Immunochemical Studies on the Components of the Pepsinogen System

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ABSTRACT Rabbit antisera to pepsin and pepsinogen were characterized by several immunological criteria. Both antisera inhibited the rennet activity of pepsin. Antipepsinogen protected pepsin from alkaline denaturation. Using antipepsinogen, precipitin analysis at pH 5.5 indicated that the native enzyme resembles the precursor more closely than did the denatured enzyme. However, all three proteins have some antigenic sites in common. Both antisera reacted more efficiently with their homologous antigens. When measured by C′ fixation, the pepsinogen-antipepsinogen system was inhibited by pepsin and to a greater degree, by the activation mixture and the pepsin-inhibitor complex. Pepsin-antipepsin was inhibited by pepsinogen. The specificity of these two antibodies toward pepsin and pepsinogen conformation was used to measure the disappearance of pepsinogen and the concomitant appearance of pepsin during autocatalytic conversion at pH 4.6. The experimental results obtained during the conversion could be duplicated by using varying proportions of pepsin and pepsinogen in the model system. The potentialities of employing these antisera to detect conformational changes such as the unmasking of the pepsin moiety in pepsinogen molecules modified by physical or chemical reagents are discussed.

INTRODUCTION

When pepsinogen is converted to pepsin according to the following scheme:

$$\text{Pepsinogen} \xrightarrow{[H^+]} \text{pepsin} + \text{inhibitor} + \text{miscellaneous peptides}$$

$$\text{inhibitor} \underset{\text{pH 5.6}}{\overset{<\text{pH 5.6}}{\rightleftharpoons}} \text{Pepsin-inhibitor complex}$$

the physical and chemical properties of the protein are altered. The evidence relating to those changes in the structure and properties of swine pepsinogen and pepsin has recently been reviewed by Herriott (10).

Since the reaction of some globular proteins with their antibodies is sensitive to conformational changes (14), it was thought that antibodies to pepsinogen and pepsin could be used to measure these alterations. Seastone and Herriott (26) and Lobachevskaya (15) have described the serological relationships of pepsinogen and pepsin. The present report is concerned with the quantitative characterization of these immune systems and their application for study of the structural interrelationships of the components of the pepsinogen system.

EXPERIMENTAL

Pepsinogen Swine gastric fundic mucosae were minced and extracted with 45 per cent saturated ammonium sulfate in 0.1 M sodium bicarbonate, followed by precipitation of the active pepsinogen with 68 per cent saturated ammonium sulfate (8 *a*). The precipitate was then dissolved in water and dialyzed against 0.005 M phosphate buffer, at pH 6.8. The crude pepsinogen was purified by chromatography on DEAE-cellulose (13, 25, 30). All three methods yielded preparations whose specific activity was comparable to that found by Herriott (8 *a*).

Pepsin Swine pepsin (3 times crystallized) was purchased from Worthington Biochemical Corporation. In experiments requiring the use of denatured pepsin, the enzyme was incubated in veronal buffer, pH 7.5, at 37°C for 2 to 24 hours.

Pepsin-Inhibitor Complex The complex was made either by the activation of pepsinogen (8 *a*, 9) or by admixture of pepsin and purified inhibitor at pH 5.6. An inhibitor preparation containing 0.016 mg N/ml in 75 per cent glycerine (from Dr. R. M. Herriott) or one that had been prepared by a modified procedure (29) was used in these studies.

Assays Peptic activity was determined by the milk clot test at pH 5.4 (8 *a*) or by using hemoglobin as substrate at pH 2 (2). Pepsinogen and inhibitor were determined using the modifications of the above assays (8 *a*, 9).

Antisera Rabbits were immunized *via* the toe pads with 20 mg of chromatographically purified swine pepsinogen in Freund's adjuvant. After 2 months, 40 ml of blood was removed. Each animal then received 5 mg of pepsinogen intravenously followed by bleeding 9 days later. The rabbits were again immunized by toe pad injection and bled 2 months later. Following this bleeding one intravenous injection of 5 mg protein was given and the animals bled 9 days later. Only the tubes in the center of the chromatographic peak were used for immunization since experience had shown that antisera produced against the whole pepsinogen fraction reacted with this antigen to give more than one band of precipitation by single diffusion analysis (22). These immunological impurities were not detected by electrophoretic, ultracentrifugal, or end-group analyses.

