J. Membrane Biol. 76, 261–268 (1983)

The Journal of
Membrane Biology

Anion Exchange in Oxyntic Cell Apical Membrane: Relationship to Thiocyanate Inhibition of Acid Secretion

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Summary. The effects of SCN^- on H^+-accumulation by inside-out gastric vesicles derived from the apical membrane of secreting oxyntic cells are reported. SCN^- inhibited the formation of pH gradients in Cl^- and isethionate media. In Cl^-, the concentration of SCN^- required to achieve a certain degree of inhibition of H^+ uptake (or dissipation of preformed gradients) was increased with the increase in Cl^- concentration, indicating some competitive phenomena between these anions. Comparison of the rates of dissipation of similar pH gradients achieved in Cl^- *vs.* isethionate suggested the existence of a fast Cl^-/SCN^- exchange. In addition, direct isotopic fluxes confirmed the existence of rapid anion exchange and K-salt transport for both Cl^- and SCN^-. The rates of anion-exchange and K-salt transport were of similar magnitude, and the rates for SCN^- in either countertransport against Cl^- or cotransport with K^+ were twice as fast as the equivalent values for Cl^-. These mediated pathways in the apical membrane provide the possible means for rapid access of SCN^- to the acidic canalicular spaces of the oxyntic cell that is implicit in recent proposals to explain SCN^- inhibition of gastric HCl secretion.

Key Words gastric secretion · anion transport · apical membrane · HCl secretion · $(H^+ + K^+)$-ATPase · gastric mucosa

Introduction

The inhibition of gastric acid secretion by thiocyanate and other pseudohalogens is a phenomenon which has occupied gastric physiologists since its discovery several decades ago (Davenport, 1940; LeFevre, Gohmann & Rehm, 1964; Forte, 1968). Using the frog gastric mucosa, Sanders et al. (1978) demonstrated that, after inhibition of H^+ secretion by SCN^-, the rate of H^+ transport could be fully restored when certain weak bases were added to the serosal solution. This observation led to the hypothesis that inhibition of H^+ secretion by SCN^- is due to a "back diffusion" of HSCN from acidic spaces, and not due to a direct inhibitory effect on H^+ transport. The ameliorative effect of weak bases would, presumably, be due to an elevation of pH within normally acidic spaces (Rehm, Carrasquer & Schwartz, 1981). The HSCN backflux hypothesis is consistent with the observations of Gutknecht and Walter (1982) who showed very high permeability of HSCN through lipid bilayer membranes.

Using the ATP-dependent H^+ transport system ($(H^+ + K^+)$-ATPase) of the classical gastric microsomal preparation, Reenstra and Forte (1983) demonstrated recently that intravesicular SCN^- acts to increase the rate of passive proton loss from the vesicle interior. The results could be explained by HSCN formation within the vesicles followed by outward diffusion, in support of the HSCN backflux hypothesis. In the gastric epithelium, SCN^- is particularly effective when added to the serosal side. Thus, it could be expected that transport pathways for the anion, capable of matching the rate of acid generation, would exist in both basolateral and apical permeability barriers. However, in the microsomes, efficient entry of SCN^- and other anions (together with K^+) required the presence of the K^+ ionophore, valinomycin (Lee, Breitbart, Berman & Forte, 1979; Reenstra & Forte, 1983). In the absence of ionophore, external SCN^- was ineffective in promoting dissipation of preformed pH gradients, suggesting a deficiency in transport mechanisms as compared to those mechanisms present in whole tissue (Rabon, Chang & Sachs, 1978; Reenstra & Forte, 1983).

The transport properties of the inside-out $(H^+ + K^+)$-ATPase-rich membranes isolated from cytosolic and apical membrane of oxyntic cells depend upon the secretory state of the tissue (Wolosin & Forte, 1981*a*, *b*). Unlike the gastric microsomes, associated with the nonsecreting cell, the $(H^+ + K^+)$-ATPase-rich vesicles isolated from stimulated tissue, which we have designated stimulation-associated (*s.a.*) vesicles, exhibit patterns of acid accumulation consistent with acid secretion by intact tissue. This would indicate that at least

the more essential apical membrane transport mechanisms responsible for *in vivo* secretion are preserved during the isolation procedure. HCl acid accumulation in these *s.a.* vesicles has been shown to result from two sequential processes, (i) fast entry of KCl through a transport system absent in gastric microsomes; and, (ii) ATP-driven uptake of H^+ in exchange for the pre-internalized K^+ (Wolosin & Forte, 1981*c*, 1983). Thus, stimulation of secretion induces the appearance of an additional transport system in the $(H^+ + K^+)$-ATPase-rich membrane. Since this system or other undiscovered ion pathways may be involved in the translocation of SCN^-, a study of the effect of SCN^- in pH gradient generation in *s.a.* vesicles was undertaken.

Materials and Methods

Membrane Preparation

Stimulation-associated (*s.a.*) vesicles were prepared from highly stimulated rabbit gastric mucosa as described by Wolosin and Forte (1983). Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Measurement of pH Gradients

The development of pH gradients (ΔpH) in *s.a.* vesicles was followed by the quenching of acridine orange fluorescence (Lee & Forte, 1978). Experiments carried out at 37 °C were started by addition of a small aliquot of membranes in 1 M sucrose to the H^+ uptake medium. At the end of any given experimental protocol, the pH gradient was fully dissipated by addition of 5 μM nigericin. The fluorescence reading at that point was taken as 100%, and all other values are reported as a % of that maximum value. We have previously shown, under similar uptake conditions, that the rate of fluorescence change is a relative measure of the H^+-efflux across the membrane (Wolosin & Forte, 1983) and that the maximal ΔpH's exceed 4.5 pH units (Wolosin & Forte, 1981*b*).

