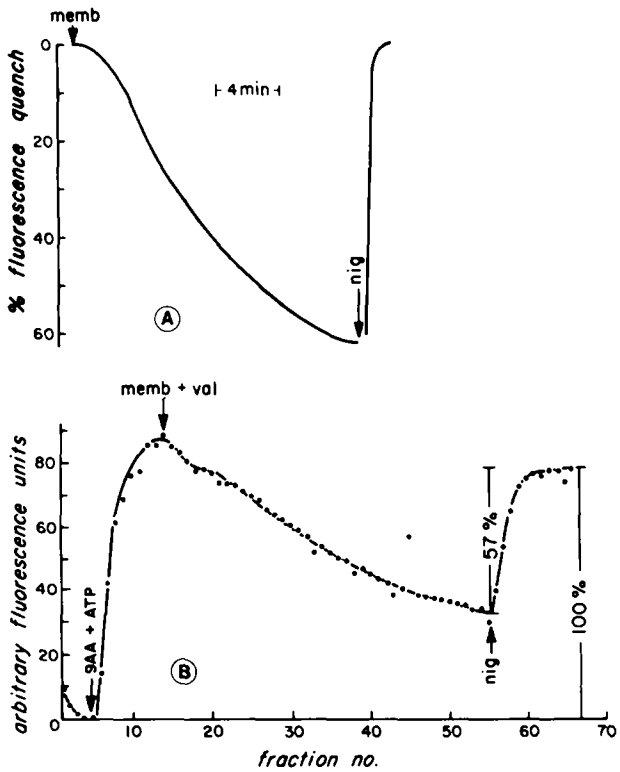

Fig. 4. Comparison between spectrofluorometric and flow dialysis measurement of 9-aminoacridine (9AA) uptake. (A). For the spectrofluorometric method, the reaction was initiated by adding gastric microsomal membranes (0.16 mg protein) to 2 ml of K^+-Mg^{2+}-medium containing 10 μM 9-aminoacridine, 1 mM ATP and 2 μM valinomycin (val). Dye uptake was reversed by adding 2 μM nigericin (nig) at the arrow indicated. (B). For the flow dialysis measurement, exactly the same conditions as in (A) were used in the upper chamber. In the lower chamber, K^+-Mg^{2+}-medium was pumped through at 5.4 ml/min and collected at 0.5 min intervals and the fluorescence monitored for each point. The nigericin dissipated fluorescence was 57% of the total which agreed well with 62% dye uptake measured by the spectrofluorometric method.