The immunization with pepsin was carried out in a similar manner. At the physiological pH of immunization, antipepsin is directed toward enzymatically inactive pepsin and antipepsinogen toward the native zymogen (26).

Immunochemical Procedures Quantitative precipitin analyses at pH 7.2 were done according to the method of Heidelberger and Kendall (7). Some precipitin analyses were done at pH 5.5 in a buffer containing 0.1 M maleate and 0.1 M tris (hydroxymethyl)aminomethane (tris). Prior to use at this lower pH, all antisera were dialyzed against this buffer. Quantitative complement (C′) fixation (17) was done according to the method of Wasserman and Levine (31) at pH 7.6. Diffusion constants were measured according to the method of Allison and Humphrey (1); and double diffusion by the method of Ouchterlony (21).

TABLE I

QUANTITATIVE PRECIPITIN ANALYSIS OF PEPSINOGEN-ANTIPEPSINOGEN

No.	Pepsinogen N added	Peptic activity recovered in precipitate	Ab N*	Mole ratio Ab:Ag	Supernatant analyses	
					+ Ab	+ Ag
	μg	*per cent*	μg			
1	2.7	100	59	6.08	−	++
2	5.3	107	113	6.00	−	++
3	10.4	111	187	5.04	−	++
4	15.7	104	238	4.25	−	+
5	21.0	114	266	3.55	−	−
6	26.6	95	290	3.05	−	−
7	54.2	48	277	2.91	+++	−

The immune precipitates were allowed to form at 2–4° for 48 hours. The washed precipitates were dissolved in 0.25 M acetic acid and the absorbancy taken at 277 mμ (18). The analyses were carried out in triplicate. Aliquots of the dissolved precipitate were brought to pH 2 for 10 minutes prior to enzyme assay (2).

* Rabbit antipepsinogen No. 21-4 (0.5 ml).

RESULTS

Immunochemical Characterization of Antipepsinogen

All antisera were examined for immunochemical homogeneity by double diffusion in agar. Solutions of crude pepsinogen and antiserum, diffusing simultaneously from different wells on an agar plate, gave a single band of precipitation over a wide dilution of antigen and antiserum. Only with Ra 21-4 (the antiserum after 4 courses of immunization) was a second band observed when crude pepsinogen was used as antigen.

One method used for the identification of the antibody was the recovery of the antigen in the washed immune precipitate. In this system, the pH necessary for the dissociation of the antigen-antibody complex (27) is also favorable for the conversion of pepsinogen to pepsin (8 *a*, *b*) and for the biological assay of the enzyme (2). The data in Table I show that all of the added pepsinogen was precipitated by the antipepsinogen in the regions of

antibody excess and equivalence. In these zones of the precipitin reaction, no pepsinogen was found in the supernatant fluid, either by immunological or biological assay. Peptic activity was, however, found in the supernatant fluid of the antigen excess zone.

Using immunodiffusion (1), pepsinogen and antipepsinogen were allowed to diffuse from troughs set at right angles to each other. The resulting band of precipitation formed an angle of 54.0°. Calculation of the diffusion constant gave a $D_{20,w}$ of 7.2 × 10^{-7} cm^2/sec. The diffusion constant obtained by another method gave a value of 7.54 × 10^{-7} cm^2/sec. (19).

If the immune complex is allowed to form at pH 5.6 antipepsinogen neutralizes the enzymatic activity of pepsin (Fig. 1).[1] While this shows that an antigen-antibody reaction has taken place, it does not necessarily prove that the antibody is directed toward the enzymatically active site. Similar

