Omeprazole, a Specific Inhibitor of Gastric (H+-K+)-ATPase, Is a H+-activated Oxidizing Agent of Sulfhydryl Groups*

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Omeprazole ([5-methoxy-2-[[4-(methoxy-3,5-dimethylpyridinyl)methyl][sulfanyl]-1H-benzimidazole] appeared to inhibit gastric (H+-K+)-ATPase by oxidizing its essential sulfhydryl groups, since the gastric ATPase inactivated by the drug in vivo or in vitro recovered its K+ dependent ATP hydrolyzing activity upon incubation with mercaptoethanol. Biological reducing agents like cysteine or glutathione, however, were unable to reverse the inhibitory effect of omeprazole. Moreover, acidic environments enhanced the potency of omeprazole. For example, in vivo pretreatment of rats with carbachol, a secretagogue, enhanced the activity of omeprazole to inhibit gastric (H+-K+)-ATPase, while pretreatment with cimetidine, an antisecretory agent, reduced its potency. In vitro, lowering pH of incubation media from 7.4 to 5.0 improved the ability of omeprazole to inhibit hog gastric (H+-K+)-ATPase almost 60-fold. The inhibitory effect of the drug was accompanied by a dose-dependently decreased amount of free sulfhydryl groups in the isolated hog gastric membranes.

The chemical reactivity of omeprazole with mercaptans is also consistent with the biological action of omeprazole. The drug, only under acidic conditions, reacted with a stoichiometric amount of ethyl mercaptan to produce omeprazole-sulfide quantitatively.

The gastric polypeptides of 100 kilodaltons representing (H+-K+)-ATPase in the rat gastric mucosa or isolated hog gastric membranes were covalently labeled with [3H]omeprazole. The radioactive label bound to the ATPase, however, could not be displaced by mercaptoethanol under the identical conditions where the ATPase activity was fully restored. These observations suggest that the essential sulfhydryl groups which reacted with omeprazole did not form a stable covalent bond with the drug, but rather that they further reacted with adjacent sulfhydryl groups to form disulfides which could be reduced by mercaptoethanol.

Omeprazole, a substituted benzimidazole, is a highly specific inhibitor of gastric (H+-K+)-ATPase (1, 2). Its gastric antisecretory activity in vivo has been ascribed to inhibition of the ATPase which represents the terminal step of the acid secretory process (3-8). The biochemical mechanism for the drug action, however, has not been reported (2). During preliminary experiments, we have observed that omeprazole is no longer inhibitory to the ATPase in the hog or rat gastric microsomal membranes which had been prepared in the presence of dithiothreitol (1 mM). This observation prompted us to explore possible interactions of the drug with sulfhydryl groups. In this report, we will present evidence that omeprazole is a H+-activated oxidizing agent of sulfhydryl groups.

METHODS AND MATERIALS1

RESULTS

The effect of pH on the inhibitory action of omeprazole on gastric (H+-K+)-ATPase was examined on isolated hog gastric membranes which were rendered permeable to ions (Fig. 1). Omeprazole, when incubated with the gastric membranes in a Tris acetate buffer, pH 5.0, for 30 min, dose-dependently blocked membrane (H+-K+)-ATPase activity with an apparent K; value of 6 μM. When the pH of the buffer was raised to 7.4, the K; increases to 200 μM. Clearly acidic environments enhanced the inhibitory action of omeprazole. As further shown in Fig. 1, the ATPase inhibitory activity of omeprazole was accompanied by a dose-dependent reduction of free sulfhydryl groups in the gastric membranes. However, adding 2 mM dithiothreitol (or 4 mM mercaptoethanol) to the media completely nullified the inhibitory effect of omeprazole regardless of the pH of the media.

We have also examined the effect of omeprazole on H+ accumulation in isolated hog gastric membrane vesicles. Typically, the distribution ratio of [14C]aminopyrine, an index used for estimating the degree of intravesicular acidification, varied only from 1500 to 1200 over the incubation period from 5 to 30 min in the presence of ATP, KCl, and valinomycin. As shown in Fig. 2, the ability of omeprazole to dissipate a pH gradient across the gastric membranes intensified as a function of incubation time; apparent K; values were 15.0, 3.0, 1.5, and 0.5 μM at the incubation times of 5, 10, 20, and 30 min, respectively. These time-dependent changes in the po...