Isotopic Fluxes

Uptake mixtures were obtained by mixing vesicles and solutions containing the isotopic label. The details for each experiment are given in the legend to the appropriate figure. Aliquots of 80–100 μl were withdrawn at appropriate times from isotope uptake mixtures containing $^{36}Cl^-$ and/or $(^{14}C)SCN^-$. The aliquots were added to 0.45 μm Gilman-Gn-6 filters, which had been prewetted and precooled by vacuum filtration of 5 ml ice cold washing buffer (100 mM choline Cl + 100 mM sucrose) just prior to sample addition. The filters were immediately (< 10 sec) washed twice with 10 ml of washing buffer and transferred to scintillation vials. Retained radioactivity was measured in 10 ml of Aquasol® (Packard) using a Packard Tri Carb scintillation counter. To obtain the blank sample (i.e., radioactivity retained as a result of binding at $t = 0$ min) membranes and labeled solution were mixed at 0 °C and two or three aliquot samples were processed within 20 sec. The mean retained radioactivity for these samples was subtracted from the value of each individual time-point, and the result was divided by the total counts present in the aliquot to obtain the fraction of trapped isotope, cpm_{in}/cpm_{out}. Unless specified, experiments were carried out at room temperature (~24 °C).

Abbreviations. Tris – Tris(hydroxymethyl)aminomethane; TES – N(tris[hydroxymethyl]methyl-2-amino methane sulfonic acid); EDTA – Ethylene diaminetetraacetic acid.

Results

The Effect of SCN^- on pH Gradients

In preliminary experiments, it was observed that addition of SCN^- (1–5 mM) to *s.a.* vesicles in which ATP-generated H^+ gradients were preformed using cytoplasmic-like concentrations of K^+ and Cl^- (100 and 30 mM, respectively) produced considerable dissipation of the H^+ gradient. On the other hand, gradients of similar magnitude obtained using the inverse concentration of ionic substrates (30 mM K^+ and 100 mM Cl^-) were only slightly affected by the same concentrations of SCN^-. This apparent interdependence between the ionic composition and the effect of SCN^- pointed to an interesting involvement of K^+ and/or Cl^- in the dissipation process; hence, a comprehensive study of SCN^--induced pH gradient dissipation as a function of K^+ and Cl^- was carried out.

Figure 1*A* depicts the time course for the development of a pH gradient as a function of the Cl^- concentration at fixed K^+ (= 100 mM) and the effect of 1 mM SCN^- on the pH gradients. At low $[Cl^-]$, the addition of SCN^- led to a relatively rapid dissipation of pH gradient; these effects were more attenuated as Cl^- was increased. A set of H^+ accumulation patterns, equivalent to that displayed in Fig. 1*A* can be achieved at high Cl^- concentration (100 mM) by varying the K^+ concentration (Fig. 1*B*). In these cases, SCN^- has a weak effect on the preformed gradient irrespective of the K^+ concentration. These results suggest that pH gradient dissipation by SCN^- is antagonized by extravesicular Cl^-.

As shown in Fig. 2, at given K^+ and Cl^- concentrations, the rates of pH gradient dissipation (and the inhibition of pH gradient development) increased with increasing $[SCN^-]$. Given the antagonistic effect of Cl^-, similar rates of dissipation could be achieved for different Cl^- concentrations by using appropriate $[SCN^-]$. For example, if $[Cl^-]$ was increased from 8 to 50 mM, the concentration of SCN^- had to be proportionally increased from 1 to 5 mM in order to achieve similar inhibitory effects. However, an additional phenomenon occurred when the concentration of SCN^- was raised above a certain threshold: when

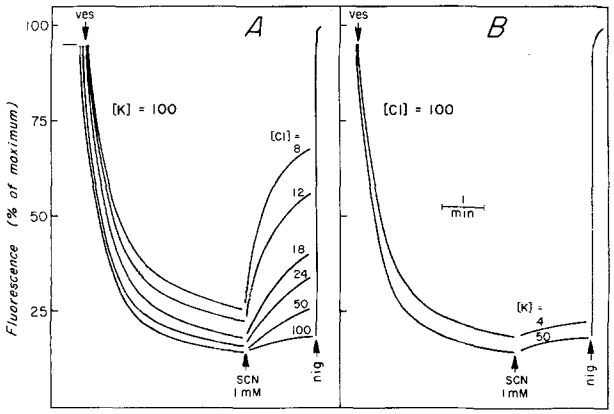

Fig. 1. The development of ATP-generated pH gradient as a function of K^+ and Cl^-, and its dissipation by SCN^-. The pH gradients and the rates of H^+ flux were monitored through the changes in the acridine orange fluorescence signal. Experiments were started by addition of vesicles (50 μg protein) to 1.25 ml of uptake medium. (*A*): The medium contained 100 mM K^+, combinations of Cl^- and gluconate adding up to 100 mM and 25 μl of complement solution (25 mM ATP, 12.5 mM Mg^{2+}, 2.5 mM EDTA, 100 μM acridine orange, and 200 mM Tris-TES, pH 7.2). The particular concentrations of Cl^- are stated in the figure. (*B*): The medium contained 25 μl complement solution and either 50 mM KCl and 50 mM choline Cl, or 4 mM KCl and 96 mM choline Cl. Additions of KSCN and 5 μM nigericin (*nig*) are shown in the figure

