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# INHIBITION OF GASTRIC $K^+$ATPase BY PHENYLBUTAZONE AND INDOMETHACIN*

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**Abstract**—Inhibition of gastric $H^+$ secretion by phenylbutazone and indomethacin was investigated by examining the effects of these agents on a putative $H^+$ transport enzyme, a $K^+$ stimulated ATPase, unique to gastric mucosa. Phenylbutazone and indomethacin were found to inhibit both the $K^+$ATPase and the $K^+$pNPPase. $K_i$s were 430 $\mu$M and 710 $\mu$M for the $K^+$ATPase and 330 $\mu$M and 670 $\mu$M for the $K^+$pNPPase for phenylbutazone and indomethacin respectively. Inhibition was not reversed by $Mg^{2+}$, ATP, pNPP, or KCl and obeyed non-competitive kinetics. Inhibition of the pNPPase suggested that the mechanism of inhibition involved the $K^+$ sensitive dephosphorylation of the phosphoenzyme. In the presence of 500 $\mu$M phenylbutazone dephosphorylation was significantly less at 3, 5, 7.5, 10 and 15 sec following KCl addition. These studies provide an alternate mechanism for inhibition of gastric $H^+$ secretion by phenylbutazone and indomethacin.

Pharmacologic agents which produce gastric mucosal injury and ulceration have also been shown to reduce $H^+$ secretion. The mechanism of reduced $H^+$ secretion could involve (1) inhibition of the $H^+$ transport enzyme, (2) inhibition of mucosal metabolism required to support $H^+$ secretion, or (3) loss of $H^+$ from the lumen by "back diffusion" through a mucosa whose permeability properties have been changed by exposure to the pharmacologic agent. The last mechanism is most frequently invoked [1, 2].

The investigations reported here were designed to determine if phenylbutazone and indomethacin affect the activity of the gastric $K^+$ATPase.

## MATERIALS AND METHODS

All chemicals used in these studies were reagent grade or the highest purity available. Disodium ATP, tris para-nitrophenylphosphate and Ficoll were purchased from Sigma. [$\gamma^{32}$P]ATP (19.4 Ci/mM), was purchased from New England Nuclear.

*Preparation of gastric cell membranes*

The $K^+$ATPase is present in both amphibian and mammalian gastric mucosa. Because of availability of mucosae and production of large quantities of membranes, hog gastric fundus was used as the source of the ATPase.

Gastric cell membranes were prepared from hog gastric fundus obtained at a local abbatoir. Stomachs were opened, washed with cold water and packed in ice for transport to the laboratory. The fractionation procedure was similar to the method reported for isolation of canine gastric cell membranes [3]. Briefly, the mucosa was scraped from the underlying muscle with glass microscope slides, suspended in 0.25 M sucrose—20 mM Tris HCl, pH 7.4, minced with scissors, and homogenized for 60 sec in a Waring blender followed by 10 strokes with a loose fitting Teflon pestle at 1500 rev/min. The homogenate was filtered through 2 layers of cheese cloth and fractionated by differential centrifugation at 1000 $g$ × 30 min, 8000 $g$ × 20 min, and 20,000 $g$ × 30 min in a RC-5 refrigerated superspeed centrifuge (Dupont–Sorvall). The final supernate was centrifuged in a No. 30 rotor (Beckman Inst.) at 27,000 rpm for 60 min; the $g$ force at $r_{ave}$ is 68,000. The pellets were resuspended in 0.25 M sucrose—20 mM Tris–HCl, pH 7.4 and injected into the center of a Ti-14 zonal rotor (Beckman Inst.) containing a linear density gradient made from 0.25 M sucrose—7.5% (w/w) Ficoll in 20 mM Tris-Cl, pH 7.4 and 37% (w/w) sucrose in 20 mM Tris, pH 7.4. Fractionation was for 5 hr at 48,000 rev/min in a Beckman L2-65 ultracentrifuge. The gradient was subsequently collected in 20 ml fractions by displacement with 60% sucrose injected at the periphery of the rotor. The membrane peaks were pooled, aliquoted, and stored in the sucrose solution at −20°. In this sucrose solution the enzymatic activity was stable for several months. Each week an aliquot of membranes was diluted to 8–9% sucrose and collected by centrifugation at 27,000 rev/min in a No. 30 rotor. The membranes were resuspended at a concentration of 103 $\mu$g/ml in the buffer used in the assays to be performed. This membrane suspension was aliquoted into tubes and frozen at −20° for daily use. The enzymatic activity declined slightly during 5 days when stored in this manner.

