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# THE SPECIFICITY OF OMEPRAZOLE AS AN ($H^+ + K^+$)-ATPASE INHIBITOR DEPENDS UPON THE MEANS OF ITS ACTIVATION

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**Abstract**—Omeprazole (OME) is a novel acid secretion inhibitor, believed to act directly on the gastric proton pump, the ($H^+ + K^+$)-ATPase. Inhibition of ATPase activity is associated with an incorporation of [$^{14}$C]OME into gastric vesicles containing the ($H^+ + K^+$)-ATPase, and both processes are greatly enhanced if the OME is exposed to acidic pH. This, and other evidence, suggests that the acidic environment of the ($H^+ + K^+$)-ATPase generates from OME a reactive intermediate which covalently inhibits the pump.

We have compared the means by which the OME was acid-activated with the specificity of inhibition (amount of incorporation of omeprazole required to produce 100% inhibition of $K^+$-stimulated ATPase activity). The stoichiometry of incorporation has been related to the number of detectable catalytic phosphorylation sites in each preparation (an index of the number of functional pumps).

In lyophilised gastric vesicles, where the membrane barriers separating the cytoplasmic and luminal faces of the enzyme are substantially destroyed, incubation with OME at pH 6.1 produced a progressive inhibition and incorporation over 120 min. Complete inhibition of $K^+$-ATPase required 13 ± 3 (SEM; N = 4) moles of OME incorporation per phosphorylation site.

In intact gastric vesicles, under conditions shown independently to result in proton pumping and the acidification of the vesicle interior (150 mM KCl, 9 μM valinomycin, 2 mM Mg–ATP pH 7.0), inhibition and incorporation occurred more rapidly (15 min). Complete inhibition of $K^+$-ATPase required only 1.8 ± 0.15 (SEM; N = 3) moles of OME per phosphorylation site.

Therefore when OME was preferentially activated on the luminal side of the vesicle membrane a more specific inhibition was observed. Intact pumping vesicles represent a useful *in vitro* model for the actions of acid activated compounds.

The substituted benzimidazoles, such as omeprazole (Fig. 1), represent a novel class of inhibitors of acid secretion. Their mechanism of action has been postulated to be by an inhibition of the gastric proton pump, the ($H^+ + K^+$)-ATPase [1].

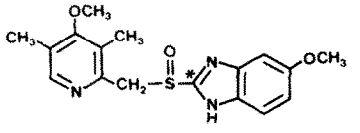

Fig. 1. Structure of omeprazole. The position of radiolabel in [$^{14}$C]omeprazole is also indicated (*).

The nature of the interaction of omeprazole with the proton pump would appear to be complex. Omeprazole itself does not interact directly with the pump, rather, under conditions of low pH, omeprazole is converted into a relatively non-selective sulphydryl-reactive agent which reacts with essential thiol groups on the pump [2–4]. Recent studies indicate that the reactive intermediate derived from omeprazole, and related compounds of this type, is a rearranged cyclic sulphenamide [5]. In gastric vesicles a 50% inhibition of ATPase activity has been reported following reaction with 5–10 nmole omeprazole per mg protein [2, 4]. Furthermore, since reaction continues after 100% inhibition has been achieved, not all of these thiol groups are essential for the inactivation process.

The biological systems used to study the actions of omeprazole on acid secretion range from the whole animal, through isolated-cell preparations, to broken-cell membrane fragments. Whilst omeprazole can be shown to be an inhibitor of acid secretion at all these levels of organisation, this is no guarantee that an identical mechanism is the cause in each case, particularly since some of the broken-cell preparations allow omeprazole to interact with the pump under conditions quite different from those *in vivo*.

In particular, omeprazole-induced inhibition of ATPase activity in gastric vesicles has been performed under conditions likely to render the vesicle membrane permeable, such as after lyophilisation [2, 3] or with hypo-osmotic incubation [4]. Under such conditions, inhibition by omeprazole is favoured by incubation at moderately low pH and the omeprazole is presumably acid activated on both sides of the vesicle membrane. *In vivo*, under conditions of proton pumping, the intact membrane will exist in a highly asymmetric environment, with the cytosolic face at neutral pH and the luminal face exposed to conditions of high acidity. In this instance, omeprazole might be expected to be selectively activated on the luminal side of the membrane.

