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Adsorption of Pepsin by Aluminum Hydroxide II: Pepsin Inactivation

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Abstract □ Pepsin adsorbed on gibbsite or boehmite, non-acid-reactive forms of aluminum hydroxide, had a significantly lower activity than pepsin in solution. IR and desorbed pepsin activity studies showed that the reduced activity of adsorbed pepsin was not due to denaturation of pepsin on adsorption. Steric occlusion of the active site, following pepsin adsorption, was responsible for the lower activity of pepsin adsorbed on gibbsite. The porous morphology of boehmite caused diffusional resistance and steric exclusion, contributing to the decreased activity of adsorbed pepsin. The specific inactivation of pepsin by adsorption on aluminum hydroxide may be important in ulcer therapy.

Keyphrases □ Aluminum hydroxide—adsorption of pepsin, pepsin inactivation □ Pepsin—adsorption by aluminum hydroxide, inactivation □ Adsorption—pepsin adsorption by aluminum hydroxide, pepsin inactivation

The first phase of this study demonstrated that aluminum hydroxide adsorbs pepsin by anionic ligand exchange and electrostatic attractive forces (1). The effect of adsorption on the activity of pepsin and the elucidation of the mechanism responsible for any specific antipepsin effect is the focus of this study.

BACKGROUND

The reduction of pepsin activity by colloidal antacid materials was initially hypothesized to be due to the precipitation of pepsin (2, 3). Berstad (4) has recently concluded that aluminum hydroxide has powerful adsorbent and precipitation effects on pepsin in human gastric juice. Aluminum hydroxide was reported to precipitate pepsin in an inactive form (2). Mutch (5) indicated that pepsin was adsorbed on kaolin, silica gel, calcium phosphate, and magnesium phosphate. Pepsin adsorbed on kaolin or silica gel remained active, but pepsin adsorbed on calcium phosphate or magnesium phosphate was inactive due to denaturation by the high local pH of the surface. Bismuth aluminate, magnesium oxide, and magnesium carbonate were effective in inhibiting pepsin following adsorption (6). Berstad *et al.* (7) assumed that pepsin adsorbed by aluminum hydroxide was inactive. Piper and Fenton (8) stated that the effect of adsorption on pepsin activity was just as important as the pH effect for aluminum hydroxide. Anderson and Harthill (9) found that pepsin adsorbed on an acid-reactive aluminum hydroxide exhibited only 21% of the theoretical activity. Liebman (10) reported that the activity of pepsin was 22% of the control following adsorption on aluminum hydroxide, 29% of the control following adsorption by magnesium hydroxide, and 46% of the control following adsorption by cholestyramine. It was concluded that pepsin was still active while adsorbed because pepsin activity, following dissolution of the three adsorbents, was 88-91% of the control.

EXPERIMENTAL SECTION

Gibbsite[1], boehmite[1], aluminum hydroxycarbonate gel[2], aluminum chlorohydrate[3], porcine pepsin[4], and bovine hemoglobin[4] were obtained commercially. The proteolytic activity of pepsin was determined by either the Anson method (11, 12), using hemoglobin as the substrate, or the dipeptide method (13), which uses the dipeptide *N*-acetyl-L-phenylalanyl-L-3,5-diiodotyrosine as the substrate. The activity of pepsin in the presence of a series of soluble cations was determined using a pepsin solution (20 μg/mL) which was 0.14 mM with respect to the appropriate cation. Likewise, the activity of pepsin in the presence of gibbsite or boehmite was determined using a pepsin solution (20 μg/mL) which contained 0.5 mg/mL of gibbsite or boehmite.

Pepsin solutions (20 μg/mL), or pepsin (20 μg/mL) and gibbsite (0.5 mg/mL) or boehmite (0.5 mg/mL) suspensions, were lyophilized and pre-

[1] Reynolds Metal Co., Bauxite, Ark.
[2] Chattem Chemical Co., Chattanooga, Tenn.
[3] Wicken Products, Huguenot, N.Y.
[4] Worthington Biochemical Corp., Freehold, N.J.

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Table I—Pepsin Activity Corrected for the Time Effect

Replicate Number	Elapsed Time to Start Replicate, s	Activity (a), AU	Mean Corrected Activity ($\overline{A}$), AU	Predicted Activity (a_p), AU	Correction Factor, AU	Corrected Activity (A), AU
1	0	3120	3152	3158	−6	3114
2	334	3180	3152	3119	33	3213
3	839	3035	3152	3060	92	3127
4	3685	2740	3152	2726	426	3166
5	4013	2675	3152	2688	464	3139
Mean		2950	—	—	—	3152
SD		±228	—	—	—	±39

pared as potassium bromide disks (1 mg of lyophilized sample/300 mg of KBr) for IR analysis. The pepsin solution was denatured by either adding 1 M NaOH to adjust the pH to 9 (base-denatured pepsin) or by heating the pepsin solution at 90°C for 1 h (heat-denatured pepsin). The denatured pepsin solutions were lyophilized and prepared for IR analysis as above. The IR spectrum was recorded using a spectrophotometer[5] interfaced with a computer. The spectrum of adsorbed pepsin was obtained by subtracting the spectrum of boehmite from the spectrum of pepsin-boehmite based on the 1070 cm^{-1} band of boehmite (1).