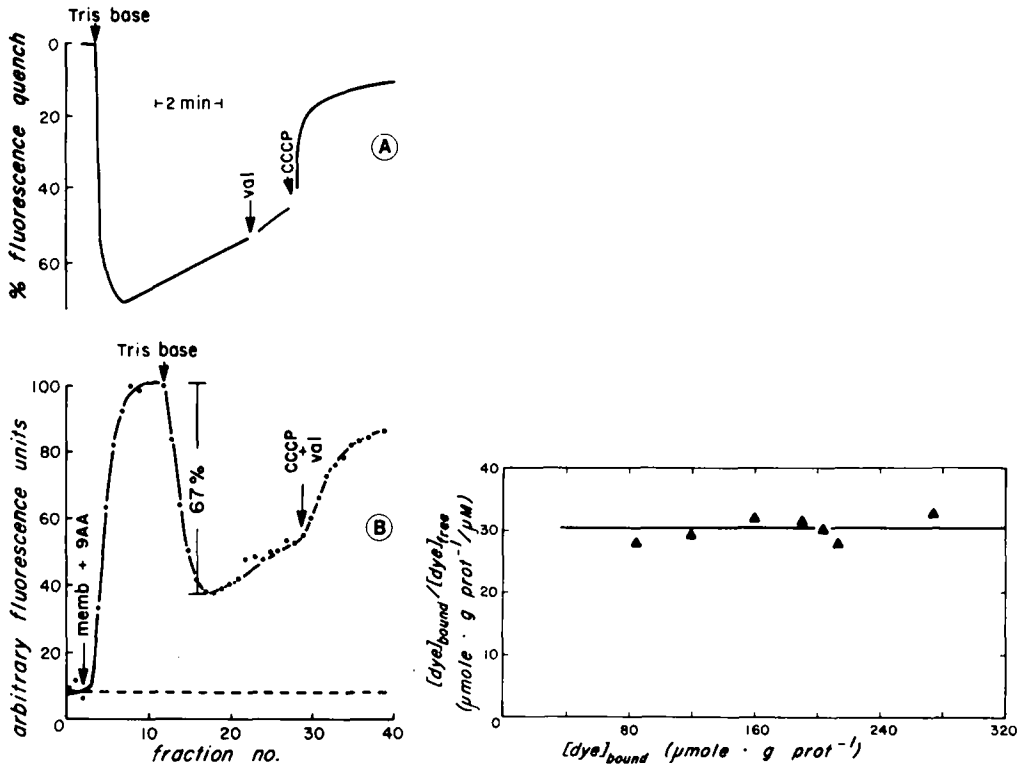

Fig. 5. ΔpH-induced 9-aminoacridine uptake as measured by spectrofluorometric and flow dialysis methods. (A). For the spectrofluorometric method, gastric microsomes (0.5 mg) were incubated in 2 ml medium containing 150 mM KCl, 10 μM 9-aminoacridine (9AA) and 10 mM succinate at pH 4.0 for 5 min. 25 mM Tris-base was added to bring the external pH to 8. Assuming that the vesicular interior equilibrated to about pH 4 in the presence of the low pH-succinate system, the addition of base bringing the external medium to pH 8 would thus impose a H^+ gradient of 4 pH units across the gastric vesicles. Valinomycin (val) and CCCP (2 μM each) were used to dissipate the H^+ gradient. (B). For the flow dialysis measurement, the conditions in the upper chamber were exactly as described in (A). In the lower chamber, 150 mM KCl solution flowed through at a rate of 7.4 ml/min and collected every 0.5 min. The change induced by the acid-base shift was 67% of the 9-aminoacridine fluorescence which agreed well with the 68% dye uptake measured by the spectrofluorometric method.

Fig. 6. Scatchard plot of 9-aminoacridine uptake induced by ATP. Spectrofluorometric method was used to measure dye uptake. For all points, 9-aminoacridine concentration was 10 μM in K^+-Mg^{2+}-medium with 0.5 mM ATP and 0.5 μM valinomycin. Amount of gastric microsomes was varied from 5.6 to 80 μg protein/ml. In other experiments, membrane protein was held constant and the dye concentration varied with results similar to that shown here.

the same as the amount of fluorescence quenched in the cuvette.

A Scatchard plot of the ATP-induced uptake of 9-aminoacridine is shown in Fig. 6. In contrast to that observed with acridine orange (cf. Fig. 3), the ratio of $[dye]_{bound}/[dye]_{free}$ is independent of $[dye]_{bound}$. This is consistent with Eqn. 8, indicating that 9-aminoacridine is accumulated within the intravesicular space. Also consistent with this interpretation, we have observed that polyanions, although very potent in quenching the fluorescence signal of acridine orange and quinacrine, are without effect on either the fluorescence or absorption spectrum of 9-aminoacridine.

From the above observations, we conclude that, although all three probes show qualitatively similar response to ATP-induced pH gradient, the specific mechanism of fluorescence quenching is different. For acridine orange of quinacrine, the binding to membrane sites represents the primary effect. On the other hand, 9-aminoacridine does not appear to show characteristics of binding, but rather it is a pH gradient induced accumulation of the dye within the intravesicular space that quenches the fluorescence.

(b) Application to physiological study on gastric microsomes

In the previous section, we established that the quenching of fluorescence for acridine orange and quinacrine in the presence of a pH gradient is due to increased binding to membrane sites. The exact nature of the binding processes is not certain, but owing to their high sensitivity (in the sense of dye bound per g of protein), they are of great use as qualitative probes for the pH gradient. On the other hand, 9-aminoacridine behaves quite differently; its fluorescence quenching is more consistent with an accumulation within intravesicular space. It was of interest to see if the distribution of 9-aminoacridine conformed to theoretical predictions of a pH gradient probe and thus to permit quantitation of the gradient. Fig. 7 shows the calibration curve for 9-aminoacridine. Different pH gradients (ΔpH) were measured by the fluorescence quenching method. Since the percentage of fluorescence quenched (Q) is a direct measure of dye