*ATPase assays* were performed at pH 7.0 in a vol. of 1 ml containing 50 $\mu$moles MES-HEPES-Tris buffer, 2 $\mu$moles disodium ATP, 2 $\mu$moles $MgCl_2$, and 10.3 $\mu$g membrane protein with or without 25 $\mu$moles KCl unless otherwise stated. The mixture was incubated for 20 min at 37°, and the reaction was terminated by addition of 1 ml of a solution prepared from 60% perchloric acid and 4.5% ammonium molybdate (1:4, v/v). The phosphomolybdic acid was extracted into 3 ml butyl acetate by vortexing, and

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the mixture was centrifuged to separate the phases. The 320 nm absorbance of the butyl acetate layer was measured in a Gilford 2400 spectrophometer [4].

*Para-nitrophenylphosphatase assays* were performed at pH 7.5 in an incubation mixture similar to that used for ATPase except that 6 $\mu$moles *p*-nitrophenylphosphate (pNPP) replaced the ATP and 6 $\mu$moles $MgCl_2$ replaced the lower concentration used in ATPase assays. The assay was incubated for 20 min at 37° and terminated with 1 ml of cold 1 M NaOH. The protein and $Mg(OH)_2$ were sedimented at 1000 $g$ × 10 min, and the 405 nm absorbance of the supernate was measured.

*Phosphorylation of $K^+$ATPase*

Phosphorylation assays were performed in a vol. of 200 $\mu$l containing 2 nmoles ATP, [$\gamma$-$^{32}$P]ATP (0.5 $\mu$Ci), 2 nmoles $MgCl_2$, 20 $\mu$moles of MES-HEPES-Tris buffer, pH 7.0, 100 nmoles phenylbutazone and 40 nmoles KCl were included where appropriate. Approximately 40 $\mu$g protein were used in these studies. The assays were performed at 37°. Time intervals are indicated in the individual experiments. The reaction was terminated with 5 ml of ice cold 5% TCA containing 10 mM phosphate. Inclusion of ATP in the TCA-$PO_4$ solution did not affect the results. The protein was collected by filtration on Millipore filters (HAWP 0.45 $\mu$) pre-soaked in 5 mM ATP. The residue was washed three times with 5 ml of 5% TCA-10 mM $PO_4$. The filters were dried, and bound phosphate was quantitated in a LS-133 liquid scintillation spectrometer (Beckman Inst.).

*Other additions to assays*

Phenylbutazone and indomethacin were dissolved in ethanol at a concentration such that no addition exceeded 10 $\mu$l. When ethanol additions were used, control tubes also contained the same vol. of ethanol.

Protein was determined by the method of Lowry *et al.* [5] using bovine serum albumin as standard.

## RESULTS

*Inhibition of $H^+$ secretion.* Mammalian gastric mucosae function poorly *in vitro*, whereas amphibian gastric mucosae remain viable and respond to stimulation of $H^+$ secretion for as long as 24–72 hr. For this reason bullfrog gastric mucosa was used for the following example.