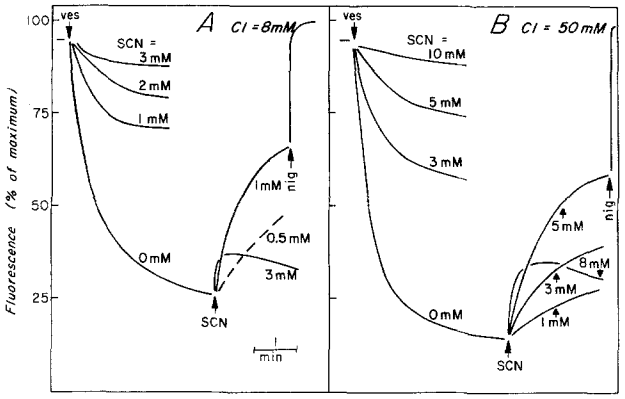

Fig. 2. Dissipation of pH gradient as a function of the SCN^- concentration. The experimental details are as in Fig. 1. KSCN was either initially present in the uptake solution or added as indicated in the figure

the initial dissipation became very rapid, an apparent "inversion" followed; i.e., the fluorescence intensity started to decrease again. The cause of this phenomenon, which does not arise from renewed intravesicular acidification, is discussed at the end of the Results section.

The Effect of SCN^- on pH Gradients in Alkylsulfonate Media

The development of the vesicular pH gradient is a function of two antagonistic processes: (i) the

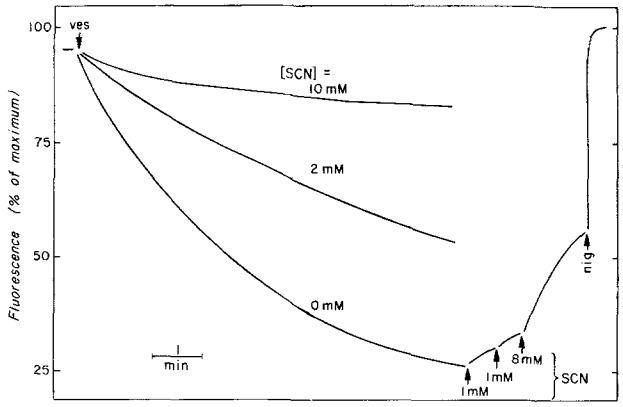

Fig. 3. Patterns of pH gradient formation in K isethionate and the dissipative effect of SCN^-. The uptake media contained 100 mM K isethionate and 25 μl of complement solution; SCN^- was present or added as indicated in the figure. All other experimental details are as in Fig. 1

ATP-driven H^+ accumulation and (ii) the leak of accumulated H^+ through dissipative pathways. Both processes are dependent on the salt composition of the medium. In the case of salts that are highly permeable such as KCl or KNO_3, fast intravesicular delivery of K^+ results in saturation of the K^+ site of the $(H^+ + K^+)$-ATPase and maximal initial rates of H^+ accumulation are achieved at relatively low anion and cation concentrations. In the case of an alkyl sulfonate, such as isethionate, the permeability of the K^+ salt is considerably lower than that of KCl or KNO_3, so that even at high anion (and K^+) concentration the net salt influx is low. This will result in subsaturation of the K^+ site of the $(H^+ + K^+)$-ATPase and thus in reduced rates of acid accumulation. For instance, the initial rate of H^+ uptake in 100 mM potassium isethionate is similar to the rate obtained in 2 mM Cl^-. However, because the ionic permeability of isethionate through the membrane is extremely low, the leak of accumulated acid (i.e., concomitant K^+ and isethionate efflux) is probably much smaller than in the Cl^- case. H^+ leak would occur also in exchange for incoming K^+, but in isethionate this K^+ would be utilized by the $(H^+ + K^+)$-ATPase so that no net H^+ loss occurs. The reduction in dissipation pathways for H^+'s can probably explain why, in spite of slow rates of H^+ uptake, large pH gradients are obtained in K^+-isethionate.

Figure 3 shows the pattern of H^+ uptake in 100 mM K-isethionate and the effect of SCN^- on the pH gradients. If 2 mM SCN^- were initially included in the uptake medium, there was some inhibition of acid accumulation but more complete inhibition required the presence of 10 mM SCN^-. When 1–2 mM SCN^- were added to preformed