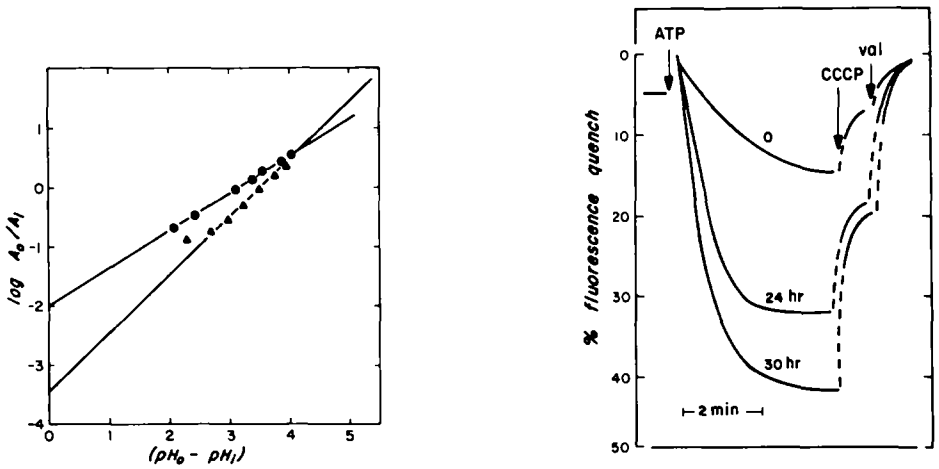

Fig. 7. Calibration curve for 9-aminoacridine and acridine orange uptake as a function of pH gradient. Data were plotted in accordance with Eqn. 9 using the log A_o/A_i as the ordinate in the pH gradient on the abscissa, where A_o is the amount of dye in the bulk external medium and A_i is the amount of dye uptake. Different pH gradients were imposed across the gastric microsomes as described in the methods section. Dye uptake was measured by the percentage of fluorescence quenching. The dye concentration was 10 μM for both 9-aminoacridine (▲) and acridine orange (●); amount of gastric microsomes used was 250 μg protein/ml for 9-aminoacridine and 50 μg protein/ml for acridine orange.

Fig. 8. Effect of preincubation in K^+-medium. Gastric microsomes were preincubated in K^+-Mg^{2+}-medium (pH 7.0) at 0—5°C for various periods of time as indicated. Small aliquots of preincubated microsomes were added to K^+-Mg^{2+}-medium at room temperature (approx. 22°C) to achieve a final protein concentration of 33 μg/ml, and the reaction was started by adding 0.25 mM ATP where indicated. Concentration of acridine orange was 1 μM. Valinomycin (val, 0.5 μM) and CCCP (0.3 μM) were used to dissipate the gradient.

uptake (cf. Figs. 4 A and B and 5 A and B), we can take the expression $Q/(100 - Q)$ as equivalent to A_i/A_o, where A_i represents the total amount of dye associated with the microsomes and A_o is the total dye in the free extravesicular solution. By taking the log on both sides of Eqn. 8 and using $A_i/V_i = [\text{dye}]_{in}$ and $A_o/V_o = [\text{dye}]_{out}$ (where V_i and V_o represent the respective internal and external volumes), we get the following:

$$\Delta\text{pH}_{(o-i)} = \log \frac{A_i}{A_o} + \log \frac{V_o}{V_i} \tag{9}$$

Therefore, a plot of $\log(A_i/A_o)$ vs. ΔpH should be a straight line with a slope of 1, and the intercept on the vertical axis should be equal to $\log(V_o/V_i)$ and can be used to estimate the vesicular volume as has been suggested for liposomes by Deamer [9]. For the case of 9-aminoacridine, shown in Fig. 7, the data approximates a straight line with a slope closely corresponding to the theoretical value of 1. From the intercept,a vesicular volume was calculated as 1.6 μl/mg protein. For the case of acridine orange, also shown in Fig. 7, the plot of $\log(A_i/A_o)$ vs. ΔpH gives a straight line, but with a slope of 0.63 instead of 1 and an intercept giving a vesicular volume 1—2 orders of magnitude higher than for 9-aminoacridine. This is, of course, consistent with the earlier suggestion that acridine orange is not simply distributing across the microsomes according to the pH gradient but is, in addition, undergoing some binding interaction. Using the H^+-space of 1.6 μl/mg protein estimated from 9-aminoacridine data and $[\text{dye}]_{bound}/[\text{dye}]_{free} \approx 30$ from Fig. 6, the ΔpH generated by ATP in these microsomes can be calculated to be approx. 4.3 pH units.