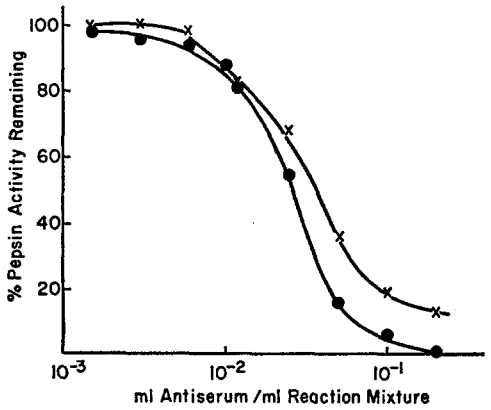


FIGURE 1. Neutralization of the enzymatic activity of pepsin by increasing quantities of antipepsinogen, ● (Ra 47 B-1) and antipepsin, × (Ra 249A-5). Pepsin (15 μg) was incubated with each dilution of antiserum at pH 5.6, 2–4°, for 20 hours in a final volume of 1 ml. Samples were withdrawn and assayed directly for rennet activity.

results may be obtained if the antibody sterically hindered the formation of the enzyme-substrate (casein) complex. The degree of inhibition observed with several antienzymes varies with the molecular weight of the substrate; inhibition is usually greatest against the larger substrate (4, 5). Unfortunately, a small substrate susceptible to pepsin at a pH where the antigen-antibody complex is formed is not readily available.

If the immune precipitate resulting from the reaction of pepsin and antipepsinogen at pH 5.6 is subjected to pH 8, the pepsin is no longer denatured (Table II). Pepsin in normal sera or pepsin present during the formation of a bovine serum albumin (BSA)–anti-BSA immune precipitate is denatured indicating that non-specific factors were not involved. The enzyme, locked in the immune complex, may be prevented from undergoing the conformational changes characteristic of the denatured state (6, 10).

Pepsinogen reacted with antipepsinogen to give a normal C′ fixation curve

[1] These results are from experiments carried out by Miss Barbara Cunningham.

(Fig. 2A). Activated pepsinogen, pepsin, inhibitor, or the pepsin-inhibitor complex did not react by direct C′ fixation under these conditions. The possibility that the products of the pepsinogen conversion did react with antipepsinogen but did not possess the multivalent properties requisite for C′ fixation (20) was investigated by inhibition techniques (31). The activation mixture completely inhibited the pepsinogen-antipepsinogen system (Fig. 2B). No inhibition was observed with the purified inhibitor or the inhibitor plus miscellaneous peptides isolated from the trichloracetic acid–soluble fraction of the conversion mixture (9, 28). Pepsin inhibited the pepsinogen-antipepsinogen reaction, but not as efficiently as did activated pepsinogen. A

TABLE II

RECOVERY OF PEPTIC ACTIVITY FROM THE PEPSIN-ANTIPEPSINOGEN IMMUNE PRECIPITATE SUBJECTED TO VARIOUS HYDROGEN ION CONCENTRATIONS

pH	Activity recovered	
	Immune serum	Normal serum
	per cent	*per cent*
5.6	93	101
6.0	101	119
6.5	102	25
7.0	106	6
7.5	107	3
8.0	98	0.3

Pepsin (240 μg in 1.0 ml 0.02 M maleate + 0.02 M tris buffer (pH 5.6), Γ adjusted to 0.15 with NaCl) was added to 2.0 ml of antipepsinogen (Ra 21-4) or normal serum which had been dialyzed against the same buffer. The reaction mixture stood at 4°C for 14 hours. NaOH (0.02 N Γ = 0.15 with NaCl) was added slowly with stirring to the desired pH. Aliquots were incubated at 37° for 30 minutes and assayed (2) after dissociation of precipitate at pH 2. Rennet assays (8) of the experimental sample at pH 5.6 showed less than 1 per cent activity while the control with normal serum showed neither neutralization nor loss of activity.

maximal inhibition of 70 to 80 per cent was obtained even with high concentrations of pepsin. On a molar basis, approximately four times more pepsin than activation mixture was required for comparable inhibition. The complex, formed by admixture of purified inhibitor and pepsin at pH 5.6 and brought to neutrality prior to assay, equaled the inhibition observed with activated pepsinogen. This suggests that the pepsin-inhibitor complex resembles pepsinogen more closely than pepsin. This difference in the immunological reactivity of pepsin and the complex can be used to determine whether combination between pepsin and the inhibitor has taken place. Such an assay would be useful in cases in which the biological activity cannot be measured.