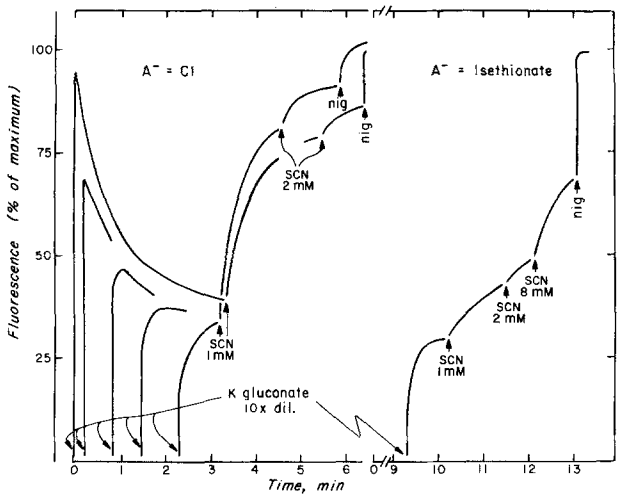

Fig. 4. pH gradient formation at reduced volume and the effect of SCN^- in the presence of 10 mM chloride or 10 mM isethionate. *Left:* The uptake mixture contained 100 mM KCl, 50 μg membranes and 25 μl of complement components in a total volume of 120 μl. The small volume did not permit a spectrometric assessment. At the indicated times, equivalent mixtures were diluted 10-fold with 1.1 ml of 100 mM K gluconate to give the final $[Cl^-]=10$ mM and the fluorescence signal was followed as shown. Comparison of these traces with the trace for which the dilution was simultaneous to the addition of vesicles (i.e., $t=0$) showed that the acid accumulation attained by both methods was very similar. *Right:* 100 mM K isethionate replaced the 100 mM KCl in the reduced volume solution prior to dilution with K gluconate. All other experimental details are as described in Fig. 1

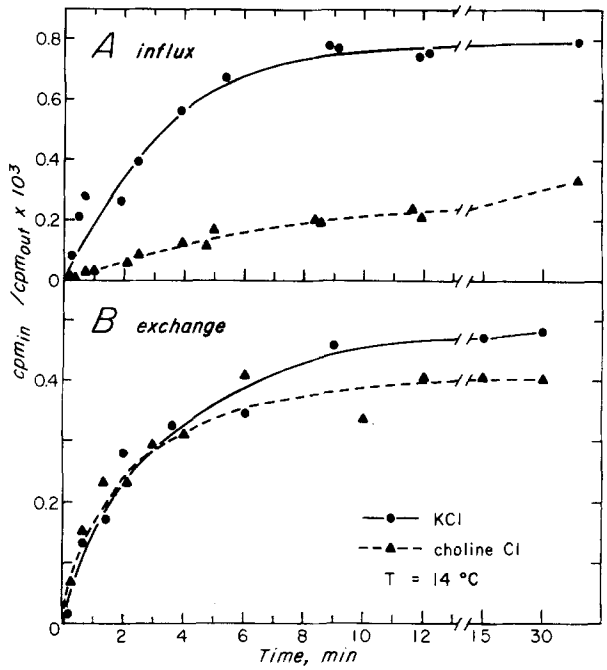

Fig. 5. Influx of Cl salt and Cl–Cl exchange in K^+ or choline media. (*A*) Salt influx. A suspension of vesicles in 300 mM sucrose (16 mg protein/ml) was mixed with an equal volume of solution containing 200 mM sucrose, 50 mM of either KCl or choline chloride, 2 mM Tris-TES (pH 7.2), and 2 μCi-^{36}Cl. Both solutions were pre-equilibrated at 14 °C. (*B*) Equilibrium exchange. The vesicles and salt solutions (without the ^{36}Cl) were mixed and pre-incubated for 72 h at 0 °C. The mixture was brought to 14 °C and ^{36}Cl (1 μCi/ml) was added. For both types of experiments, aliquots (80–100 μl) were withdrawn at various times for filtration. Details for the membrane filtration and counting procedure are described in Methods

gradients, a very small pH gradient dissipation was seen. Faster dissipation was achieved only at substantially higher concentrations of SCN^-. Since, as shown previously (*cf.*, Fig. 1*a*), no significant effect of 2 mM SCN^- was observed in 100 mM Cl^-, it might be argued that, although the permeation of the isethionate salt through the membrane is slow, the presence of 100 mM isethionate acts much like the presence of high Cl^-, inhibiting the SCN^--induced dissipation. Hence, the effect of SCN^- in the presence of lower isethionate should be tested.

Because it is impossible to develop large pH gradients when low concentrations of isethionate are used, a variation in the method was introduced to test the possibility of isethionate/SCN^- antagonism. All the components of the uptake media, except for the salt, were concentrated several-fold, and the formation of H^+ gradient was carried out in reduced volume. At the appropriate time, the reaction mixture was diluted with salt to the volume and concentration normally used in the assay. The diluting medium could then be selected as desired, using the impermeable anion, gluconate (Wolosin & Forte, 1983), as the indifferent anion. In Fig. 4 it is demonstrated that the time course of dye uptake is relatively unaffected by this volume reduction technique. By using K-gluconate in the diluting medium, it was possible to achieve large pH gradients in a medium containing only 10 mM isethionate. Addition of SCN^- at this point produced fast dissipation in Cl^-, but failed to do so in isethionate. This suggests that the specific anion accumulated within the vesicle plays an important role in SCN^--induced H^+ dissipation; presumably anion exchange occurs.

Radioisotopic Fluxes of Cl^- and SCN^-

The transport properties of KCl and KSCN were studied with radioisotopes. Influx of KCl into *s.a.* vesicles was fast; equilibration of $K^{36}Cl$ medium at 14 °C was characterized by half times shorter than 2 min (Fig. 5*A*) and of about 40 sec at 24 °C. In contrast, when K^+ was replaced by choline, the half time of equilibration at 14 °C exceeded 1 hr. On the other hand, when the rate of isotopic equilibration was measured under equilibrium exchange conditions, using vesicles pre-equilibrated in KCl or choline Cl, it was found that the rate of ^{36}Cl

equilibration was independent of the cation (Fig. 5*B*). The half times for equilibration of ^{36}Cl were, within the experimental error, equal to the half time for influx in KCl. Since choline Cl permeates the membrane very slowly, the ^{36}Cl equilibration can only occur through anion exchange.