1 Portions of this paper (including "Methods and Materials," part of "Results," Table I, Figs. 1-8 and 10, and Schemes 1 and 2) are presented in miniprint at the end of this paper. The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethylether)-N,N',N'-tetraacetic acid; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 8650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-2789, cite the authors, and include a check or money order for $6.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
tency of omeprazole could be due to either a progressive inhibition of (H+—K+)-ATPase or a time-dependent interference with aminopyrine accumulation by omeprazole as a competing weak base of pKₐ 4.0 (19). The aminopyrine movement is not likely to be subject to interference by omeprazole, however, since omeprazole sulfide, a similar weak base but not a potent inhibitor of (H+—K+)-ATPase, had no effect on the intravesicular acidification at the micromolar doses. Furthermore, the low Kᵢ values of omeprazole suggest that the compound interacted with the ATPase under acidic environments of the intravesicular space, where the luminal side of the ATPase is accessible in these inside-out gastric membrane vesicles (20, 21).

Omeprazole, when administered subcutaneously in the rat, dose-dependently reduced (H+—K+)-ATPase activity in the gastric mucosa (Fig. 3). The dose to inhibit 50% of the ATPase activity as obtained 3 h after dosing was 1.5 ± 0.4 mg/kg. However, when rats were induced to a resting state of acid secretion with cimetidine treatment 30 min prior to the dosing of omeprazole, then the drug even at 10 mg/kg blocked only 20% of the ATPase activity in the gastric mucosa. With the rats stimulated to secrete acid by carbachol treatment, on the other hand, the dose of omeprazole to inhibit 50% of the ATPase activity was less than 1 mg/kg. These observations are consistent with the above studies in vitro showing acid activation of omeprazole.

We have studied the effect of sulfhydryl reducing agents on gastric (H+—K+)-ATPase inactivated by omeprazole in vivo or in vitro. For instance, the gastric microsomes which were prepared from the rats treated with omeprazole (5 mg/kg) lost 70% of their (H+—K+)-ATPase activity as compared to those from the untreated rats. Upon incubation with β-mercaptoethanol, the omeprazole-treated microsomes recovered their ATPase activity as a function of incubation time (Fig. 4) and of concentration of the reducing agent (Fig. 5). With a saturating dose of mercaptoethanol (200 mM), it took about 45 min of incubation time to recover half of the lost ATPase activity. With a fixed incubation time of 3 h, half-maximal activation occurred at 25 mM mercaptoethanol. Dithiothreitol was as effective as mercaptoethanol in activating the omeprazole-inhibited ATPase activity, while cysteine and reduced glutathione were not effective. Similar studies were carried out with intact isolated hog gastric membrane vesicles incubated with omeprazole (50 μM) under the conditions developed by us (23). More than 70% of (H+—K+)-ATPase activity was lost during incubation for 30 min. Again, the (H+—K+)-ATPase activity was restored by mercaptoethanol (Fig. 6) with similar kinetics as in vivo. Cysteine and glutathione were not effective (data not shown).

[14C]Omeprazole was used in order to study labeling patterns of gastric (H+—K+)-ATPase in vivo or in vitro. The doses of the labeled drug, 1.5 mg/kg in the rat and 5 × 10⁻⁵ M in intact isolated hog gastric membrane vesicles developing a pH gradient, were sufficient to produce 50–70% inhibition of (H+—K+)-ATPase activity. Table I shows the amounts of ¹⁴C radioactivity tightly bound to the hog and rat gastric membranes, that is, the radioactivity in the membranes after freeze-thawing several times and filtration over a Millipore filter (HAWP, 0.45 μm). The specific ¹⁴C activity associated with the hog membranes as labeled in vivo (35,800 cpm/mg of protein) was much higher than that of the rat membranes labeled in vivo (13,500 cpm/mg of protein). It would be expected, however, that the in vitro ¹⁴C labeling involved higher levels of nonspecific binding of the drugs. Table I also shows that the amounts of ¹⁴C radioactivity associated with the hog and rat membranes were not significantly affected by incubation of the membranes with 200 mM mercaptoethanol for 3 h.