In order to determine whether native or denatured pepsin resembles the

zymogen, precipitin analyses were carried out at pH 5.5. Pepsinogen, which reacted with antipepsinogen to the same extent at pH 5.5 as it did at pH 7.2, precipitated more antibody nitrogen than did either native or denatured pepsin (Fig. 3). Native pepsin precipitated more antibody nitrogen than denatured pepsin, indicating that the pepsin moiety of the zymogen is more like native than denatured pepsin. The lower precipitating activity of denatured pepsin may reflect the alteration in conformation which results when the enzyme is inactivated.

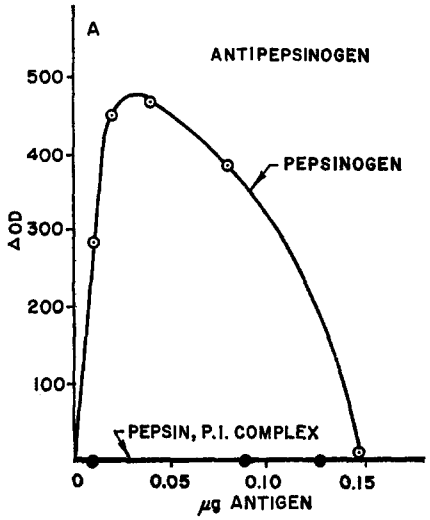
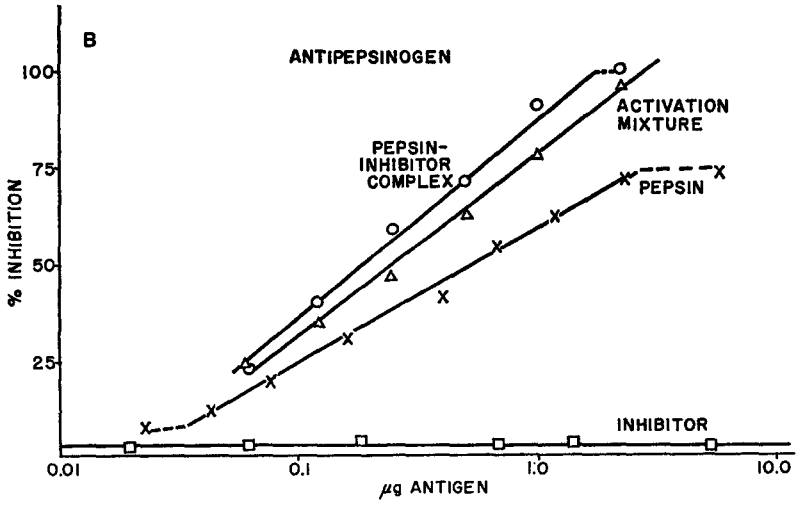


FIGURE 2. A. Fixation of C′ by increasing quantities of pepsinogen, ⊙, pepsin, ●, and the pepsin inhibitor complex, ●, with antipepsinogen (Ra 21-3) diluted 1:3300.
B. C′ fixation inhibition of pepsinogen-antipepsinogen by activated pepsinogen, △, pepsin-inhibitor complex, ○, pepsin, ×, and inhibitor, □. Pepsinogen concentration 0.02 μg; Ab (Ra 21-3) diluted 1:3300.



Immunochemical Characterization of Antipepsin

The antibodies produced by rabbits immunized with pepsin could also be identified by immunodiffusion analysis of the precipitating antigen (1). The $D_{20,w}$ of denatured pepsin reacting with antipepsin was found to be 8.05 × 10^{-7} cm^2/sec. Free diffusion measurements have yielded values of 8.7 × 10^{-7} cm^2/sec. for native pepsin and 6.9 × 10^{-7} cm^2/sec. for denatured pepsin (6). The latter value is questionable in view of the fact that the pepsin preparation had undergone a decrease in molecular weight.