Desorbed-pepsin studies were performed by initially interacting pepsin (25 $\mu g/mL$) and aluminum hydroxycarbonate gel (1.7 mg equivalent of aluminum oxide/mL) at pH 2.3 in a pH-stat titrator[6]. Preliminary studies indicated immediate adsorption under these conditions. The aluminum hydroxycarbonate was dissolved under pH-stat[6] conditions at pH 2.3 and 5°C. After complete dissolution of the aluminum hydroxycarbonate gel, the activity of pepsin was determined by the modified Anson method.

The activity of desorbed pepsin was also determined by adsorbing pepsin (20 $\mu g/mL$) on gibbsite (0.5 mg/mL) or boehmite (0.5 mg/mL) at pH 2.3. After adsorption, a 0.12-g/mL phosphate solution (pH 2.3) was added. The supernatant was analyzed at 280 nm to monitor the desorption of pepsin. The activity of the desorbed pepsin was determined by the modified Anson method.

Samples of gibbsite and boehmite were prepared for scanning electron microscopy[7] by air-drying a sonicated 0.2% suspension. The samples were coated with gold and platinum[8]. A microdrop of a 0.2% sonicated gibbsite or boehmite suspension was placed on a 300-mesh copper grid which was coated with polyvinyl formal[9] and stabilized with evaporated carbon. After air-drying, the sample was examined by transmission electron microscopy[10] at 80 keV.

RESULTS AND DISCUSSION

To improve the precision of the Anson method, which has a *CV* of ~5% (14), five replicates were run rather than three. Pepsin activity tended to decrease with each replicate (Table I). The decrease appeared to be related to time and is believed to be due to the autodigestion of pepsin in the pepsin stock solution. The autodigestion of pepsin was slowed by maintaining the pepsin stock solution at 5°C, but a variation in pepsin activity (a) with time was still observed. A plot of pepsin activity *versus* time for the data in Table I showed that time accounts for the major part of the variation in activity. Equation 1 was used to correct for time to obtain the mean corrected pepsin activity, $\overline{A}$:

$$\overline{A} = r^2y + (1 - r^2)\overline{a} \quad \text{(Eq. 1)}$$

where r^2 is obtained from the linear regression analysis and y represents the intercept of the optimal straight line. The experimental mean pepsin activity, $\overline{a}$, is influenced by the time dependence of the replicates. Thus, $\overline{A}$ represents the mean activity that would be observed if autodigestion did not occur. However, if $r^2 = \sim 0$ (indicating only slight time dependence), then $\overline{A} \approx \overline{a}$. If a_p represents the predicted activity at time t, obtained from the optimal straight line, then each individual activity, a, may be corrected to give a corrected pepsin activity, A:

$$A = a + (\overline{A} - a_p) \quad \text{(Eq. 2)}$$

Thus, the procedure to correct for the effect of time adjusted the mean pepsin activity from 2950 to 3152 activity units and decreased the *SD* from 228 to 39 activity units. The correction for autodigestion of the stock pepsin solution reduced the *CV* ~1%. *Perhaps some of the conflicting reports on pepsin inactivation are due to the large CV associated with the unmodified Anson method.* All results in this study were obtained using the aforementioned modificiation of the Anson method.

The pH-activity profile of pepsin obtained by the modified Anson method is shown in Fig. 1, line A and is similar to previously reported profiles (15, 16). The optimal pH range for pepsin activity is pH 2.2–2.6. Pepsin activity approaches zero at pH 4, which supports one of the goals of peptic ulcer therapy, *i.e.*, to raise the gastric pH to minimize pepsin activity.

The activity of pepsin in the presence of soluble forms of aluminum was determined because numerous investigators have reported an inhibitory effect by aluminum (3, 8, 17, 18). However, Anderson and Harthill (9) reported that aluminum ions had no effect on pepsin activity. Two forms of soluble aluminum (aluminum chloride and aluminum chlorohydrate) were tested for an antipepsin effect at pH 2.4 by the modified Anson method. Aluminum chlorohydrate was chosen because it is an aluminum complex with a +7 charge (19). A concentration ratio of 250 ions per pepsin molecule was chosen to provide a substantial excess of aluminum ions, because pepsin contains ~43 carboxylate groups on the surface. The results (Table II) indicate a slight enhancement of pepsin activity (~4%) by aluminum cation and a slight inhibition (~4%) by aluminum chlorohydrate. These differences are statistically significant at an α level of 0.05. Even though statistically significant differences in pepsin activity were observed in the presence of aluminum ion and aluminum chlorohydrate, these differences are not believed to be large enough to produce any significant *in vivo* effects. Furthermore, aluminum cation did not alter the pH-activity profile of pepsin (Fig. 1, line B).