Intact gastric vesicles can be demonstrated to generate an asymmetric pH gradient under the appropriate conditions [3] and might therefore provide a broken-cell preparation more closely resembling conditions *in vivo*.

In this study, we have compared the amount of

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omeprazole required to produce inhibition of ATPase activity with the means by which the omeprazole was acid activated. The results suggest that omeprazole acts as a more specific inhibitor when activated within gastric vesicles that have generated a pH gradient by active proton pumping.

## METHODS

*Chemicals.* Omeprazole was synthesised by SK&F. Stock solutions (10–100 mM) were prepared in DMSO.*

[Benzimidazole-2-$^{14}$C]Omeprazole (50 mCi/mmole) was synthesised at SK&F [6], film dried and stored at −20°. For each set of experiments film dried material was taken up in DMSO and was stored for up to 1 month at −20°. Radiochemical purity was greater than 96%.

Ionophores and ATP (disodium salt) were obtained from Sigma Chemical Co (Poole, U.K.)

*Preparation of gastric vesicles.* Lyophilised gastric vesicles were prepared as described previously [2]. The same method was used to prepare intact gastric vesicles except that the material from the isolation medium/9% Ficoll interface of the discontinuous density gradient was mixed with an equal volume of 60% sucrose before being flash frozen and stored at −75°.

The specific activity of ATPase, stimulated by 10 mM KCl and 5 μg/ml nigericin was 2.1 ± 0.1 I.U./mg protein (SEM; N = 5 preparations) for lyophilised vesicles and 1.6 ± 0.3 I.U./mg (SEM; N = 5 preparations) for intact vesicles.

The proportion of broken or leaky vesicles in the intact vesicle preparation was assessed by measuring the effect of the K/H-ionophore nigericin (5 μg/ml) on the activation of ATPase activity by 10 mM KCl. Only 21% of the total potassium-stimulated ATPase activity was observed in the absence of nigericin indicating that almost 80% of the vesicles were intact.

*Determination of ATPase activity.* ATPase activity was determined as follows. In lyophilised vesicle preparations, enzyme sample (3 μg protein) was incubated in a volume of 1 ml containing 100 mM Pipes/Tris buffer pH 7.0, 10 mM KCl, 2 mM $MgCl_2$, 2 mM ATP and 5 μg nigericin. For intact vesicle preparations, enzyme sample (3 μg protein) was incubated in a volume of 0.75 ml containing 10–50 mM Pipes/Tris buffer pH 7.0, 300 mM sucrose, 2 mM $MgSO_4$, 10 mM KCl, 2 mM ATP and 5 μg nigericin (except where otherwise stated). After incubation at 37° for 15–17 min the inorganic phosphate released was determined by the method of Yoda and Hokin [7]. All activities were corrected for the amount of ATP hydrolysis occuring in the absence of enzyme sample.

*Determination of phosphoenzyme.* H/K ATPase preparations (100 μg protein/ml) were incubated at 20° in 200 μl of medium containing 80 mM Pipes/Tris buffer pH 7.4, 300 mM sucrose, 2 mM $MgCl_2$ and 5 μM [$^{32}$P]ATP. After 15 sec the reaction was terminated by adding 1 ml of ice-cold stopping solution (10% (w/v) perchloric acid, 5 mM non-labelled ATP and 40 mM $Na_2HPO_4$) and filtered on pre-soaked Whatman GF/B filters. Each filter was washed 5 times with 10 ml ice-cold washing solution (5% (w/v) perchloric acid and 10 mM $Na_2HPO_4$) and was transferred to a scintillation vial containing 10 ml Picofluor-15 scintillant. Radioactivity was counted in a Beckman LS1800 liquid scintillation counter. Phosphorylation levels were corrected for background filter-associated radio-activity, by subtracting the values obtained in the absence of enzyme sample. Background levels were less than 3% of maximum incorporation levels in the case of lyophilised vesicles and 20% of maximum incorporation levels in the case of intact pumping vesicles.