It is clear from the above studies that both acridine orange and 9-aminoacridine are probes capable of responding to ΔpH across membranes. We will now proceed to use them in the study of H^+ transport processes in the gastric vesicles.

The effect of preincubating gastric microsomes in KCl on the ATP-induced quenching of acridine orange fluorescence is shown in Fig. 8. Marked stimulation both on the initial rate and the maximum amount of quenching was observed with preincubation. Since preincubation in NaCl did not show the enhancing effect, this suggests that internal K^+ is important in the development of the H^+ gradient.

Another way to increase internal K^+ is to use the K^+-ionophore, valinomycin. Fig. 9 shows the stimulation of fluorescence quenching by valinomycin on all three dyes, indicating the increased vesicular acidification produced by the K^+ ionophore. This is possible only if these microsomes have a relatively low H^+ permeability. Otherwise, an increase in K^+ permeability with valinomycin can only dissipate the pH gradient by enhancing $H^+ : K^+$ exchange.

To investigate the role of H^+ permeability in this stimulatory effect of valinomycin, the H^+-ionophore, CCCP, was used to selectively vary H^+ permeability of the microsomes. The effects of adding increasing amounts of CCCP to the vesicles prior to activating the pump by ATP is shown in Fig. 10. At 10^{-8} M CCCP the stimulatory effect of valinomycin was abolished; at $2 \cdot 10^{-8}$ M CCCP valinomycin partially dissipates the gradient, and at $3 \cdot 10^{-7}$ M complete dissipation occurs with the addition of valinomycin. Thus, valinomycin stimulation of the pH gradient is eliminated by increasing permeability to H^+.

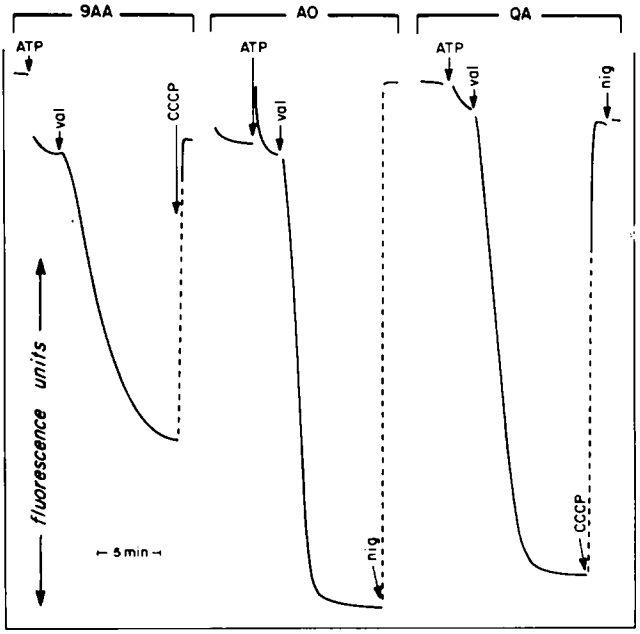

Fig. 9. Valinomycin (val.) stimulation effect on fluorescence quenching of 9-aminoacridine (9AA), acridine orange (AO) and quinacrine (QA). Dye concentration was 10 μM in K^+-Mg^{2+}-medium with 80 μg/ml gastric microsomes. Reaction was started by adding 0.5 mM ATP. Valinomycin was added at 2.5 μM. Fluorescence quenching was reversed by either 2.5 μM CCCP or 2.5 μM nigericin (nig.).

Valinomycin can increase internal K^+ without dissipating the gradient only if the entry of K^+ is followed by an anion, most possibly Cl^-. If this is true, then valinomycin should increase Cl^- influx. It is clear from Fig. 11 that valinomycin increases the rate of passive Cl^- uptake by the vesicles; the half time for $^{36}Cl^-$ equilibration was reduced from 31 min to 12 min in the presence of valinomycin. We can also use Cl^- uptake data as another means of estimating the vesicular volume if we assume that an equilibrium was reached at 180 min such that $[Cl^-]$ inside was the same as outside. The Cl^- space can be calculated from the counts associated with the microsomes (cpm/μl medium), i.e., Cl^- space (μl/mg protein) = external volume $\times$ microsome activity/medium activity. For five experiments similar to that shown in Fig. 11, we have calculated vesicular volumes (Cl^- space) between 1.6 and 2.5 μl/mg protein. These values are consistent with those obtained by the pH gradient method (cf. earlier) and are also in good agreement with the value of 2 μl/mg protein published by Sachs et al. [18].