The first part of this series demonstrated that pepsin was specifically adsorbed by aluminum hydroxide through anionic ligand exchange, involving carboxylate groups of pepsin and surface aluminum ions, and electrostatic attraction (1). Therefore, the effect of adsorption on pepsin activity was examined by determining pepsin activity in the presence of gibbsite or boehmite, non-acid-reactive forms of aluminum hydroxide. Under normal physiological conditions, a 600-mg dose of aluminum hydroxide will produce a pepsin to aluminum hydroxide ratio in the stomach of 0.02:1 to 0.16:1 (20). Thus, a ratio of pepsin to aluminum hydroxide of 0.04:1 was used. The activity of pepsin in the presence of either gibbsite or boehmite was lower than the control (Table

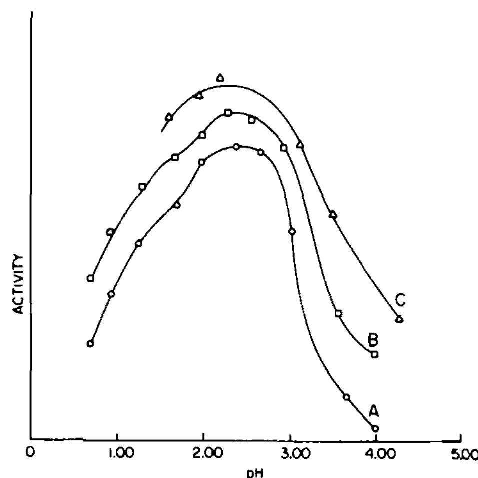

Figure 1—*pH-activity profile. Key: (A) pepsin solution using modified Anson method; (B) pepsin solution in presence of aluminum ion using modified Anson method; (C) pepsin solution using the dipeptide method.*

[5] Model 180; Perkin-Elmer Corp., Norwalk, Conn.
[6] PHM 62, TTT 60, ABU 12, TTA 60, and REA 60; Radiometer, Copenhagen, Denmark.
[7] JSM-U3; Japan Electron Optics Laboratory Co., Ltd., Tokyo, Japan.
[8] Hummer II Sputter Coater; Technics, Alexandria, Va.
[9] Formvar; Ladd Research Industries, Burlington, Vt.
[10] EM-400; Philips, Mahwah, N.J.

Table II—Effect of Soluble Forms of Aluminum on Pepsin Activity

	Aluminum Chloride				Aluminum Chlorohydrate			
	I		II		I		II	
	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control
Mean corrected activity, AU	3152	3030	3155	3034	3017	3170	3097	3186
SD	±39	±83	±46	±59	±136	±66	±31	±25
pH	2.26	2.27	2.56	2.58	2.42	2.42	2.37	2.39

Table III—Effect of Gibbsite or Boehmite on Pepsin Activity

	Gibbsite		Boehmite	
	Treatment	Control	Treatment	Control
Mean corrected activity, AU	2518	2999	1571	2753
SD	±100	±148	±40	±79
pH	2.43	2.42	2.64	2.61
Percent Adsorbed	50	—	74	—
Activity of adsorbed pepsin, %	68	—	42	—

III). However, the observed activity of pepsin in the aluminum hydroxide suspensions was not due to adsorbed pepsin alone since not all of the enzyme was adsorbed. Activity due to adsorbed pepsin will be termed immobilized pepsin activity. To calculate the immobilized pepsin activity, the extent of adsorption had to be determined. Also, it was assumed that free pepsin in the aluminum hydroxide suspension had the same activity as pepsin in the control solution. For example, UV analysis of the supernatant from the pepsin-boehmite suspensions indicated that 26% of the pepsin was in solution. Thus, the activity contributed by the free pepsin was calculated to be 716 AU, *i.e.*, 26% of the activity of the control. Since the total activity of the pepsin-boehmite suspension was 1571 AU, the adsorbed pepsin (which comprised 74% of the pepsin in the suspension) had an activity of 855 AU. However, 74% of the activity of the control is 2037 AU. Thus, the adsorbed pepsin exhibited 42% as much activity as unadsorbed pepsin. The adsorbed pepsin in the pepsin-gibbsite suspension was only 68% as active as free pepsin.

The decreased antiproteolytic activity of adsorbed pepsin was directly measured by adsorbing pepsin on gibbsite or boehmite, washing, and separating the solid phase to obtain only immobilized pepsin. Adsorption was strong enough that desorption did not occur when the immobilized pepsin was resuspended (1). Pepsin immobilized on gibbsite was 59-67% as active as free pepsin while pepsin adsorbed on boehmite was only 31-39% as active as the control, as determined by the modified Anson method (Table IV). These results agree with the 68 and 42% activity calculated for pepsin adsorbed on gibbsite and boehmite, respectively, based on the activity of the unwashed suspension (Table III).