*Acid pretreatment of omeprazole.* For some experiments, 1–2 mM omeprazole was pretreated with acid by incubating in the presence of 0.1 M HCl for 15 min at 37°. The resulting orange solution was then added directly to gastric vesicle preparations containing sufficient buffer to neutralise the added acid.

*Incubation of gastric vesicles with omeprazole.* Radiolabelled omeprazole or acid-pretreated omeprazole (10 μM) was incubated with enzyme preparations as follows. Lyophilised vesicle preparations were incubated at 37° at a protein concentration of 30–150 μg/ml in 10–50 mM Pipes/Tris buffer, at pH 6.1 or 7.4. Intact vesicles were incubated at 20° and 60 μg protein/ml in medium containing 10 mM Pipes/Tris buffer pH 6.1 or 7.0, 300 mM sucrose or 150 mM KCl, 2 mM ATP, 2 mM $MgSO_4$ pH 6.1 or 7.0, and 10 μg/ml valinomycin (unless otherwise stated). At timed intervals, 50–100 μl samples were taken for the determination of ATPase activity and incorporation of [$^{14}$C]omeprazole.

*Incorporation of [$^{14}$C]omeprazole into gastric vesicle preparations.* For lyophilised gastric vesicles, aliquots (100–200 μl) of the incubated preparation were transferred to tubes containing an excess of non-radioactively labelled omeprazole (100 nmol) followed by 750 μl dimethyl sulphoxide. The presence of dimethyl sulphoxide was found to reduce the filter blanks obtained when acid-pretreated [$^{14}$C]omeprazole was used.

Each tube was rapidly vortexed and the contents were filtered on individual Whatman GF/B filters pre-soaked in 5% (w/v) perchloric acid. Each filter was washed 3 times with 15 ml of ice-cold 5% (w/v) perchloric acid, and transferred to a scintillation vial containing 10 ml Picofluor-15 scintillant. Radioactivity was counted in a Beckman LS1800 scintillation counter. All incorporation values were corrected for the amount of filter-associated radioactivity from parallel incubations in the absence of of enzyme and were expressed per mg vesicle protein.

In the case of intact vesicle preparations, it was found that the presence of dimethyl sulphoxide prior to filtration reduced the recovery of material by the filter. This was not the case for lyophilised preparations. Consequently, in experiments involving intact vesicles, the 750 μl dimethyl sulphoxide was replaced with distilled water.

*Protein.* Protein was determined by the method of Lowry [8].

* Abbreviations used: Pipes, piperazine; *N, N'*-bis[2-ethanesulphonic acid]; DMSO, dimethylsulphoxide.

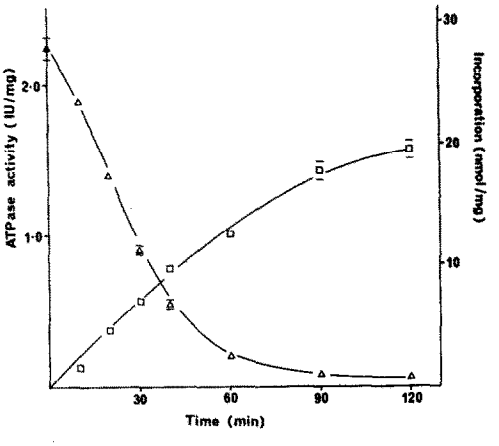


Fig. 2. Effects of [$^{14}$C]omeprazole on lyophilised gastric vesicles at pH 6.1. Lyophilised gastric vesicles (30 $\mu$g protein/ml) were incubated with 10 $\mu$M [$^{14}$C]omeprazole in 10 mM Pipes/Tris buffer pH 6.1 at 37° for the times indicated after which aliquots were taken for the determination ATPase activity (△) and incorporation (□) as described in the text. Values are means ± range from duplicate determinations.