Experiments of K^+ salt uptake and anion-anion exchange were conducted with SCN^-, in a comparative approach against Cl^-. Figure 6*A* describes the time course of K^+-driven influx of $^{14}C(SCN)$ and ^{36}Cl using a K^+/A^- (where A^- = permeable anion) ratio of 100:2 mM. The initial rates of isotope uptake showed that the influx of $^{14}C(SCN)$ was at least twofold faster than that of ^{36}Cl. The use of K^+/A^- ratios larger than 1.0 should promote the intravesicular accumulation of anions above their concentration in the medium. For example, at equilibrium $[K^+]_{out}/[K^+]_{in} = [A^-]_{in}/[A^-]_{out}$, and since $[K^+]_{in} = [A^-]_{in}$, one derives $[A^-]_{in}/[A^-]_{out} = ([K^+]_{out}/[A^-]_{out})^{1/2}$. To obtain an estimate of $[A^-]_{in}/[A^-]_{out}$, an isotopic uptake experiment using equimolar concentration of K^+ and Cl^- (25 mM) was carried out and results are also shown in Fig. 6*A* (open circles). For this case at equilibrium, $[A^-]_{in}/[A^-]_{out} = 1$. Under the simplistic assumption that the intravesicular volumes are identical in all three cases, the ratio between the equilibrium cpm_{in}/cpm_{out} should be equivalent to the $[A^-]_{in}/[A^-]_{out}$ ratios. As shown on the right-hand side of Fig. 6*A*, setting the cpm_{in}/cpm_{out} ratio for the $[K^+]_{out} = [Cl^-]_{out}$ case as equal to 1, $[A^-]_{in}/[A^-]_{out}$ ratios of between 2 and 3 are obtained for the 100:2 (K^+/A^-) cases. These experimental estimations should be contrasted against a theoretically expected value of 7. Differences in the vesicular volume at equilibrium and/or permeation of the indifferent anion, gluconate, could lead to such deviations. In any event, external $[K^+]$ does influence the rate and relative accumulation of Cl^- and SCN^-.

Anion-anion exchange was studied using vesicles containing a high intravesicular concentration of Cl^-, trapped in the form of choline Cl. Since choline Cl cannot rapidly leave the vesicles, anion internalization could only occur in exchange for intravesicular Cl^-. When the exchangeable isotopic anion is present at low concentration in the extravesicular space, it should accumulate within the vesicle. Under such conditions, the time course of isotopic uptake will be extended and will result in large accumulation ratios. Figure 6*B* describes the result of such an experiment. When the extravesicular Cl^- concentration was 50 mM, isotopic equilibration was reached rapidly. Because choline is impermeable, no significant net salt flux (and hence no volume change) should occur; hence