Three factors may be involved in the decreased immobilized pepsin activity. A structural change in pepsin may result from adsorption on the aluminum hydroxide surface. The properties of the active site of an enzyme depend on the tertiary structure and any modification of the three-dimensional structure could have an effect on activity. A second factor might involve steric occlusion of the active site due to adsorption. Depending on the orientation of the adsorbed pepsin molecule, the accessibility of the active site may be reduced or blocked altogether. This effect would reduce the rate of proteolysis. A third possible mechanism is the development of a substrate concentration gradient between the bulk solution and the microenvironment of the adsorbed pepsin. The substrate concentration gradient can be due to diffusional resistance of the substrate or steric exclusion of the substrate from reaching the adsorbed pepsin.

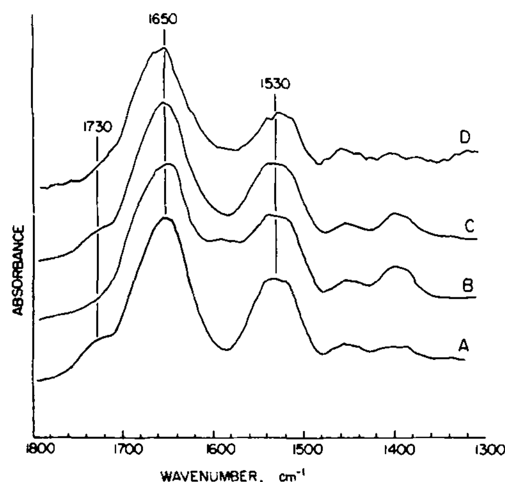

Figure 2—*IR spectra. Key: (A) pepsin; (B) base-denatured pepsin; (C) heat-denatured pepsin; (D) pepsin adsorbed on boehmite.*

The possibility that adsorption produces a structural change in pepsin was investigated by IR spectroscopy and by determining the activity of pepsin which had been desorbed from gibbsite or boehmite. The IR spectra of pepsin, base-denatured pepsin, and heat-denatured pepsin are compared in Fig. 2. The major bands for pepsin occur at 1530 cm^{-1} (amide II) and 1650 cm^{-1} (amide I) (21). The shoulder at 1730 cm^{-1} is due to the C=O stretching vibrations from the carboxylic acid groups of glutamic and aspartic acids (22). The spectra of the base-denatured and heat-denatured pepsin also had bands at 1530 and 1650 cm^{-1}. The base-denatured pepsin had a band at ~1600 cm^{-1} which was due to the strong asymmetric stretching vibrations from the ionized carboxylic acid groups of glutamic and aspartic acids. Since the carboxylic acid groups will be ionized at the pH required for base denaturation, the shoulder at 1730 cm^{-1} was not present in the spectrum of the base-denatured pepsin. Both the base-denatured and the heat-denatured pepsin possess a unique band at 1400 cm^{-1}. This band has not been assigned but is believed to be associated with the denaturation of pepsin.

The IR spectrum of pepsin adsorbed on boehmite was obtained by computer subtraction of the boehmite spectrum from the pepsin-boehmite spectrum and is shown as Fig. 2, line D. The amount of pepsin adsorbed by gibbsite was too low to obtain an IR spectrum. The absence of the band at 1400 cm^{-1} suggests that pepsin was not denatured by adsorption. The amide I band at 1650 cm^{-1} shifted to a slightly higher frequency, possibly due to a slight deformation of the structure of pepsin as a consequence of adsorption. The similarity of the spectra of pepsin and pepsin adsorbed on boehmite suggests that pepsin was still active even when adsorbed on aluminum hydroxide.

Pepsin activity following desorption was determined to further establish whether pepsin was denatured due to adsorption. A dissolution method and an exchange method were used to desorb pepsin. Aluminum hydroxycarbonate gel, the acid-reactive form of aluminum hydroxide, was used as the adsorbent for the dissolution method. A low ratio of pepsin to aluminum hydroxide was used so that the majority of pepsin would be adsorbed. Following dissolution of the aluminum hydroxycarbonate gel, the activity of the desorbed pepsin

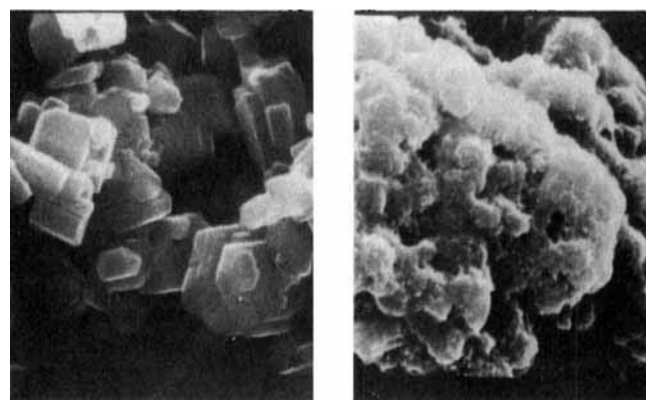

Figure 3—*Scanning electron micrographs of gibbsite (left) and boehmite (right) at 30,000×.*