Another test for the requirement of a permeable anion for the valinomycin-stimulatory effect is to replace Cl^- by other impermeable anions, such as sulfate or isethionate, and thus abolish the effect. Fig. 12 shows that when Cl^- is partially replaced by isethionate, stimulation by valinomycin is reduced, and, in fact, was nearly abolished when Cl^- concentration was down to 7.5 mM. It is also clear that the rate of dye uptake was dependent on $[Cl^-]$ even before the addition of valinomycin. Similar results were obtained using SO_4^{2-} as the impermeable anion.

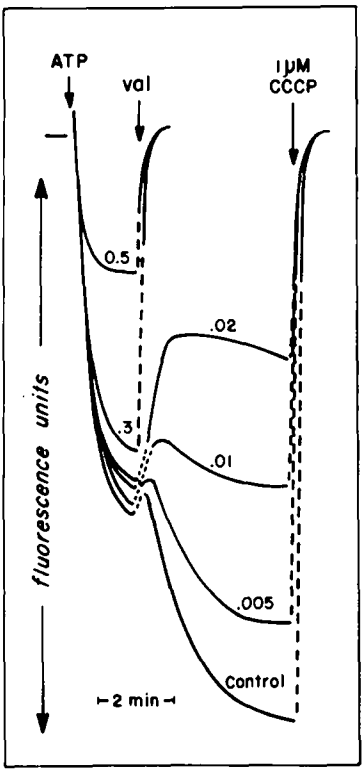

Fig. 10. Effects of increasing H^+-permeability by titration with a protonophore. Gastric microsomes were preincubated at 0—5°C for 4 days in K^+-Mg^{2+}-medium (pH 7.0). Various concentrations of CCCP, as indicated on each curve in μM, were added before starting the reaction with 0.25 mM ATP. Concentration of valinomycin was 0.5 μM.

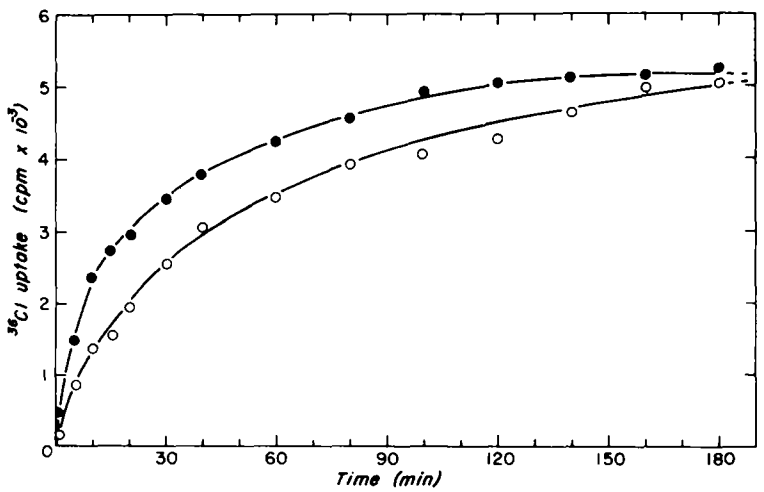

Fig. 11. The effect of valinomycin on $^{36}Cl^-$ uptake by gastric microsomes. Experimental details are described in the methods section. The points represent the mean of 4 separate experiments comparing preparations run without (○) and with (●) 10 μM valinomycin.