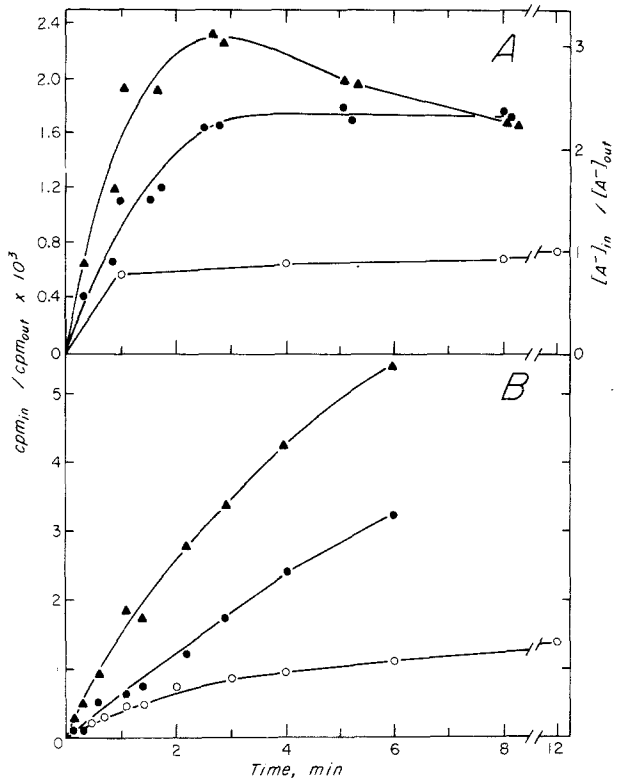

Fig. 6. Comparison of Cl^- and SCN^- influx and exchange. (*A*): Salt influx. Experiments were started by adding 100 µl of membranes (6.4 mg/ml) to 900 µl of solution. The final reaction mixture contained 1 mM Tris-TES (pH 7.2) and either 2 mM KCl–100 mM K gluconate (●), 2 mM KSCN–100 mM K gluconate (▲) or 25 mM KCl–150 mM sucrose (○). The Cl solutions were labeled with 1 µCi ^{36}Cl and the thiocyanate solutions with 1 µCi $^{14}C(SCN)$. The intra- to extravesicular ratio for the anions ($[A^-]_{in}/[A^-]_{out}$) was determined by assuming that the intravesicular volume is identical in all cases and that for the $[K^+]_{out} = [Cl^-]_{out}$ case, at equilibrium, $[Cl^-]_{in} = [Cl^-]_{out}$. (*B*): Exchange fluxes. Vesicles (0.7 mg/ml) were pre-incubated at 0 °C for 72 hr in 100 mM ChCl–100 mM sucrose and 2 mM Tris-TES (pH 7.2) and concentrated fivefold by centrifugation for 5 min in an Eppendorf centrifuge and resuspension. For the double-label experiment, 20 µl of these concentrates were added to 1 ml solution to give a final concentration of 2 mM Cl^-, 2 mM SCN^-, 2 mM Na^+, 2 mM choline, 300 mM sucrose, 1 mM Tris-TES and 1 µCi each of ^{36}Cl and $(^{14}C)SCN$. Serial aliquots were taken for filtration and counting of ^{36}Cl (●) and $(^{14}C)SCN$ (▲). For the single-label experiment, the dilution was made to give a final concentration of 50 mM choline Cl, 200 mM sucrose, 1 mM Tris-TES and 1 µCi of ^{36}Cl (○). Ratios of cpm_{in}/cpm_{out} were determined as described in Methods

when isotopic equilibrium is reached, $[Cl^-]_{in}$ is still 100, and $[Cl^-]_{out} = 50$ mM. The experimental cpm_{in}/cpm_{out} ratio for this case represents a $[^{36}Cl]_{in}/[^{36}Cl]_{out}$ ratio of 2. When the external concentrations of SCN^- and Cl^- were 2 mM each, the time course of tracer uptake (measured simultaneously) remained linear for several minutes and large isotopic accumulation developed. The initital rate of SCN^- uptake was faster than that of Cl^- by a factor of at least two.

ATP-Dependent Accumulation of Cl^- and SCN^-

Parallel experiments were carried out to measure the effects of SCN^- on ATP-dependent H^+ uptake (by the dye accumulation method) and ^{36}Cl uptake (Fig. 7*A* and *B*). In order to measure the changes in intravesicular Cl^- which occur as the pH gradient developed, conditions of the H^+-uptake experiments were slightly modified as compared with those described earlier, e.g., Fig. 1. Protein and acridine orange concentrations were increased eightfold, and the vesicles were added to the medium 1 min before the ATP. Addition of ATP induced in about 2 min a large pH gradient which was significantly dissipated by 2 mM SCN^- (Fig. 7*A*). During the 1-min incubation in the ATP-free media, ^{36}Cl reached equilibration (Fig. 7*B*). With $[K^+]_{out}=100$ and $[Cl^-]_{out}=8$ mM, the intravesicular Cl^- calculated from the estimated intravesicular volume was 14 ± 2 mM. Addition of ATP triggered a four- to five-fold increase in trapped radioactivity. Thus, under the simplistic assumption that no volume change occurred, the final Cl^- concentration reached in the presence of ATP would be five- to 10-fold higher than $[Cl^-]_{out}$. (If only a fraction of the vesicles participate in the ATP-dependent Cl accumulation, the actual concentration of accumulated Cl^- might be even higher. On the other hand, the concentration will be decreased by concomitant vesicular volume expansion). When 2 mM SCN^- was added, a large fraction of the accumulated Cl was rapidly dissipated. It can be seen in Fig.7 that the time course for the accumulation and dissipation was similar for Cl^- and acid.

Intravesicular Precipitation of Acridinium Thiocyanate – a Precautionary Note

The "inversion" phenomenon observed in Fig. 2 is most probably not associated with re-initiation of acid accumulation. This conclusion is based on the observation that, following the "inversion", the recovery of fluorescence induced by nigericin is gradually eliminated (Fig. 8*A*). Normally, the addition of nigericin, an effective H^+/K^+ exchange ionophore, results in the instantaneous dissipation of pH gradients (and hence full recovery of fluorescence as shown in Fig. 1). Since it seems unlikely that any significant pH gradient will continue to exist with high external K^+ in the presence of nigericin, the entrapment of dye has to be attributed to a different process. It could be due to precipitation of acridinium thiocyanate within the vesicle; the formation of the salt would decrease the free