The ¹⁴C-labeled membranes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. Then autoradiographs of the gels were obtained (Fig. 7). With the hog membranes, we have detected the incorporation of ¹⁴C radioactivity into the polypeptides of 100 kilodaltons corresponding to (H+—K+)-ATPase. Addition of excess nonradioactive omeprazole during the in vitro incubation only marginally reduced the intensity of radiation associated with the ATPase. It should be noted, however, that a majority of the membrane-bound radioactivity was located ahead of the dye front, clearly representing nonspecific binding of the labeled omeprazole to the membranes. In the rat membranes labeled in vivo, we have barely detected the incorporation of ¹⁴C radioactivity into the polypeptides of 100 kilodaltons. No noticeable radioactivity was detected in the lower molecular mass regions of the gels.

The chemical reactivity of omeprazole with β-mercaptoethanol was examined in the presence or absence of 2 molar equivalents of HCl (Fig. 8). Omeprazole only under acidic conditions diminished free sulfhydryl groups with a t₅₀ of 7 min at 22 °C. Analysis of the reaction mixtures by thin-layer chromatography (TLC) indicated conversion of omeprazole to a new compound (Compound I); TLC RF values (chloroform/methanol, 5:1) were 0.47 and 0.73 for Compound I and omeprazole, respectively. The amount of Compound I formed was inversely proportional to the level of free sulfhydryl groups remaining in the reaction mixture; at an incubation time of 40 min, omeprazole was almost quantitatively transformed to Compound I. The reaction occurred with ethyl and benzyl mercaptans as well to give related products. Timoprazole, the unsubstituted parent compound of omeprazole, also reacted in the same manner with the various mercaptans, although at somewhat slower rates. On the other hand, the N-methyl analog of timoprazole (1-methyl-2-[2-pyridinylmethylsulfinyl]benzimidazole) failed to react under the identical reaction conditions and could be recovered unchanged.

Fig. 9 shows the 200-MHz ¹H NMR spectrum of Compound IA. Compound IA differs from Compound I in that ethyl mercapatan was employed in place of β-mercaptoethanol. With the exception of a singlet which appeared at δ 4.90 (S-OCH₃), the remaining ¹H resonances of Compound IA appeared as double lines in the 200-MHz spectrum. This notable feature in line positions suggested Compound IA was a mixture of two closely related regio-isomers. The appearance of a triplet at δ 1.02 and a quartet at δ 2.30 clearly indicated the incorporation of an ethyl mercaptaryl group into the omeprazole structure. Interestingly, the methylene hydrogens at δ 4.90 were exchangeable as evident by the addition of D₂O. Under the same conditions, the methylene hydrogens of omeprazole or the sulfide of omeprazole did not exhibit this behavior.

The infrared spectrum of Compound IA showed the absence of strong absorption bands at 1070–1030 cm⁻¹, indicating the loss of the sulfoxide oxygen. The mass spectrum (fast atom bombardment) of Compound IA showed (M + H) as double lines in the 200-MHz spectrum. This notable feature in line positions suggested Compound IA was a mixture of two closely related regio-isomers. The appearance of a triplet at δ 1.02 and a quartet at δ 2.30 clearly indicated the incorporation of an ethyl mercaptaryl group into the omeprazole structure. Interestingly, the methylene hydrogens at δ 4.90 were exchangeable as evident by the addition of D₂O. Under the same conditions, the methylene hydrogens of omeprazole or the sulfide of omeprazole did not exhibit this behavior.

Three sites of the benzimidazole moiety of omeprazole can be considered for substitution with the mercaptetyl group, N-1, C-2, and N-3. At the present time, we believe Compound IA is a mixture of N-1 and N-3 sulfonylated compounds. The exchangeability of the methylene hydrogens can be rational-
The spectrum was obtained using the spectrum of Compound IA in CDCl₃. Varian XL-200 (200 MHz) NMR Spectrophotometer.