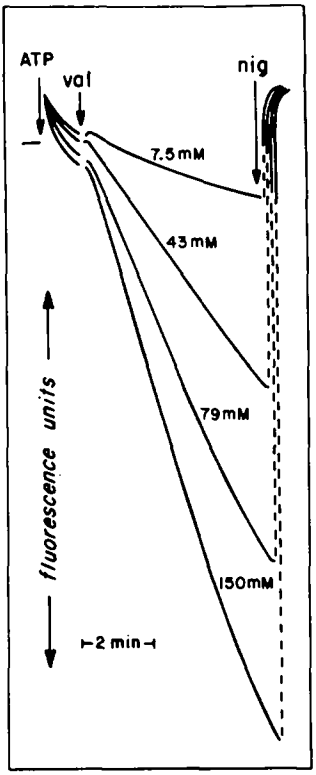

Fig. 12. Influence of [Cl^-] on the ATP-induced acidification of gastric microsomes. Concentrated membranes were preincubated in 150 mM KCl medium (pH 7.0) for 12 h at 0—4°C. Small aliquots of these membranes were added to reaction mixtures containing different concentration of Cl^- as indicated in mM on the figure; isethionate was used as the balance anion. Ionic strength and K^+ concentration were fixed constant in all cases at 0.16 and 150 mequiv., respectively. The reaction mixtures also contained 1 mM $MgSO_4$, 10 mM Pipes (pH 7.0) and 5 μM acridine orange. The reaction was started by the addition of 0.5 mM ATP; ionophores, valinomycin and nigericin were added as indicated to a final concentration of 2.5 μM. Final protein concentration was 35 μg/ml.

Discussion

The most direct method to follow H^+ movement is to measure extra-vesicular pH changes with a pH sensing electrode. However, with a preparation of biological material, such as described here, there are some limitations with this method. The most severe is to account for net pH change of the medium which would be produced by hydrolysis of ATP. To avoid this pH interference, H^+ translocation measurement must be done under very specialized conditions [2,4,18,19], e.g. weakly-buffered media at pH 6.1—6.2 with 2 mM Mg^{2+} and ionic strength of approx. 0.2. The uncertainty of the buffering power of intravesicular medium also limits the estimation of the pH gradient across the membrane. To overcome some of these difficulties associated with the pH electrode method, we turned to the use of ΔpH sensitive probes, such as acridine orange, neutral red, quinacrine and 9-aminoacridine. In this report, we demonstrated the applicability of these probes in monitoring the development of a pH gradient by gastric microsomes. Although all of these probes are sensitive to ΔpH

across the membrane, specific mechanisms associated with the ΔpH-sensitive optical changes are certainly different. In fact, we showed that acridine orange neutral red and quinacrine show increased binding to membrane sites which resulted in quenching of fluorescence, a shift in absorption spectrum and an increase in immobilization. On the other hand, 9-aminoacridine behaves more closely to an ideal ΔpH probe. It distributes itself across the membrane in accordance with the pH gradient, thus permitting the use of its distribution as a quantitative measure of ΔpH. Since all these probes are sensitive to the pH gradient, the extra-vesicular pH interference due to ATP hydrolysis can be eliminated by increasing the buffering power of the medium. Thus, using these probes, the development of pH gradient can be monitored over a wide range of ionic concentrations and external pH.

However, because of the indirect nature of the method and the perturbation of the system by the introduction of probes, there are some shortcomings. Firstly, the basic assumption of the probe method is that the charged form of the probe is non-permeable while the uncharged form is freely permeable. This assumption is reasonable; however, because of the relatively high probe concentration achieved intravesicularly, any finite permeability to the charged form of the probes will introduce leakage of the probes. Thus, there is a conceivable shunt pathway that might diminish the effective "power" of the H^+ pump via a leakage of the protonated form of the probe. Secondly, the solubility of the probes might introduce a limit on the amount of accumulated probe. Thirdly, since the probes penetrate the membrane in the uncharged form and become protonated once they get inside, the achievement of high internal concentration of probes might introduce a significant buffer power to the intravesicular medium. All of these shortcomings are due essentially to the high absolute internal concentration of probes. However, given a fixed ΔpH, the absolute internal concentration of probes is a direct function of external probe is a direct function of external probe concentration. Therefore, by choosing an appropriate external probe concentration, we can effectively eliminate some of the shortcomings. Because of the high sensitivity of the fluorescence technique, the external probe concentration can be varied over at least three orders of magnitudes. Further experiments are in progress to quantitatively evaluate the limitation of this probe method.