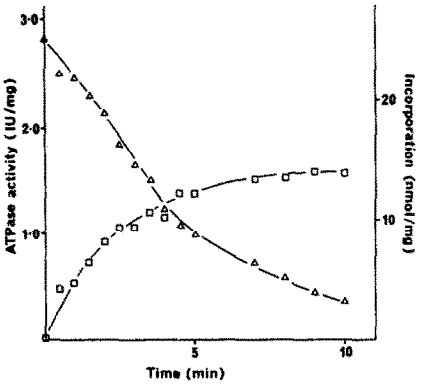


Fig. 3. Effects of acid-pretreated [$^{14}$C]omeprazole on lyophilised gastric vesicles at pH 6.1. Lyophilised gastric vesicles (30 $\mu$g protein/ml) were incubated with 10 $\mu$M acid-pretreated [$^{14}$C]omeprazole in 50 mM Pipes/Tris buffer pH 6.1 at 37° for the times indicated after which aliquots were taken for the determination of ATPase activity (△) and incorporation (□) as described in the text. Values are single determinations at each time point.

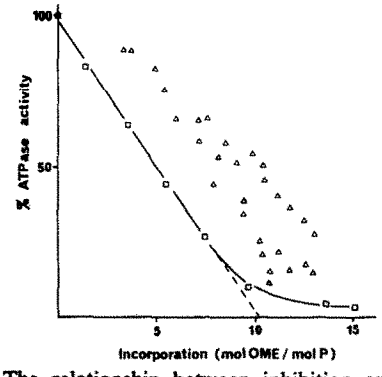


Fig. 4. The relationship between inhibition and incorporation in lyophilised gastric vesicles at pH 6.1 produced by [$^{14}$C]omeprazole (□) and acid-pretreated [$^{14}$C]omeprazole (△). Data from Fig. 2, pooled data from Fig. 3 and an identical experiment are shown. The amount of incorporation of omeprazole (OME) into the gastric vesicles has been corrected for the level of phosphorylation in that preparation and is expressed as moles of omeprazole per mole phosphorylation sites.

## RESULTS

The ($H^+ + K^+$)-ATPase becomes phosphorylated from ATP as part of its catalytic cycle. In the absence of potassium the rate of phosphorylation is 140 times faster than the rate of dephosphorylation and hence under these conditions the enzyme can be trapped in the phosphorylated form [9]. When the intact vesicle preparations were incubated with 5 $\mu$M [$^{32}$P]ATP for 15 sec phosphoenzyme formation was detected at a level of 1.02 ± 0.03 nmol/mg protein (SEM; N = 7) and 0.62 ± 0.08 nmol/mg protein (range; N = 2) for the two preparations used in this study. This level of phosphoenzyme was not increased by longer incubation times or by higher concentrations of ATP up to 20 sec and 12.5 $\mu$M respectively. Under the same conditions the level of phosphoenzyme in the lyophilised vesicle preparation was 1.3 nmol/mg protein. These levels of phosphoenzyme were taken to represent the number of phosphorylation sites present in these preparations and as such an index of the number of functional pumps.

Incubation of lyophilised gastric vesicles (30 $\mu$g protein/ml) with 10 $\mu$M [$^{14}$C]omeprazole at pH 6.1 resulted in a time dependent loss of ATPase activity with a corresponding incorporation of radiolabel into the preparation (Fig. 2). After 120 min more than 95% of the ATPase activity had been inhibited. During this time the level of incorporation increased steadily to almost 20 nmol/mg protein but did not reach a plateau. These data have been replotted in Fig. 4 to show the relationship between % inhibition of enzyme activity and level of incorporation per phosphorylation site. Complete inhibition of activity occurred at a stoichiometry of 10 moles per phosphorylation site. Pooled data from this and 3 similar experiments, in which the protein concentration ranged from 100 $\mu$g/ml to 150 $\mu$g/ml gave an average stoichiometry of 13 ± 3 (SEM; N = 4) moles per phosphorylation site. The results of a similar experiment using 10 $\mu$M acid-pretreated [$^{14}$C]omeprazole are shown in Fig. 3. Both inhibition of enzyme activity and incorporation occurred over a much shorter time course with plateau levels of incorporation (16 nmol/mg protein) being reached within 10 min. To produce a 50% inhibition of ATPase activity required approximately twice as much incorporation of acid pretreated omeprazole as non-pretreated omeprazole (Fig. 4).