Table IV—Immobilized Pepsin Activity

	Gibbsite				Boehmite			
	I		II		I		II	
	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control
Mean corrected activity, AU	1854	3124	1996	3002	938	3046	1163	2992
SD	±32	±41	±30	±32	±20	±63	±80	±42
pH	2.41	2.42	2.50	2.51	2.48	2.49	2.50	251
Immobilized pepsin activity, %	59.3	—	66.5	—	30.8	—	38.9	—

was determined by the modified Anson method (Table V). The desorbed pepsin was 96% as active as the control indicating that adsorption by aluminum hydroxide did not affect the structure of pepsin enough to alter its activity. This result corresponds well with an earlier study which reported a 94% recovery of activity following dissolution of the adsorbent, aluminum hydroxide (9).

Pepsin was also desorbed from gibbsite or boehmite by exchange with phosphate. As seen in Table VI, almost all of the adsorbed pepsin was desorbed from either gibbsite or boehmite by exchange with phosphate. The activity of the desorbed pepsin, as determined by the modified Anson method, was virtually identical to the controls. However, the mean corrected activity after phosphate exchange includes both desorbed pepsin and pepsin which had not been adsorbed. When Eq. 3 was applied to calculate the activity of only desorbed pepsin, the pepsin desorbed from gibbsite and boehmite was 91 and 96% as active as the control, respectively.

$$D = \frac{\bar{A}_t - \left(\bar{A}_c \frac{F_f}{F_r}\right)}{\bar{A}_c - \left(\bar{A}_c \frac{F_f}{F_r}\right)} \times 100 \quad \text{(Eq. 3)}$$

where D is the activity of desorbed pepsin, %; $\bar{A}_t$ is the mean corrected activity after phosphate exchange; $\bar{A}_c$ is the mean corrected activity of control; F_f is the fraction of pepsin in the supernatant initially; and F_r is the fraction of pepsin in the supernatant after phosphate exchange. Thus, IR and desorption studies indicate that adsorption of pepsin by aluminum hydroxide does not denature pepsin.

To determine whether steric effects were involved in reducing the activity of adsorbed pepsin, the activity of pepsin against a smaller substrate was studied. Hemoglobin (Hb), the substrate used in the Anson method, is a macromolecule with a mol. wt. of ~35,000 (23). The low-molecular-weight substrate selected was *N*-acetyl-L-phenylalanyl-L-3,5-diiodotyrosine, a dipeptide with a mol. wt. of ~600. The smaller size of the dipeptide should enable it to reach the active site of adsorbed pepsin more easily, and increased activity against the dipeptide should be observed if steric effects are important. The autodigestion of pepsin was observed using the dipeptide, and the mathematical procedure used to correct for the autodigestion of pepsin during the Anson method was also used for the dipeptide method. This method is reported to have a *CV* of 6% (13). When the modifications developed for the Anson method were applied to the dipeptide method, the *CV* was reduced to 2%. The pH-activity profile of pepsin using the dipeptide (Fig. 1, line C) was similar to the pH-activity profile using hemoglobin (Fig. 1, line A).

Table V—Activity of Pepsin Desorbed From Aluminum Hydroxycarbonate Gel by Acid Dissolution

	Treatment	Control
Mean corrected activity, AU	1951	2037
SD	±39	±29
pH	2.63	2.68
Activity of desorbed pepsin, %	96	—

Table VI—Activity of Pepsin Desorbed from Gibbsite and Boehmite by Phosphate Exchange

	Gibbsite		Boehmite	
	Treatment	Control	Treatment	Control
Pepsin in supernatant initially, %	27	—	15	—
Pepsin in supernatant after phosphate exchange, %	96	—	82	—
Mean corrected activity after phosphate exchange, AU	2363	2521	2822	2918
SD	±41	±39	±64	±107
pH	2.49	2.49	2.30	2.30
Activity of desorbed pepsin, %	91	—	96	—

The activity of pepsin adsorbed on gibbsite or boehmite was determined in two separate studies using the modified Anson and the modified dipeptide methods. As seen in Table VII, pepsin adsorbed on gibbsite was less active than the control, but the Student's t test, at an α level of 0.05, showed that the immobilized pepsin activity was statistically the same for both substrates. Thus, both the dipeptide and hemoglobin interacted with the active site of pepsin to the same extent, indicating that both hemoglobin and the dipeptide experienced the same inhibitory steric effect. This steric effect is probably due to steric occlusion of the active site of pepsin resulting from adsorption. Anderson and Harthill (9) also suggested that some active site occlusion occurs when pepsin is adsorbed by aluminum hydroxide.