Using the 9-aminoacridine distribution, we can calculate the pH gradient generated by activation of the K^+-ATPase with ATP. From experiments such as that shown in Fig. 6, we measure a dye accumulation ratio of about $1.5 \cdot 10^4$-fold, which, from Eqn. 8, also approximates the ΔpH. Thus, at an external pH of 7.0 the observed ΔpH of 4.2 pH units indicates an intra-vesicular pH of 2.8. Although these may be the actual conditions of pH gradient in the presence of 9-aminoacridine, from the above-mentioned limitations we might expect an even larger gradient (lower intravesicular pH) in the absence of the probe.

From the model that we would propose, the maximum H^+ gradient would be a function of the power or driving force of the ATP-driven pump in the ideal case where there were no leaks in the system. In actual fact, the maximum gradient is determined by the turnover or flux of H^+ through the pump and the loss of H^+ as HCl or in exchange for a cation, e.g. K^+. This pump-leak system

would thus achieve a steady state at some pH gradient where the net ionic flux, summed through pump and the leaks would be zero.

Application of these probes to gastric microsomes reveals the importance of internal K^+, as can be seen from the preincubation experiment and the valinomycin stimulation effect. This internal requirement of K^+ can be interpreted as evidence for the $H^+ : K^+$ non-electrogenic exchange pump as proposed by Sachs et al. [18]; i.e., K^+ is being actively pumped out in exchange for H^+ in a one-for-one fashion. On the basis of the present evidence, we are inclined to favor a $H^+ : K^+$ exchange type of pump; however, we can not be certain on the stoichiometry of the exchange. The electrogenicity of the pump would then rest on the stoichiometry between H^+ and K^+ specifically through the pump, that is, some mechanism similar to the $Na^+ : K^+$ pump in red blood cells [20].

Several lines of evidence indicate that these gastric microsomes are derived from tubulovesicular structures of oxyntic cells [1—3,21]. The existence of the K^+-ATPase in the microsomes, which accumulates H^+ upon activation by ATP in the presence of K^+ and Mg^{2+}, suggests some importance in gastric HCl secretion. Electron microscopic observations indicate that upon stimulation by histamine the tubulovesicular structures of the oxyntic cells fuse with the plasma membrane [22,23]. If the isolated gastric microsomes had the same orientation as in the intact cell, the intravesicular space would correspond to the extracellular space. The internal K^+ requirement for the activation of microsomal H^+ pump would therefore correspond to an extracellular (luminal side) requirement for K^+ which is well known not to be the case under normal ionic bathing conditions. To incorporate the $K^+ : H^+$ exchange transport into an hypothesis of gastric HCl secretion, we would have to postulate the existence of a fairly tight recycling mechanism of K^+. From the analysis above, it is clear that in the presence of valinomycin and a $H^+ : K^+$ exchange pump the result is the transport of HCl into intravesicular space. There is no evidence for the existence of a valinomycin-type of ionophore in gastric mucosa, but perhaps there is some cytoplasmic mechanism of control which is lost during our purification procedure, and which might account for the tight recycling of K^+.

In response to the pH gradient across gastric microsomes, acridine orange binding to membrane sites increases. Similar behavior was observed in the energization of submitochondrial particles [16]. Analogous phenonomena have also been reported for quinacrine with energized chloroplasts and submitochondrial particles [17]. We would like to speculate on the reason for this increase in binding. From the Scatchard analysis of binding data, it was shown that the number of binding sites would be about 250 μmol/g protein (Fig. 6). The major protein band in these gastric microsomes has a molecular weight of about 100 000 [24]. If all the acridine orange binding sites were on protein molecules, there would be about 25 sites per protein molecule, which would seem to be too high. This leads us to suspect that negative sites on lipids may play a role. To explain the increase in binding induced by a pH gradient, we would like to put forward the following postulate. Acridine orange, like 9-aminoacridine, has a pK_a around 10. At any given pH, two species of acridine orange exist in equilibrium with each other, the charged and uncharged forms. If the uncharged form were permeable and the charged form were not, then acridine orange would accumulate in accordance with the pH gradient that existed

across the membrane. If we further postulate that there were negative sites on the internal face of the membrane (either on protein, lipids, or both) and that acridine orange has high affinity for these sites, then mass action law would predict an increase in binding to these sites with increasing ΔpH due to higher internal concentration. When all internal sites are saturated by binding, then the characteristics of dye uptake would be that of accumulation into internal vesicular space. Therefore, the major difference between 9-aminoacridine and acridine orange would be the affinity for the membrane sites and the number of sites available.

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