The observation that acid pretreated omeprazole reached plateau levels of incorporation after 10 min suggested depletion of one or more components of the reaction. To investigate this further, lyophilised gastric vesicles (30 $\mu$g protein/ml) were incubated at pH 6.1 with 10 $\mu$M acid pretreated [$^{14}$C]omeprazole until stable plateau levels of incorporation were obtained. At this point an additional aliquot of either gastric vesicles or of freshly prepared acid-pretreated

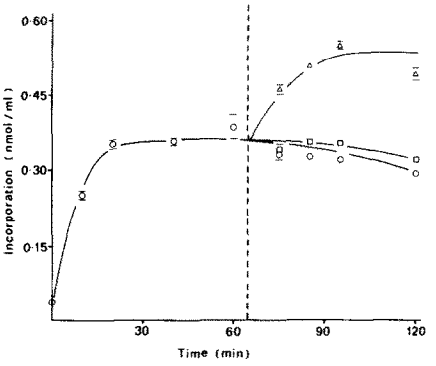


Fig. 5. Addition of further reactants to the incorporation of acid-pretreated [$^{14}$C]omeprazole into lyophilised gastric vesicles. Lyophilised gastric vesicles (30 $\mu$g protein/ml) were incubated with 10 $\mu$M [$^{14}$C]omeprazole at pH 6.1 until plateau incorporation levels were reached. At the time indicated by the dotted line further additions were made of either 10 $\mu$M freshly prepared acid-pretreated [$^{14}$C]omeprazole ($\triangle$) or 30 $\mu$g protein/ml lyophilised gastric vesicles ($\square$). The subsequent timecourses of incorporation were followed and compared with that when no further additions were made ($\bigcirc$). Values shown are means ± range of duplicate determinations.

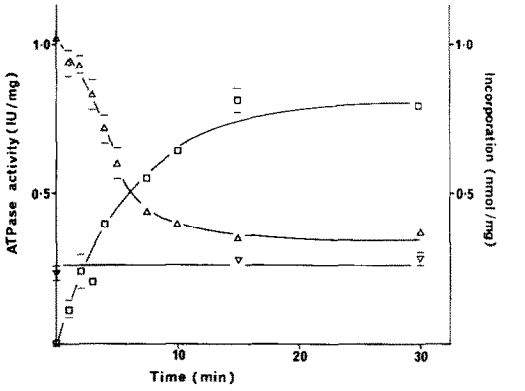


Fig. 6. Effect of [$^{14}$C]omeprazole on intact pumping gastric vesicles. Intact gastric vesicles (60 $\mu$g protein/ml) were incubated with 10 $\mu$M [$^{14}$C]omeprazole at room temperature under conditions shown to result in proton pumping (150 mM KCl, 9 $\mu$M valinomycin 2 mM Mg ATP and 10 mM Pipes/Tris buffer pH 7.0). At the times indicated 50 $\mu$l aliquots were taken for the determination of ATPase activity in the presence of nigericin ($\triangle$). Additional aliquots were taken for the determination of incorporation ($\square$). A parallel incubation was performed in the absence of valinomycin. At the times indicated 50 $\mu$l aliquots were taken for the determination of ATPase activity in the absence of nigericin ($\nabla$). Values shown are means ± range from duplicate determinations.

[$^{14}$C]omeprazole was added. The resulting time course of incorporation is shown in Fig. 5. It would appear therefore that the short time course observed for acid-pretreated omeprazole was due to the depletion, under these conditions, of the active species derived from omeprazole.