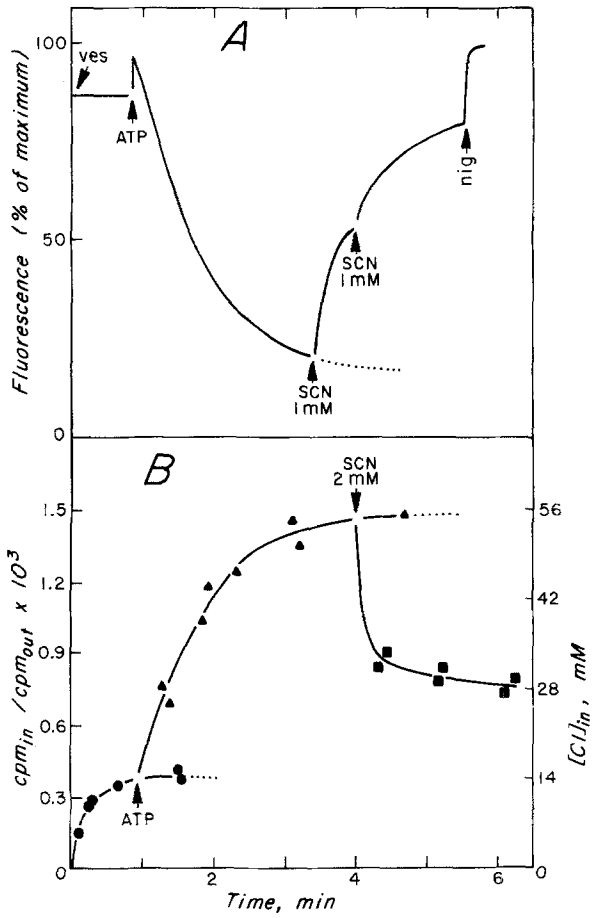

Fig. 7. Simultaneous ATP-dependent H^+ and Cl^- accumulation and dissipation by SCN^-. (*A*): Proton accumulation. Experiment was started by addition of 50 µl membranes to 1.2 ml of 92 mM K gluconate, 8 mM KCl, 0.05 mM EDTA, 16 µM acridine orange, and 4 mM Tris-TES (pH 7.2); $T=36$ °C. After 0.9 min, 12 µl of 100 mM ATP–50 mM Mg^{2+} were added, and the resulting H^+ accumulation was followed spectrofluorimetrically. When the system had approached a steady state, two consecutive additions of 1 mM KSCN induced a large dissipation of ΔpH. Finally, total dissipation was accomplished by 5 µM nigericin (*nig*). (*B*): Chloride uptake. Experiment was started by adding 100 µl of membranes (8 mg/ml) to 2.4 ml of the same solution as in *A* but including 2.5 µCi ^{36}Cl. At 0.9 min, 1.8 ml were transferred to a tube containing 18 µl of 100 mM ATP–50 mM Mg, and at 4 min, 800 µl from that tube were transferred to a tube containing 8 µl of 200 mM KSCN. Aliquots of 100 µl solution were withdrawn at intervals from each one of the reaction tubes for filtration, and determination of the cpm_{in}/cpm_{out} ratio as described in Methods. Different symbols indicate results before addition of MgATP (●), in the presence of ATP (▲); and results in the presence of ATP and SCN^- (■). The intravesicular Cl^- concentrations ($[Cl^-]_{in}$) were calculated from the intravesicular volume of the preparation determined from the equilibrium cpm_{in}/cpm_{out} ratios for ^{36}Cl in 25 mM KCl–150 mM sucrose

acridine orange and thiocyanate concentrations and trigger further uptake of both. A number of observations are consistent with such an hypothesis: (i) When the membrane barrier was eliminated by addition of detergent, the fluorescence was im-

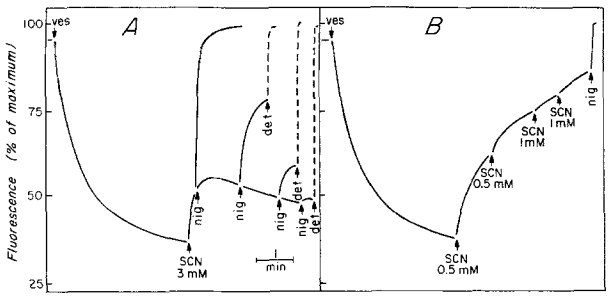

Fig. 8. Evidence for intravesicular precipitation of acridine orange hydrothiocyanate. pH gradients were formed as in Fig. 1 *A* using 100 mM K^+ and 8 mM Cl^-. (*A*): Addition of thiocyanate induces a very fast dissipation followed by an "inversion." Addition of 5 mM nigericin (*nig*) before the "inversion" induces a rapid fluorescence recovery. After the "inversion," the effectiveness of nigericin decreases gradually. The full fluorescence is recovered when 0.05% Triton X-100 (*det*) is added. (*B*): If the addition of SCN^- is performed stepwise so that the intravesicular acridine orange concentration is decreased before the next increase in SCN^- concentration, the "inversion" does not occur and a large dissipation of the gradient is achieved

mediately recovered. (ii) The threshold concentration of thiocyanate increased as the concentration of dye in the medium decreased (and thus the concentration accumulated within the vesicles). (iii) If the final SCN^- concentration was increased in steps, so that partial dissipation of the accumulated acridine orange could occur at each step, the appearance of the "inversion" was eliminated (Fig. 8*B*). In this case a stable state was reached, and addition of nigericin induced full recovery of the fluorescence signal. (iv) The solubility product for the salt is very low (*see* below). Since the concentration of acridine orange within the vesicles could be as high as 50–60 mM, a fast accumulation of a small amount of SCN^- within the vesicle could trigger a vigorous precipitation. The matter is further complicated because a concentration of reactants well above the solubility product of the salt is required to start the fast precipitation. When up to 2 mM SCN^- was gradually added to a solution of 1 mM acridine orange in 20 mM KCl, no precipitate formed; however, when the concentration of SCN^- reached 2.60 mM, strong precipitation occurred. By removing the precipitate and measuring the concentration of acridine orange in solution, it was possible to estimate a solubility product of about 0.3×10^{-6} M^2, i.e., about 10-fold lower than the concentration product that triggered the precipitation. The precipitation was independent of the KCl concentration.