Figure 1 was taken from an experiment in which bullfrog gastric mucosa, stripped of the external muscle layer, was mounted in a plexiglas chamber and bathed with solutions in which $Cl^-$ had been replaced with sulfate. The mucosal solution was an isotonic solution of $Na_2SO_4$ and $K_2SO_4$; the serosal solution was a modified amphibian Ringer's solution. Under these conditions the short circuit current ($I_{sc}$) has been shown to approximate the prevailing $H^+$ secretory rate [8]. Measurement of $H^+$ secretory rate by this technique was necessitated by the buffering capacity of phenylbutazone which was added to the mucosal solution.

Figure 1 shows that the pd was initially positive. The calculated short circuit current, 40 $\mu$AMP/cm$^2$, corresponded to an $H^+$ secretory rate of 1.5 $\mu$moles/cm$^2$-hr. Addition of phenylbutazone at a final concentration of 1 mM reduced the $H^+$ secretory rate to zero 60 min following addition. During inhibition the transmucosal resistance increased from 400 $\Omega$cm$^2$ to 900 $\Omega$cm$^2$. One possible mechanism underlying these changes was investigated by determining the effect of phenylbutazone and indomethacin on the gastric $K^+$ATPase, a putative $H^+$ transport ATPase [9–11].

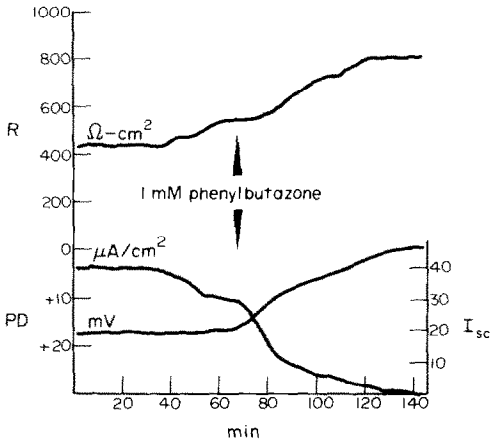


Fig. 1. Frog gastric mucosa mounted in a Ussing chamber was exposed to 1 mM phenylbutazone in the mucosal solution. The mucosa was bathed with $Cl^-$ free solution. The resistance of the mucosa and the short circuit current are shown.

*pH Optimum.* The ATPase was shown to have optimum activity over the range of 6.5–7.25 and the pNPPase optimum was skewed slightly toward higher pHs with optimum activity between pH 7.5–7.75. ATPase assays were performed at pH 7.0 and pNPPase at pH 7.5.

*Effect of phenylbutazone and indomethacin.* The $K^+$ATPase and the $K^+$pNPPase activities were found to be inhibited by both indomethacin and phenylbutazone. Figure 2 shows the ATPase activity when increasing concentrations of these inhibitors were included in the assay mixture. The $k_i$ for indomethacin was 710 $\mu$M and $K_i$ for phenylbutazone was 430 $\mu$M as determined from the slope [$1/v_m(1 + i/K_i)$] of the Eadie-Hofstee plot in Fig. 4. The $K^+$pNPPase activity (Fig. 3) was similarly inhibited by both of these compounds and $K_i$s were 670 $\mu$M and 330 $\mu$M for indomethacin and phenylbutazone respectively.

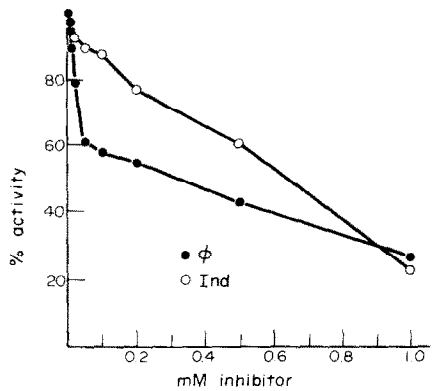


Fig. 2. Inhibition of the $K^+$ATPase by phenylbutazone (●) and indomethacin (○).