The immobilized pepsin activity depended on the substrate when pepsin was adsorbed on boehmite (Table VII). Pepsin adsorbed on boehmite was less active than the control, but greater activity occurred with the dipeptide substrate. The t test indicated that the difference in activity against hemoglobin and the dipeptide was statistically significant at an α level of 0.05. These results suggest that a steric factor related to the adsorbent is important, in addition to steric occlusion.

It is possible that the substrate may interact directly with the adsorbent and that the degree of adsorption may differ between hemoglobin and the dipeptide. An adsorption effect occurring between the dipeptide and boehmite can be considered negligible, since the activity of pepsin adsorbed on either boehmite or gibbsite was similar when the dipeptide was used as the substrate. However, hemoglobin may be adsorbed by boehmite since the activity of pepsin adsorbed on boehmite decreased when compared with pepsin adsorbed on gibbsite with hemoglobin as the substrate. This lower activity may be caused by a decrease in hemoglobin concentration in the microenvironment of the adsorbed pepsin due to adsorption of hemoglobin on boehmite. This possibility was discounted when calculation showed that the hemoglobin could form a monomolecular layer 400 times as great as the surface area of the boehmite used in the test. Thus, even if adsorption of a hemoglobin bilayer occurred, the concentration of hemoglobin in solution would not be substantially reduced.

Adsorption of hemoglobin could also affect the activity of adsorbed pepsin if the adsorbed pepsin was "buried" by the adsorption of hemoglobin on the adsorbed pepsin. This mechanism was also discounted because both hemoglobin and the dipeptide were affected equally by pepsin adsorbed on gibbsite.

Diffusional resistance may be partially responsible for the reduced activity of adsorbed pepsin. Hemoglobin has a diffusion coefficient of $6.3\text{-}6.7 \times 10^{-7}$ cm^2/s (24). The diffusion coefficient of the dipeptide has not been determined, but it is expected to be at least 10 times larger than hemoglobin since there is a 58-fold difference in molecular weight. Thus, the dipeptide would be more likely to maintain an equal concentration between the bulk and the mi-

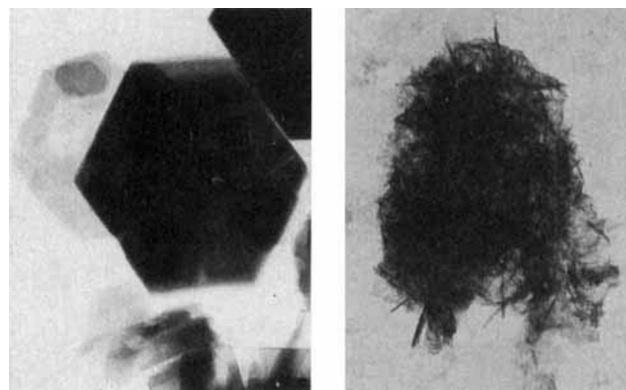

Figure 4—*Transmission electron micrographs of gibbsite (left) and boehmite (right) at 170,000×.*

Table VII—Immobilized Pepsin Activity Using Hemoglobin and Dipeptide Substrates

	Gibbsite								Boehmite							
	I				II				I				II			
	Hemoglobin		Dipeptide		Hemoglobin		Dipeptide		Hemoglobin		Dipeptide		Hemoglobin		Dipeptide	
	T[a]	C[b]	T	C	T	C	T	C	T	C	T	C	T	C	T	C
Mean corrected activity, AU	1854	3124	12.8	24.8	1996	3002	14.0	20.8	938	3046	30.8	66.7	1163	2992	35.9	64.0
SD	±32	±41	±1.2	±1.4	±30	±32	±3.1	±3.3	±20	±63	±1.9	±1.4	±80	±45	±0.8	±1.0
pH	2.41	2.42	2.11	2.11	2.50	2.51	2.16	2.17	2.48	2.49	2.10	2.10	2.50	2.51	2.21	2.22
Immobilized pepsin activity, %	59.3	—	51.6	—	66.5	—	67.3	—	30.8	—	46.2	—	38.9	—	56.1	—

[a] Treatment. [b] Control.

croenvironment of the adsorbed pepsin. In addition to the size of the diffusing species, the morphology of the surface may be an important factor in diffusional resistance. Scanning electron micrographs of gibbsite and boehmite at 30,000X magnification are shown in Fig. 3. Gibbsite is platy with smooth faces and sharp edges, while boehmite is porous. Because the boehmite particles did not have the well-defined edges of gibbsite, the scanning electron micrographs are not as well resolved.