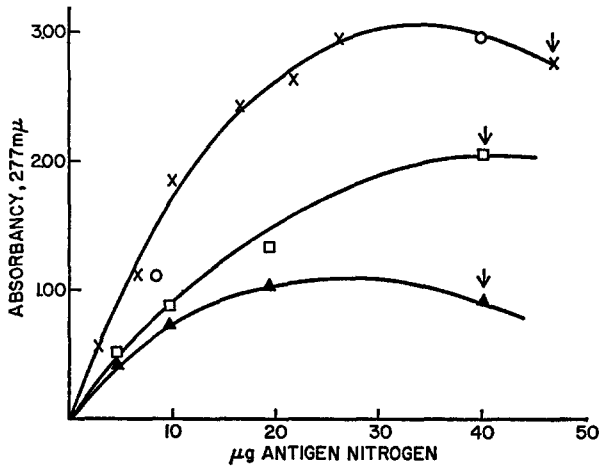


FIGURE 3. Analysis of immune precipitates of pepsinogen, ×, with 0.5 ml of antipepsinogen (Ra 21-4) at pH 7 and of pepsinogen, ○, native pepsin, □, and denatured pepsin, ▲, with 0.5 ml of antipepsinogen (Ra 21-4) at pH 5.5. The absorbancy obtained from washed immune precipitates dissolved in 2.0 ml of 0.25 M acetic acid measured at 277 mμ is plotted (18). The vertical arrows, ↓, indicate regions of antigen excess.

The addition of increasing amounts of antipepsin sera to a constant amount of pepsin at pH 5.6 progressively neutralized the enzymatic activity of the antigen, yielding the sigmoid curve shown in Fig. 1. In contrast to the antipepsinogen sera, complete neutralization of enzymatic activity was never achieved. Although both antisera neutralize the enzymatic activity of pepsin, only antipepsinogen protects pepsin from alkaline denaturation at pH 8.0 (Table II). These differences may be a function of the potency of the antisera and/or the character of the immune complex. The pepsin antisera were in general weaker, did not form immune precipitates with pepsin at pH 5.5, and did not neutralize the last 10 per cent of peptic activity.

Since pepsin is denatured at neutrality, experiments designed to identify the antibody by recovery of peptic activity in the immune precipitate formed at pH 7 could not be done. Therefore, the immune precipitation was per-

formed at pH 5.5 at 2–4°C for 3 days. Despite the range of antibody to antigen concentrations, precipitation was not obtained with either native or denatured pepsin using antipepsin although in some cases over 80 per cent of the enzyme activity had been neutralized and full peptic activity was recoverable after dissociation at pH 2. Pepsin added to normal sera still possessed full activity at pH 5.5 after standing 3 days at 4°C.

The inability of an antigen to form an immune precipitate with an antibody at pH 5.5 is not characteristic of all immune systems. Pepsinogen-antipepsinogen as well as BSA–anti–BSA gives superimposable precipitin curves in the tris-maleate buffer at pH 5.5 and in phosphate buffer at pH 7.2. Singer (27), using ultracentrifugal analysis of BSA–anti–BSA immune precipitates, has