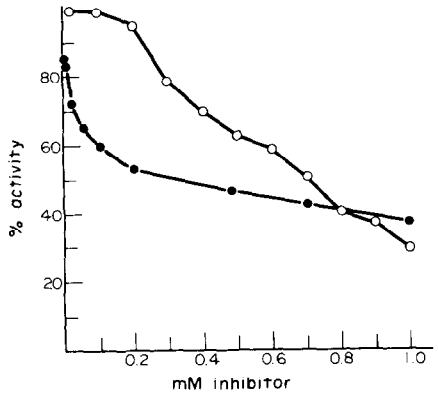


Fig. 3. Inhibition of the $K^+$pNPPase by phenylbutazone (●) and indomethacin (○).

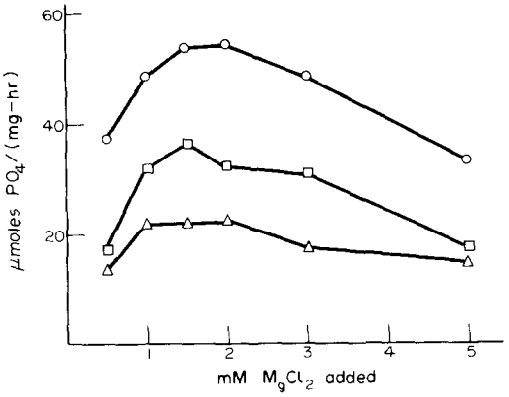


Fig. 5. $K^+$ATPase activities determined at 2 mM with various additions of $MgCl_2$. $K^+$ATPase without additions (○), with 400 μM phenylbutazone (△) or with 700 μM indomethacin (□).

*Type of inhibition.* The type of inhibition was investigated in experiments in which the inhibitor concentration was fixed and the concentrations of Mg-ATP or $Mg^{2+}$-pNPP, $Mg^{2+}$ and $K^+$ were varied individually. Figure 4 shows the effect of increasing concentration of ATP. In the absence of inhibitor the $K_m$ for ATP was 0.2 mM and was not altered by inclusion of either inhibitor. Figure 4 shows substrate inhibition by [ATP] greater than 2 mM. The inhibition due to phenylbutazone and indomethacin was not reversed by greater [ATP]; the $V_{max}$ was reduced. Thus inhibition was noncompetitive.

Varying $Mg^{2+}$ concentration indicated a 1:1 ratio for $Mg^{2+}$ and ATP was optimal and was unaffected by inclusion of indomethacin and phenylbutazone. Figure 5 shows these findings; there is no suggestion that greater concentrations of $Mg^{2+}$ reverse inhibition by these agents.

Figure 6 shows the enzyme activity and the Eadie-Hofstee plot obtained when $Mg^{2+}$-pNPP were varied at a fixed ratio of 1:1; the plot of $S/V$ vs $S$ is non-linear at low concentrations (closed circles). The data points shown as open circles were obtained when $Mg^{2+}$ was constant (5 mM); the non-linearity was corrected under this condition. When [$Mg^{2+}$] was fixed and pNPP concentration was varied, the $K_m$ was 1.0 mM. Like the ATPase, the $K_m$ for pNPP was not altered by indomethacin or phenylbutazone, and the $V_m$ was reduced. Thus inhibition of the pNPPase was non-competitive with respect to pNPP.

$K^+$ greatly stimulated hydrolysis of both ATP and pNPP (Fig. 7 and 8). The $K_a$ for $K^+$ stimulation was

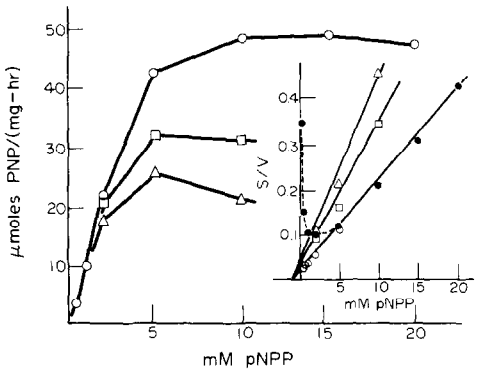


Fig. 6. pNPPase activities determined at pNPP concentrations from 0.5–20 mM. $K^+$pNPPase without addition (●), with 40 μM phenylbutazone (△) and with 700 μM indomethacin (□). The insert is the Eadie–Hofstee plot. (●) $K^+$pNPPase determined with [$Mg^{++}$] = [pNPP]. (○) $K^+$pNPPase determined when $MgCl_2$ was constant at 5 mM.