Timoprazole, picoprazole, and omeprazole, which are all substituted benzimidazoles, are known as inhibitors of gastric (H⁺-K⁺)-ATPase and clinically effective antisecretory agents (1-8, 19, 21-23). Wallmark et al. (2) have reported that low pH facilitated the inhibitory activity of picoprazole on gastric (H⁺-K⁺)-ATPase. It has not been determined, however, whether the pH effect was due to a change in the reactivity of the inhibitor or to the protein. In this study, we have presented several lines of biochemical evidence that omeprazole acted as an oxidizing agent of sulfhydryl groups under acidic conditions, for example, the reversibility of the omeprazole effect by mercaptoethanol, the facile oxidation of acidic conditions, for example, the reversibility of the omeprazole sulfide was postulated on the basis of 1) the UV spectral shift of omeprazole from 300 to 357 nm under acidic conditions, and 2) the disappearance of the absorption peak at 357 nm upon stoichiometric addition of mercaptans and its conversion to Compound IA. Compound IA is the only product formed under acidic conditions, and this pathway probably represents the mode of interaction of omeprazole with essential sulfhydryl groups of gastric (H⁺-K⁺)-ATPase. Furthermore, the N-methyl analog of timoprazole had no effect on rat gastric (H⁺-K⁺)-ATPase in vivo. This underscores the importance of the benzimidazole nitrogen in interaction with the enzyme. In the literature (24, 25), N-sulfenylated benzimidazoles have been prepared previously by reaction of 2-benzylthiobenzimidazoles with electron-deficient reagents, e.g. CCl₃SH and CF₃SH. It should be noted, however, that Compound IA cannot be formed from omeprazole sulfide under our experimental conditions. The HCl salt of Compound IA was very stable in aqueous media. Its free base, on the other hand, was stable in CHCl₃ but decomposed fairly rapidly to unknown products in aqueous environments. Apparently, essential sulfhydryl groups of gastric (H⁺-K⁺)-ATPase which react with omeprazole are not likely exposed directly to aqueous environments, since polar reducing agents like cysteine and glutathione could not interact with them. Nevertheless, the probable N-sulfenylated complex between omeprazole and essential —SH groups of the ATPase did not appear to survive neutral conditions employed during isolation of gastric microsomes, since we could not detect any displacement of the ¹⁴C label bound to the ATPase in vivo by mercaptoethanol under the conditions where the ATP hydrolyzing activity of the enzyme was fully restored. Certainly, the specific activity of [¹⁴C]omeprazole (35 mCi/mmol) appeared to be sufficiently high enough to detect any such changes. For example, if we assume that (H⁺-K⁺)-ATPase represents at least 2.5% of the microsomal proteins, a considerable underestimation judging from the intensity of 100-kDa protein in the sodium dodecyl sulfate-gel pattern (26), one molecule of [¹⁴C]omeprazole bound to a 100-kDa unit of (H⁺-K⁺)-ATPase would be equivalent to 14,000 cpm/mg of the microsomal protein. Two possibilities may be cited: 1) the ¹⁴C label bound to the ATPase rather represents the interaction of omeprazole with other than essential sulfhydryl groups via mercaptoethanol-insensitive

**DISCUSSION**

Chemically, omeprazole undergoes two different types of reactions with mercaptans depending on the state of protonation of the drug. A pathway shown in Scheme 2 is proposed for the reaction of the free base of omeprazole in the presence of a 20-fold excess of ethyl mercaptan. Isolation and identification of Compounds II and III support the scheme. This reaction pathway is only noticeable in the presence of excess sulfhydryl groups under neutral conditions. This route, therefore, is not likely to represent the major mode of reaction of omeprazole with essential sulfhydryl groups of gastric (H⁺-K⁺)-ATPase. However, this reaction is probably responsible for abolishing the inhibitory effect of omeprazole by excess dithiothreitol (or mercaptoethanol) when incubated together, since a mixture of Compounds II and III is not inhibitory to (H⁺-K⁺)-ATPase.