As shown in Fig. 2, the intravesicular precipitation occurred only when the rate of H^+-dissipation was overwhelmingly fast. At moderate rates of dissipation, which were appropriate to perform this study, no "inversion" phenomena occurred. It is possible that this phenomenological interaction between SCN^- and acridine orange might account for the wide variation in effects of SCN^- reported by Rabon et al. (1978).

Discussion

The possibility that SCN^- inhibits H^+ secretion by intact gastric mucosa via the "backflux" of HSCN has gained recent experimental support (Sanders et al., 1978; Gutknecht & Walter, 1982). It is noted that SCN^- is effective as an inhibitor when added to either the serosal or mucosal bathing solution. Although pathways for SCN^- translocation across the luminal membrane have been postulated (Gutknecht & Walter, 1982), they have not been heretofore rigorously identified. Experiments with isolated gastric microsomes have generally been consistent with an hypothesis whereby intravesicular SCN^- dissipates accumulated H^+ via an HSCN leak pathway (Rabon et al., 1978; Reenstra & Forte, 1983). However, in the absence of valinomycin (and K^+), entry of SCN^- to these vesicles could not account for the ion access from the cytosol to acidic spaces in the intact system.

Previous experiments have suggested that the *s.a.* vesicle is a more appropriate model for the apical membrane of the secreting oxyntic cell. In this system, addition of SCN^- to the outer medium induced the immediate dissipation of pH gradients in Cl^- and isothionate medium. The existence of a fast Cl^-/SCN^- exchange was suggested by the observation that SCN^- effectivity was decreased upon replacement of intravesicular Cl^- by isethionate. Pathways for SCN^- translocation consistent with the concept of rapid movement of the ion across the luminal barrier were confirmed by isotopic flux studies. The comparative experiments of Cl^- and SCN^- demonstrated that: (i) thiocyanate and Cl^- can rapidly enter the *s.a.* vesicles in conjunction with K^+ or in exchange for pre-internalized Cl^-, (ii) the rates of influx and exchange are of similar magnitude, and (iii) both in the cases of influx as K salts and in exchange versus Cl^-, the rates for thiocyanate are at least twice as large as the rates for Cl^-. The source of these transport processes, whether the result of facilitated (conductive or nonconductive) mechanisms displaying specific kinetic properties or of passive ion "leaks" remain to be investigated.

In isotopic flux studies, all sealed vesicles present in the membrane fraction contribute to the experimental results, whereas ATP-dependent acid accumulation occurs solely in a specific population of inside-out $(H^+ + K^+)$-ATPase-rich vesicles.

Thus, projections of the isotopic flux measurements to the patterns of H^+ accumulation-dissipation requires a demonstrated correlation between both processes. The striking similarity between the patterns of ATP-induced accumulation and SCN^--induced dissipation for Cl^- and H^+ as shown in Fig. 7 indicate that at least a substantial fraction of the sealed volume present in the membrane suspension is contributed by acid-accumulating vesicles.

The patterns of ΔpH dissipation can be explained in terms of the observed permeabilities and the HSCN backflux hypothesis. Consider the case of $[Cl^-]_{out}=8$ mM, $[K^+]=100$ mM (e.g., Fig. 7). When a large pH gradient is achieved, intravesicular $[Cl^-]$ is quite high, probably above 50 mM, then efficient uptake of the small concentration of SCN^- present in the medium will occur (similar to the uptake shown in Fig. 6B). Once inside the vesicle, some SCN^- will combine with H^+ forming the highly permeable HSCN, which diffuses out of the vesicle. If the concentration of Cl^- in the medium is increased, the competitive effect of the external Cl^- induces a decrease in the rate of SCN^- entry and hence of H^+ dissipation. This, in turn, can be compensated by a comparable increase in SCN^-, generating the observed Cl^-/SCN^- antagonism depicted in Fig. 2.

When the intravesicular anion is isethionate, anion exchange is presumably impeded, hence thiocyanate could enter only as the K salt. Although it is true that under our working hypothesis each internalized SCN^- will be able to combine with and carry out one H^+, the accompanying K^+ could be utilized by the pump (which in isethionate is clearly working below its maximal capacity) to accumulate one additional H^+. At the low concentrations of SCN^- applied (1–2 mM), it is most likely that the rate of KSCN entry does not exceed the maximal rate of the ATPase pump so that net change achieved per cycle is minimal. The maximal rate of KCl entry and hence of KSCN exceeds the V_{max} of the pump (Wolosin & Forte, 1983). Thus, when the concentration of SCN^- is sufficiently high, the unidirectional flux of KSCN will exceed the maximal rate of H^+ pumping. Dissipation of pre-accumulated H^+ will then occur because incoming SCN^- could carry out H^+ at a rate exceeding the rate at which the pump can utilize the incoming K^+.

The SCN^-/Cl^- exchange activity demonstrated in the isolated *s.a.* membrane vesicle is a rapid process. It will provide a convenient pathway for transport of SCN^- across the apical membrane of oxyntic cells. If the HSCN backflux is an efficient process, a relatively small concentration of cytoplasmic SCN^- might be able to recycle and "return" a large number of secreted H^+ (and Cl^-) to the cytoplasm. Such a mechanism might be able to explain the observation that while reducing H^+ and associated Cl^- transport in bullfrog gastric mucosa, SCN^- had only a slight inhibitory effect on Cl^- exchange diffusion (Forte, 1968).

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Received 10 January 1983; revised 16 May 1983