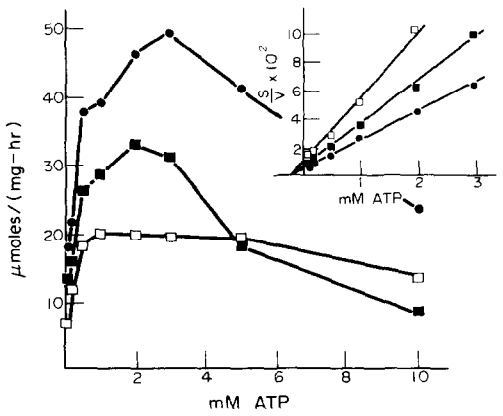


Fig. 4. Activities of $K^+$ATPase (●) and $K^+$ATPase in the presence of 400 μM phenylbutazone (□) or 700 μM indomethacin (■) at [ATP] from 0.25–10 mM. The Eadie–Hofstee plot is the insert.

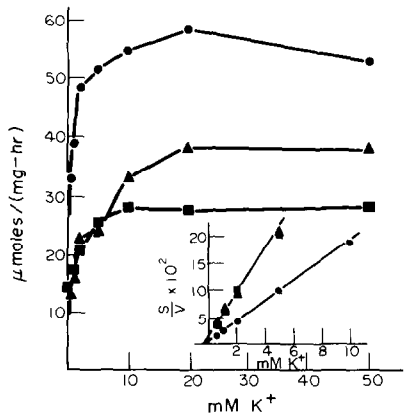


Fig. 7. Stimulation of $K^+$ATPase activity by various concentrations of KCl. $K^+$ATPase activity without additions (●), with 400 μM phenylbutazone (■) or 700 μM indomethacin (▲). The insert is the Eadie–Hofstee plot.

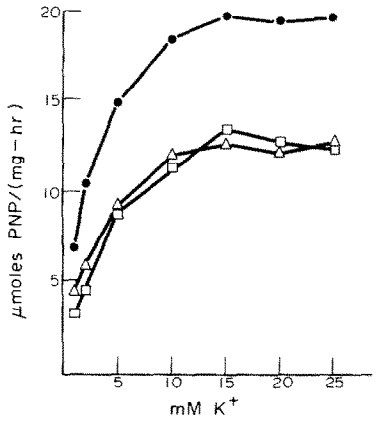


Fig. 8. Stimulation of pNPPase activity by various concentrations of KCl. Activities were determined in the absence of inhibitor (●) or in the presence of 400 μM phenylbutazone (△) or 700 μM indomethacin (□).

0.4 mM and 2.5 mM for the ATPase and pNPPase respectively. These figures also show the effect of indomethacin and phenylbutazone. The $K_a$ was unchanged and greater concentrations of $K^+$ did not reverse the reduction of $V_{max}$ Thus inhibition was non-competitive with respect to $K^+$.

The non-competitive inhibition of both the ATPase and the pNPPase further suggested that the second partial reaction, the $K^+$ sensitive dephosphorylation of a phosphoenzyme [12, 13], was the indomethacin and phenylbutazone sensitive reaction. Studies were performed to determine if phenylbutazone inhibited dephosphorylation.