Scheme 1 shows a plausible pathway for the reaction of omeprazole with mercaptans under acidic conditions. Formation of an extensively conjugated sulfonium salt of omeprazole sulfide was postulated on the basis of 1) the UV spectral shift of omeprazole from 300 to 357 nm under acidic conditions, and 2) the disappearance of the absorption peak at 357 nm upon stoichiometric addition of mercaptans and its conversion to Compound IA. Compound IA is the only product formed under acidic conditions, and this pathway probably represents the mode of interaction of omeprazole with essential sulfhydryl groups of gastric (H⁺-K⁺)-ATPase. Furthermore, the N-methyl analog of timoprazole had no effect on rat gastric (H⁺-K⁺)-ATPase in vivo. This underscores the importance of the benzimidazole nitrogen in interaction with the enzyme. In the literature (24, 25), N-sulfenylated benzimidazoles have been prepared previously by reaction of 2-benzylthiobenzimidazoles with electron-deficient reagents, e.g. CCl₃SH and CF₃SH. It should be noted, however, that Compound IA cannot be formed from omeprazole sulfide under our experimental conditions. The HCl salt of Compound IA was very stable in aqueous media. Its free base, on the other hand, was stable in CHCl₃ but decomposed fairly rapidly to unknown products in aqueous environments. Apparently, essential sulfhydryl groups of gastric (H⁺-K⁺)-ATPase which react with omeprazole are not likely exposed directly to aqueous environments, since polar reducing agents like cysteine and glutathione could not interact with them. Nevertheless, the probable N-sulfenylated complex between omeprazole and essential —SH groups of the ATPase did not appear to survive neutral conditions employed during isolation of gastric microsomes, since we could not detect any displacement of the ¹⁴C label bound to the ATPase in vivo by mercaptoethanol under the conditions where the ATP hydrolyzing activity of the enzyme was fully restored. Certainly, the specific activity of [¹⁴C]omeprazole (35 mCi/mmol) appeared to be sufficiently high enough to detect any such changes. For example, if we assume that (H⁺-K⁺)-ATPase represents at least 2.5% of the microsomal proteins, a considerable underestimation judging from the intensity of 100-kDa protein in the sodium dodecyl sulfate-gel pattern (26), one molecule of [¹⁴C]omeprazole bound to a 100-kDa unit of (H⁺-K⁺)-ATPase would be equivalent to 14,000 cpm/mg of the microsomal protein. Two possibilities may be cited: 1) the ¹⁴C label bound to the ATPase rather represents the interaction of omeprazole with other than essential sulfhydryl groups via mercaptoethanol-insensitive
Omeprazole, a Specific Inhibitor of Gastric (H⁺-K⁺)-ATPase

covalent bond, and 2) essential sulfhydryl groups which probably reacted with omeprazole via N-sulfenylation may further react with adjacent —SH groups to form disulfide bonds.

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Omeprazole, a Specific Inhibitor of Gastric (H\(^{+}\)-K\(^{+}\))-ATPase

**METHODS AND MATERIALS**

Preparation of HCl Gastric Membranes Enriched in (H\(^{+}\)-K\(^{+}\))-ATPase

The mucosal slippings from hog stomachs were suspended in about 10 vol. Buffer (250 ml, HCl buffered to pH 1.2, 2 M), and were homogenized with 30 strokes of a motor-driven (1,500 rpm) Teflon pestle in a Potter-Elvehjem homogenizer. The membrane slurries were collected by centrifugation at 2,000 g for 30 min. The membrane pellets were washed twice by centrifugation at 2,000 g for 30 min.

**Tissue Preparation**

To render the membrane vesicles freely permeable to cations, the gastric membranes were either lyophilized (10 g) or homogenized in 3 m with a Sonnle Dimin Mixer and freeze-thawed twice in the absence of glycerol.

**Treatment of Rats and Preparation of Rat Gastric Membrane Vesicles**

Rats were killed by a blow on the head, and the stomachs were removed and placed in ice-cold HCl buffer (pH 1.2, 2 M) within 1-2 min. The stomachs were washed twice by centrifugation at 2,000 g for 30 min.

**Labeling Gastric (H\(^{+}\)-K\(^{+}\))-ATPase with [14C]-Omeprazole**

[14C]-Omeprazole (2-methyl-3-(1H)-indolyl-3-methylsulfonimidoyl)-4-phenylbutyrate) was used to label gastric (H\(^{+}\)-K\(^{+}\))-ATPase (1). After homogenization, the gastric membrane vesicles were incubated with [14C]-omeprazole (80 nmol/mg protein) for 5 min. The membrane vesicles were recovered by centrifugation at 2,000 g for 30 min at 4°C. The membrane-bound [14C]-omeprazole was subsequently washed with 5 mM Mg\(^{2+}\)-ATP, 10 mM KCl, and 150 mM KCI. The membrane vesicles were washed twice by centrifugation at 2,000 g for 30 min at 4°C. The membrane vesicles were used for subsequent experiments.