Transmission electron micrographs (Fig. 4) provide more information on the morphology of the adsorbents. Gibbsite particles again appear to have a well-defined structure with distinct edges. The material is platy which can be seen by close examination of the edges of the hexagonal units. The darker particles are a collection of stacked plates. The individual boehmite particles are shown to be smooth flat plates. The plates tend to roll up and form an aggregate of interpenetrating scrolls. The tubular nature of the boehmite particles was deduced because the cores appear to be less dense. Gibbsite particles and boehmite aggregates are approximately the same size, but the porous nature of the boehmite aggregate results in a larger surface area.

The lower activity against hemoglobin of pepsin adsorbed on boehmite when compared with gibbsite can be explained by the porous nature of the boehmite aggregate. Pepsin adsorbed on gibbsite is similar to adsorption on a smooth flat plate, whereas adsorption on boehmite is similar to adsorption in a long narrow channel (Fig. 5). In the case of pepsin adsorbed on gibbsite, the substrate only has to diffuse to the surface. In this situation, the size of the substrate would not be expected to be a factor. Thus, the observation that pepsin adsorbed on gibbsite was equally active against either hemoglobin or the dipeptide is consistent with the morphology of the gibbsite particle.

The porous boehmite morphology requires that the substrate must diffuse through channels in the porous structure to reach adsorbed pepsin. The dipeptide has a diffusion coefficient high enough to maintain the same concentration throughout the channels as in the bulk solution. The dipeptide is also more likely to reach all of the adsorbed pepsin due to its small size. Thus, the lower activity against hemoglobin of pepsin adsorbed on boehmite can also be related to the morphology of boehmite. The lower diffusion coefficient of hemoglobin would make it difficult to maintain the same concentration of hemoglobin in the channels as in the bulk solution. The continuous digestion of hemoglobin as it diffuses through the channels will also lower the concentration of hemoglobin in the microenvironment of the adsorbed pepsin. Thus, pepsin adsorbed at the opening of a channel will have a faster rate of proteolysis than pepsin adsorbed in the interior of a channel. The large size of hemoglobin may even sterically exclude hemoglobin from narrow pores.

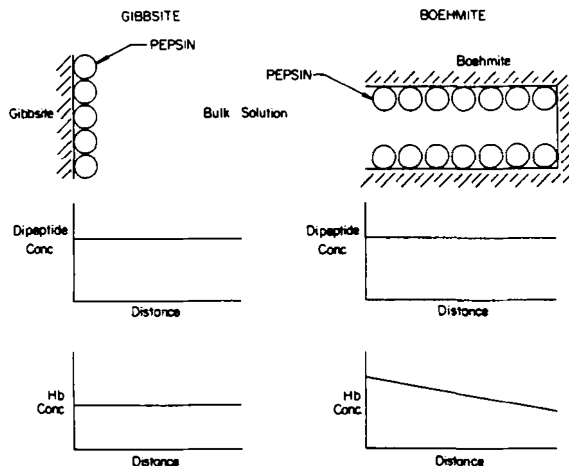

Figure 5—*Schematic diagram of pepsin adsorbed on gibbsite and boehmite.*

The conclusions regarding the importance of the morphology of the adsorbent are consistent with reported differences in activity between low- and high-molecular-weight substrates when an enzyme was adsorbed on a porous material. Immobilized papain on a copolymer had a lower activity using casein than benzoyl-L-arginine ethyl ester as the substrate (25). Pronase adsorbed on a diazotized copolymer had different activities, depending on the molecular weight of the substrate (26). The results were explained on the basis of impedance of substrate access to the catalytic site by the supporting solid matrix. The activity of trypsin adsorbed on aminoethylcellulose also depended on the molecular weight of the substrate (27, 28). It was concluded that the lower activity against the larger substrate was due to the inaccessibility of the adsorbed enzyme.

Thus, pepsin is not denaturerd by adsorption on boehmite or gibbsite. However, the orientation of the adsorbed pepsin results in steric occlusion of the active site, thereby reducing the activity of adsorbed pepsin. In addition to steric occlusion, the activity of pepsin adsorbed on boehmite is also reduced by diffusional resistance and steric exclusion due to the porous morphology of boehmite.

Antacids such as sodium bicarbonate, calcium carbonate, and magnesium hydroxide react very rapidly with acid. pH-Stat titration of these antacid compounds at pH 3 and 37°C showed that the time required to neutralize 50% of the theoretical acid, t_{50}, is <1 min. Therefore, these antacid compounds are expected to be present in the gastric fluid as their soluble ions soon after ingestion. To determine if these antacid compounds exhibit a specific antipepsin effect in addition to the general pH effect, the activity of pepsin in the presence of sodium, calcium, and magnesium ions was determined. Concentrations of ions and pepsin were selected to provide 250 ions per pepsin molecule. As seen in Table VIII, no statistically significant ($\alpha = 0.05$) change in pepsin activity was observed in the presence of sodium, calcium, or magnesium ions.