*Effect on the $K^+$ ATPase phosphoenzyme.* Figure 9 shows the results of incubation of membranes with [$\gamma$-$^{32}$P]ATP in the absence of $K^+$. The membranes were rapidly phosphorylated, and phosphorylation reached a plateau. Inclusion of 500 μM phenylbutazone did not significantly reduce the amount of phosphate incorporated. In these studies the last three time points were averaged and taken as the steady state phosphate incorporation. Control membranes

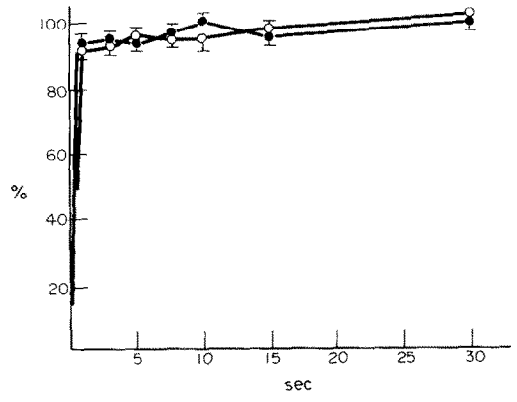


Fig. 9. Phosphorylation of the $K^+$ ATPase by [$\gamma$-$^{32}$P]ATP at 37 in the absence (●) and in the presence of 500 μM phenylbutazone (○). The points are the means of triplicate determination in three separate assays. 100% was taken as the mean phosphate incorporation at 10, 15 and 30 sec of each experiment. In control membranes mean phosphate incorporated was 1690 pmoles/mg protein and in the presence of phenylbutazone was 1787 pmoles/mg protein.

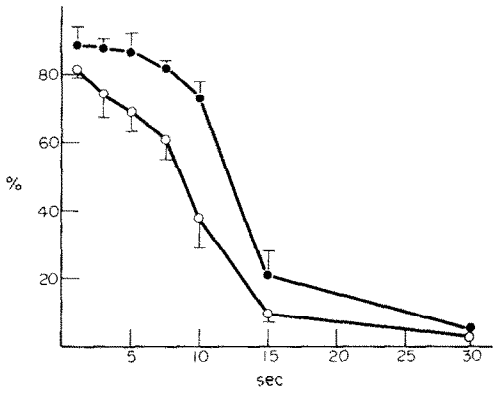


Fig. 10. Dephosphorylation of the phosphoenzyme which was phosphorylated by [$\gamma$-$^{32}$P]ATP in the absence (●) or presence (○) of 500 μM phenylbutazone is shown. 40 nmoles of KCl were added after 10 sec of phosphorylation. Zero time corresponds to KCl addition. Differences were statistically significant ($0.025 > P > 0.01$) through 15 sec.

incorporated 1690 pmoles $PO_4$/mg protein; in the presence of 500 μM phenylbutazone membranes incorporated 1787 pmoles $PO_4$/mg protein (means of 3 experiments). The effect of phenylbutazone on kinetics of phosphorylation cannot be determined from these studies since the 1 sec determination was approximately 90 per cent of the steady state level. Using manual techniques, shorter time intervals are not easily accomplished or duplicated and are probably unreliable.

For studies of dephosphorylation, the enzyme was allowed to phosphorylate for 10 sec in the presence or absence of 500 μM phenylbutazone. KCl was then added and the reaction was terminated at various times after KCl addition. Figure 10 shows the results of these experiments. Addition of 0.2 mM $K^+$ to the phosphorylated enzyme initiated rapid dephosphorylation to a very low steady state level. Inclusion of phenylbutazone reduced the rate of dephosphorylation. The phosphoenzyme present was statistically significantly greater at 1, 3, 5, 7.5, 10 and 15 sec after addition of KCl. The steady state level of phosphorylation following KCl addition was very low and the difference between phenylbutazone and control was statistically insignificant. Thus, phenylbutazone appeared to inhibit the $K^+$ ATPase by interfering with the rate of dephosphorylation of the phosphoenzyme intermediate.