**Effect of Reducing Agents on (H\(^{+}\)-K\(^{+}\))-ATPase Inhibited by Omeprazole**

Various sulfhydryl compounds were tested for their ability to reverse the inhibitory effect of omeprazole on the ATPase. In all cases, the gastric membranes were treated with 5 mM ASC at pH 7.4. The membrane vesicles were then incubated for 5 min at 37°C in the presence of 100 mM KCl. The membrane vesicles were then washed twice by centrifugation at 2,000 g for 30 min at 4°C.

**Acid Preparations**

(H\(^{+}\)-K\(^{+}\))-ATPase activity in various gastric membranes was determined in 1 ml incubation media containing 40 mM tris-acetate buffer (pH 7.0), 2 mM Mg\(^{2+}\)-ATP, and 50 mM KCl. After 5 min of incubation, the reaction was terminated with 5% trichloroacetic acid, and the membrane vesicles were recovered by centrifugation at 2,000 g for 30 min. The membrane vesicles were washed twice by centrifugation at 2,000 g for 30 min at 4°C. The membrane vesicles were used for subsequent experiments.

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Omeprazole, a Specific Inhibitor of Gastric (H⁺-K⁺)-ATPase

Fig. 3. Effect of cimetidine- or carbachol-pretreatment on the potency of omeprazole to inhibit gastric (H⁺-K⁺)-ATPase in vivo in rats. The rats were given a saturating dose of cimetidine (100 mg/kg) or carbachol (150 µg/kg) 30 min before subcutaneous injection of omeprazole at the indicated doses. The rats were sacrificed 3 hr after dosing and the gastric mucosal microsomes were analyzed for (H⁺-K⁺)-ATPase activity. The data represent average values of three determinations. Experimental errors observed with several experiments were less than 25%.

Fig. 4. Mercaptoethanol-induced recovery of (H⁺-K⁺)-ATPase activity in the rat gastric membrane from the omeprazole treated animals. The gastric mucosal membranes were prepared from rats treated with omeprazole (15 mg/kg) or water; they were incubated with mercaptoethanol at a final concentration of 100 mM (open symbols) or 500 mM (closed symbols) for 3 hr. After incubation for an indicated time period, an aliquot of the mixtures was analyzed for (H⁺-K⁺)-ATPase activity as described before (6). Each point represents an average of triplicate measurements.

Fig. 5. Comparison of various sulphydryl compounds for their ability to reverse the ATPase-inhibitory effect of omeprazole. The gastric mucosal membranes were prepared from the rats treated with omeprazole (10 mg/kg) and were incubated with mercaptoethanol (8), dithiothreitol (D), cysteine (C) or reduced glutathione (G) at 4°C for 3 hr. Their concentrations were varied from 2 to 100 mM

Fig. 6. Mercaptoethanol-induced restoration of (H⁺-K⁺)-ATPase activity in isolated hog gastric membranes treated in advance with omeprazole. The isolated hog membranes enriched in (H⁺-K⁺)-ATPase were incubated with (0) or without (x) omeprazole, 5x10⁻⁷ M at 4°C for 30 min. The incubation media also contained 150 mM KCl, 5 mM MgCl₂, ATP 10 µg valinomycin. Then the membranes were washed free of omeprazole and incubated with (closed symbols) or without (open symbols) 200 mM mercaptoethanol at 4°C for 3 hr.