Incubation of intact gastric vesicles (60 $\mu$g protein/ml) at pH 6.1 with 10 $\mu$M [$^{14}$C]omeprazole produced a similar result to that obtained with lyophilised gastric vesicles. Total ATPase activity was reduced by 50% after approximately 50 min in association with a progressive increase in the level of incorporation. However, when intact gastric vesicles were incubated with 10 $\mu$M [$^{14}$C]omeprazole at pH 7.0 with 150 mM KCl, 9 $\mu$M valinomycin and 2 mM MgATP (conditions previously shown to support proton pumping by the aminopyrine accumulation technique, [3] and unpublished data this laboratory), a rapid inhibition of approximately 75% of the ionophore-stimulated ATPase activity was observed (Fig. 6). Higher concentrations of omeprazole produced complete inhibition of the ionophore-stimulated ATPase activity (data not shown). This inhibition was associated with a much lower degree of incorporation (Fig. 6). Extrapolation of the replotted data relating inhibition to incorporation confirmed this increase in the specificity of the inhibition process (Fig. 7). Analysis of three such experiments indicated that complete inhibition was produced at a stoichiometry of 1.8 ± 0.15 (SEM) moles of omeprazole per phosphorylation site.

No time-dependent inhibition of ATPase activity or incorporation was observed when intact vesicles were incubated at pH 7.0 with omeprazole under equivalent conditions but in the absence of valinomycin.

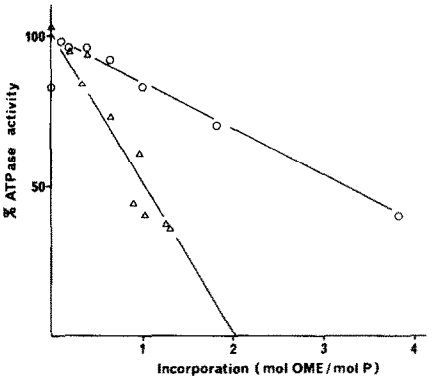


Fig. 7. The relationship between omeprazole-induced inhibition and incorporation in pumping ($\triangle$) and non-pumping ($\square$) intact gastric vesicles. The amount of incorporation of omeprazole (OME) has been corrected for the level of phosphorylation in that preparation and is expressed as moles of omeprazole per mole phosphorylation sites.

## DISCUSSION

*In vitro* studies on the inhibition of the gastric ($H^+ + K^+$)-ATPase by omeprazole are complicated by its apparent activation under conditions of acidic pH [2, 10]. We have therefore compared the actions of omeprazole in three model systems each of which produces acid activation in a different way. The interactions of omeprazole with the ($H^+ + K^+$)-ATPase proved to be highly dependent on the model used.

In the first model, omeprazole was activated at pH 6.1 in the presence of lyophilised gastric vesicles. Under these mildly acidic conditions omeprazole is relatively stable having a half-life of approx. 2.5 hr

(unpublished data). Compatible with this, both the inhibition of ATPase activity and the incorporation of radiolabel occurred progressively over a 60 min period. The degree of incorporation required to produce 100% inhibition of ATPase activity was in the range 10–15 nmol/mg protein, in agreement with our previous findings [2] and in reasonable agreement with those of others [4].

This stoichiometry can be related to the number of catalytic phosphorylation sites in each preparation and is equivalent to $13 \pm 3$ moles omeprazole per phosphorylation site. Assuming that each functional pump has one phosphorylation site and that complete phosphorylation of these sites was achieved in these experiments, then 100% inhibition of ATPase activity required the incorporation of 13 molecules of omeprazole per pump. Whilst this stoichiometry cannot be taken as exact, since it is not known to what extent omeprazole incorporated into other proteins in this preparation, it serves to make the point that the interaction of omeprazole with these membranes was not a selective process under these conditions.