Aluminum hydroxide reacts more slowly with acid; even amorphous aluminum hydroxycarbonate gel, the acid-reactive form of aluminum hydroxide, does not react as quickly as sodium bicarbonate, calcium carbonate, or magnesium hydroxide. A recent survey of antacid suspensions containing aluminum hydroxide gel and magnesium hydroxide gel showed that the most rapidly reacting product neutralized 96% of the theoretical amount of acid in 15 min at pH 3 and 37°C, while the slowest reacting product neutralized only 31%

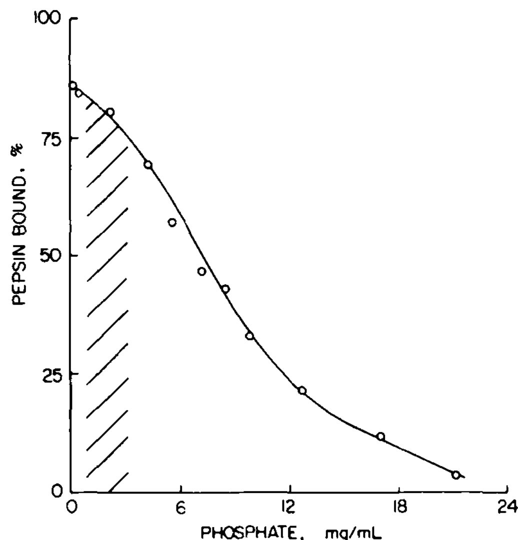

Figure 6—*Effect of phosphate on the fraction of pepsin adsorbed by boehmite. The shaded area indicates the phosphate concentration of gastric fluid.*

Table VIII—Effect of Sodium, Calcium, and Magnesium Ions on Pepsin Activity

	Sodium		Calcium		Magnesium	
	Treatment	Control	Treatment	Control	Treatment	Control
Mean corrected activity, AU	3099	3114	2921	3003	2929	2965
SD	±86	±63	±67	±173	±42	±155
pH	2.43	2.43	2.41	2.41	2.43	2.43

under the same conditions (29). Thus, a solid phase, which can adsorb and inactivate pepsin, will be present in the gastric fluid for a longer time when aluminum hydroxide is used as an antacid in comparison with sodium bicarbonate, calcium carbonate, or magnesium hydroxide.

Before the adsorption and inactivation of pepsin by aluminum hydroxide can be extrapolated to *in vivo* conditions, the possibility that solutes in the gastric fluid (such as phosphate) will compete with pepsin for adsorption by aluminum hydroxide must be considered. Phosphate is also specifically adsorbed by aluminum hydroxide (30, 31). The extent of pepsin adsorption in the presence of phosphate is shown in Fig. 6. The shaded area of the curve indicates the phosphate concentration which is found in gastric fluid (32). Pepsin was 85% bound in the absence of phosphate. The extent of pepsin adsorption gradually decreased with increasing phosphate concentration. Pepsin was 75% bound at the phosphate concentration of the gastric fluid. Therefore, even in the presence of other solutes which are strongly adsorbed by aluminum hydroxide, a major fraction of pepsin would be expected to be adsorbed by aluminum hydroxide.

Non-acid-reactive forms of aluminum hydroxide have been used in ulcer therapy. In 1941 it was reported that non-acid-reactive alumina produced nearly the same symptomatic relief as acid-reactive alumina (33). The effect of non-acid-reactive alumina was attributed to the coating of the gastric mucosa, which provides a physical barrier to gastric acid. Antacid products were marketed that contained a non-acid-reactive aluminum hydroxide. Two gastroscopic studies concluded that non-acid-reactive aluminum hydroxide coated ulcers and acted as an inert physical barrier (34, 35). The results of these studies were contradicted by Hoon (36) who observed no coating, and by Morrissey *et al.* (37) who found approximately one-third of the ulcers completely coated, but the coatings remained for only a short time. Thus, recent thinking has been that acid neutralization is the chief function of antacids. Accordingly, the recent trend in formulating antacid products has been to incorporate only acid-reactive materials. The results of the present study of the adsorption and inactivation of pepsin by gibbsite and boehmite suggest that earlier antacid products containing non-acid-reactive aluminum hydroxide may have promoted ulcer healing by adsorbing and temporarily inactivating pepsin, rather than by coating the ulcers.

Based on this study, it is hypothesized that acid reactive aluminum hydroxycarbonate promotes ulcer healing by neutralizing gastric acid, inactivating pepsin by raising the pH of the gastric contents, and specifically adsorbing and inactivating pepsin. The specific adsorption and inactivation of pepsin by aluminum hydroxide may be responsible for the wide use of aluminum hydroxide in ulcer therapy. The specific adsorption and inactivation of pepsin in ulcer therapy may be enhanced by the use of a non-acid-reactive form of aluminum hydroxide that has a high surface area, such as boehmite.

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