Table 1: Effect of mercaptoethanol on rat or hog gastric membrane-bound [¹⁴C] omeprazole. Isolated hog gastric membranes enriched in (H⁺-K⁺)-ATPase were labeled with [¹⁴C] omeprazole (final H⁺-K⁺)-ATPase activity ATPase activity. The isolated rat gastric membranes were prepared from the animals treated with omeprazole and incubated with mercaptoethanol at a final concentration of 100 mM (open symbols) or 500 mM (closed symbols) as described under Methods and Materials. The total [¹⁴C] radioactivity associated with the hog gastric membranes was 98,000 cpm/mg protein for the hog gastric membranes and 23,000 cpm/mg protein for the rat membranes. Then the membranes were frozen, thawed several times using liquid nitrogen and filtered over a Millipore filter (HA WP 04 µm) to determine [¹⁴C] radioactivity tightly bound to the gastric membranes. The membranes after free-thawing were incubated for 3 hr in the presence of 200 mM mercaptoethanol at 4°C.

|  | in vitro labeling | in vivo labeling |
|---|---|---|
| Hog gastric membranes | 35,800 ± 2,800 | 29,800 ± 1,300 |
| Rat gastric membranes | 13,500 ± 100 | 13,900 ± 680 |

Fig. 7. Autoradiograph of [¹⁴C] omeprazole labeled polypeptides in rat or hog gastric membranes. Isolated hog gastric membranes reacted with [¹⁴C] omeprazole at 1x10⁻⁷ M (A), 5x10⁻⁸ M (B) and/or 2 mM nonradioactive omeprazole (C). Two similar preparations of the rat mucosae (D and E) were obtained from the animals treated with [¹⁴C] omeprazole (1.5 mg/kg). 20 µg of the membrane proteins were applied to 12% SDS lab polycrylamide gel and subjected to electrophoresis and analyzed as described in Methods.
Fig. 8. Chemical reactivity of omeprazole with 8-mercaptoethanol in the presence or absence of HCl. Typically, 10 mmoles of mercaptoethanol were added to 10 ml of H2O containing 10% ethanol. Aliquots (1 ml) were taken at room temperature. Mercaptoethanol alone (B) or with omeprazole (A, A) in the presence (B, a) or absence (B, 0) of two molar equivalents of HCl. At an indicated time, an aliquot of 1 ml was taken from the mixture and was analyzed for free sulfhydryl group as described under "Methods and Materials."

Chemical Reactions of Omeprazole with Sulfhydryl Groups

A mechanism for the formation of Compound 1A is presented in Scheme 1. Attachment of the mercapto group at C-2 to give the iodosubstituted ring system 3 is another possible structure, but is unfavored in light of the following observations: first, replacement of the ethyl mercapto group with benzylic mercapto in the reaction with omeprazole gave a single product. Addition of DCI to the H-NMR sample only resulted in loss of the S-CH2 signal while the S-CH2 signal remained intact. If the S-CH2 group was attached to the same C-2 carbon, as shown in 1B, R = CH2Ph, and exchange occurred via the previously discussed pathway, one should have also observed loss of the S-CH2 hydrogen following D2O addition. Second, the structural assignment of Compound 1A was consistent with its UV spectrum which closely resembled the spectrum of the starting material. Although it cannot be excluded, the presence of an extra absorption maximum at 357 nm in the UV spectrum of Compound 1A (Fig. 9) showed double line absorption. This observation best explained by assignment of Compound 1A as a mixture of N-1 and N-3 sulfenylated compounds.

Acidic conditions and mercaptans also drastically affected the U V spectrum of omeprazole (Fig. 10). The free base of the drug showed two absorption maxima at 300 and 275 nm, which were shifted to 272 and 271 nm in the presence of 0.1 N HCl. Addition of an equimolar concentration of 8-mercaptoethanol instantaneously brought about the disappearance of the absorption maximum at 317 nm and the formation of a new, broad peak at 285 nm. Analysis of the reaction mixture on TLC indicated a complete conversion of omeprazole to Compound 1A.

As shown in Fig. 8, the free base of omeprazole was not very reactive with mercaptans. However, in the presence of an excess amount of mercaptans, e.g., 20 molar equivalents of ethylmercaptan, omeprazole was converted to two compounds, which were identified as Compounds II and III, on the basis of their NMR, infrared and mass spectra. The structure of Compound II was firmly established by comparison to an authentic sample prepared by independent chemical synthesis.

![Diagram](attachment:image1.png)

Fig. 10. Absorption spectral changes of omeprazole. The free base of omeprazole was dissolved in H2O (0.1 N HCl) (---) to a final concentration of 70 μM. The spectra was taken 30 min after mixing. Then, the acidic medium containing omeprazole was mixed with an equimolar concentration of 8-mercaptoethanol and the spectrum (•) was taken immediately.