The use of lyophilised gastric vesicles has certain disadvantages. Most notably, no information can be obtained on the side of the gastric vesicle membrane at which the omeprazole acts, since, due to lyophilisation, the permeability barrier between the cytosolic and luminal faces of the membrane will have been in part destroyed. Hence activation and incorporation of omeprazole may have occurred at both faces. These circumstances are unlikely to pertain *in vivo* for two reasons. The pH on the cytosolic side of the pump membrane is approximately neutral, which will not favour the activation of omeprazole. Secondly, since mercaptanes have been shown to protect the pump from inhibition by preferentially reacting with any activated omeprazole [4], the high concentrations of intracellular glutathione *in vivo* should prevent inhibition from the cytoplasmic side.

In the second model, omeprazole was activated in strong acid (pH 3) prior to incubation with gastric vesicles. Pre-treatment at this pH overcame the rate limiting activation of omeprazole at pH 6.1 and a more rapid time course of inhibition and incorporation was observed. However, the observation that incorporation ceased after less than 5% of the available radiolabel had been incorporated, but could be restarted by the addition of fresh acid-treated omeprazole, implied that the active product of omeprazole was not stable under these conditions.

The degree of incorporation required to produce 50% inhibition of ATPase activity using acid-pretreated omeprazole was approximately twice that in the absence of acid pretreatment, suggesting that the mechanism of inhibition was less specific in this case. Omeprazole has been proposed to inhibit the ATPase by the reaction of an acid-generated intermediate with essential thiol groups on the enzyme [4]. Indeed, reaction with a relatively small proportion of the total available thiol groups can produce complete inhibition [2, 4]. It remains possible that when the active product of omeprazole is present in relatively high concentrations, such as after acid pretreatment, reactions with non-essential thiols occur in greater proportion. Alternatively, the acid activation of omeprazole in the absence of enzyme might lead to the accumulation of different intermediates, which show altered specificity for the available thiol groups in this preparation. As in experiments performed without pretreatment of the omeprazole, the use of lyophilised vesicles would allow the activated omeprazole access to both faces of the membrane.

In the third model, intact gastric vesicles were incubated with omeprazole under conditions more closely resembling those in the stimulated gastric mucosa. The hydrolysis of ATP by intact vesicles in the presence of high concentrations of KCl and the ionophore valinomycin can be shown to result in the formation of large pH gradients as the interior of the vesicle becomes acidic ([3] and unpublished data, this laboratory). In intact pumping vesicles one might expect to observe a rapid activation of omeprazole confined to the vesicle interior. Indeed, the time course of inhibition of ATPase activity was rapid and comparable with that of acid-pretreated omeprazole. However, in contrast to the second model, this activation of omeprazole occurred in the presence of the enzyme.

The observation that omeprazole inhibited only the ionophore-stimulated ATPase activity supported the idea that inhibition by omeprazole could only occur in intact vesicles which were capable of establishing a pH gradient. Thus the acid-generated intermediate of omeprazole would have immediate access to the luminal face of the enzyme, but depending on its ability to cross biological membranes may not have access to the cytoplasmic face.

The inhibition by omeprazole in intact pumping vesicles was associated with lower levels of incorporation. Although 100% inhibition of enzyme activity could not be obtained because of the proportion of broken vesicles present, a linear extrapolation of replots as shown in Fig. 8 would indicate complete inhibition of ATPase activity required an incorporation stoichiometry of $1.8 \pm 0.15$ nmole omeprazole per phosphorylation site. This inhibition appears much more specific than that observed in non-pumping vesicles and may reflect the fact that in this model the activated omeprazole has access only to the luminal face of the pump.

In conclusion, the inhibition of the $(H^+ + K^+)$-ATPase by omeprazole in lyophilised vesicles is unlikely to resemble that *in vivo* where the cytosolic face of the enzyme is most probably protected from inhibition by both the neutral pH and by the high concentration of intracellular thiols. A model more closely resembling the situation *in vivo* is that of intact pumping vesicles where omeprazole is activated in the acidic luminal space. Under these conditions the specificity of omeprazole is greatly increased.

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