## DISCUSSION

The mechanism of gastric $H^+$ secretion remains undefined but many recent experiments suggest that a $K^+$ ATPase, uniquely found in gastric mucosa, may be the transport enzyme [9–11]. In investigating the mechanisms of inhibition of $H^+$ secretion and injury to the gastric mucosa, *in vivo* models have been used extensively. Indomethacin and phenylbutazone inhibit $H^+$ secretion by the *in vitro* gastric mucosa as shown above. *In vivo* both agents can produce gastric mucosal ulceration [14, 15]. The usual explanation for reduced $H^+$ secretion relates increased mucosal $H^+$ permeability to loss of $H^+$ by diffusion [1, 2]. No studies have reported interaction of phenylbutazone

or indomethacin with the presumptive $H^+$ transport enzyme.

The membranes studied were fractionated by zonal density gradient centrifugation. The membranes isolated at 25% sucrose contained greater $K^+$ATPase and $K^+$pNPPase activity than another membrane fraction isolated at 11% sucrose. The ATPase and the pNPPase activities of the membranes isolated at 25% sucrose were stimulated by $K^+$ (Fig. 7 and 8). AMPase activity was found predominately in the membrane fraction at 11% sucrose.

We have used concentrations of phenylbutazone and indomethacin ranging from 1–1000 $\mu$M. The $K_i$ determined for phenylbutazone is slightly greater than therapeutic blood concentrations in man, about 150–300 $\mu$M [6]. The $K_i$ for indomethacin is substantially greater than therapeutic blood concentrations (about 5 $\mu$M) in man [7]. The gastric mucosa (following oral administration) is exposed to lumenal concentrations which are much greater than blood concentration. The acid environment of the stomach and the lipid solubility of these agents favors absorption from the stomach and exposure of the mucosal cells to a large concentration. To our knowledge no measurements of mucosal concentration have been reported. Assuming 100 mg of phenylbutazone were ingested in 100 ml $H_2O$, the gastric mucosa would be exposed to approximately 3 mM phenylbutazone (about 10 times the $K_i$ determined above).

Both $K^+$ATPase and $K^+$pNPPase activities were inhibited by indomethacin and phenylbutazone. The inhibition was not reversed by $Mg^{2+}$, ATP, pNPP or KCl in greater concentrations and the $K_m$ for each of these substrates was not altered by the inhibitors. Thus the inhibition appeared to be non-competitive.

The gastric $K^+$ATPase has been shown to form a phosphoenzyme intermediate [12, 13] which is dependent only on the presence of ATP. Dephosphorylation is $K^+$ dependent (Fig. 10).

$$\text{ATP} + \text{enzyme} \rightarrow E \sim \text{PO}_4 \xrightarrow{K^+} E + P_i$$

The ATPase reaction encompasses the overall reaction while the $K^+$pNPPase is thought to probe the second partial reaction. Since both activities are inhibited to a similar extent with very similar $K_i$s, the site of inhibition would appear to be the dephosphorylation reaction.

Inhibition of dephosphorylation was directly measured in membranes phosphorylated in the presence or absence of phenylbutazone. Dephosphorylation was initiated by KCl addition. The rate of dephosphorylation was reduced in membranes incubated with phenylbutazone. The steady state level was, however, not significantly different from the control. The most likely explanation for similar steady state phosphorylation in control and phenylbutazone-treated membranes is that the level of phosphorylation in the presence of KCl was very close to the non-specific binding (retention) on the filter. The variation of non-specific retention between filters may have been such that real differences could not be detected by the filtration method. More complex explanations such as multiple states of the enzyme similar to the complex partial reactions of the $Na^+ - K^+$ ATPase [16] may however be valid. The partial reactions for the gastric $K^+$-ATPase have not been defined in detail.

We have thus shown that two agents which produce mucosal injury and inhibit *in vitro* gastric $H^+$ secretion are inhibitors of gastric $K^+$-ATPase. Analysis of the kinetics of this interaction indicate that inhibition in non-competitive and involves the $K^+$ sensitive dephosphorylation of the phosphoenzyme. These findings provide an alternate mechanism for inhibition of $H^+$ secretion by these agents.

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