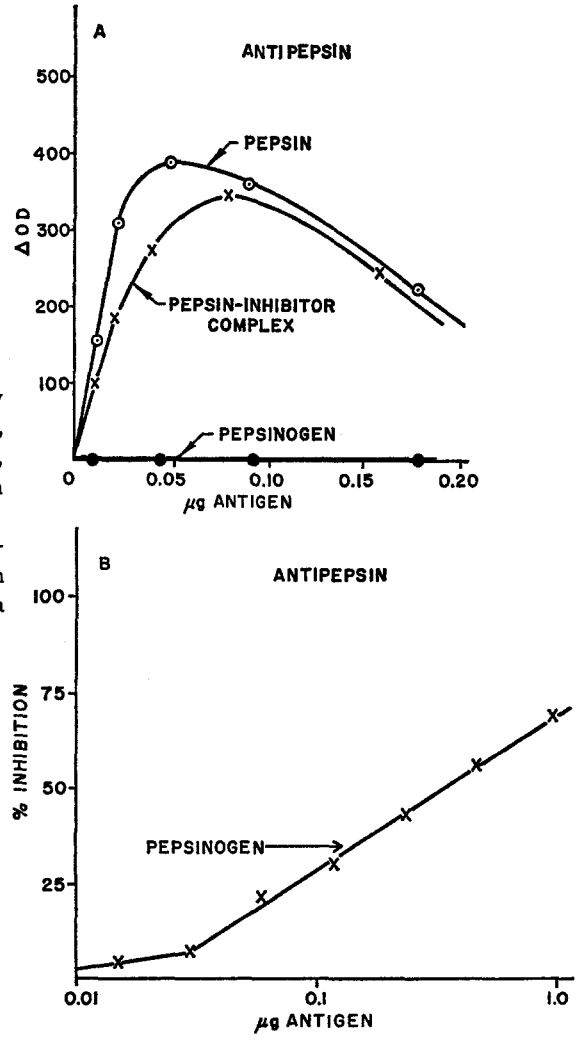


FIGURE 4. A. Fixation of C′ by increasing quantities of pepsin, ⊙, pepsin-inhibitor complex, ×, pepsinogen, ●, with antipepsin Ra 250A-5) diluted 1:450. B. C′ fixation inhibition of pepsin-antipepsin by pepsinogen, ×. Pepsin concentration 0.06 μg; Ab (Ra 250A-5) diluted 1:450.

shown that there is no effect of pH on the antigen-antibody equilibrium in the range of 4.5–7.5.

Since antipepsin will not precipitate pepsin at pH 5.5, it can be inferred that either the antibody and/or the antigen-combining sites are affected by this change in pH so that a lattice can no longer be sufficiently built up to

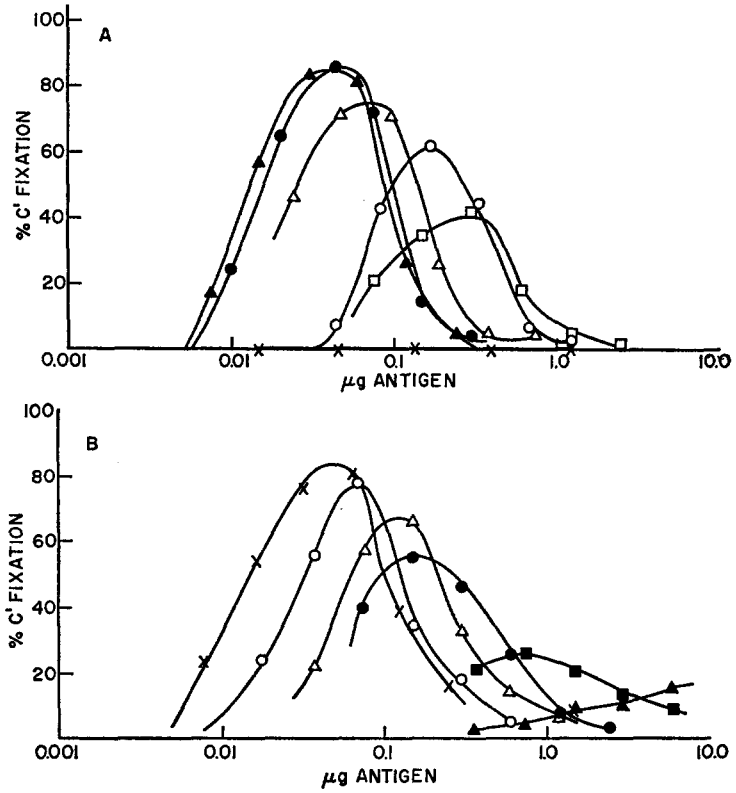


FIGURE 5. C′ fixation of pepsinogen and the products produced during its autocatalytic conversion. A. Antipepsinogen; B. Antipepsin. Eight mg of pepsinogen was dissolved in 5.0 ml of 0.1 M acetate buffer, pH 4.6. Samples were withdrawn at various intervals and diluted into cold water or veronal buffer for enzyme (8*a*) and immunologic assay respectively. ×, 100 per cent pepsin; □, 80 per cent pepsin, 20 per cent pepsinogen; ○, 58 per cent pepsin, 42 per cent pepsinogen; △, 40 per cent pepsin, 60 per cent pepsinogen; ●, 20 per cent pepsin, 80 per cent pepsinogen; ■, 4 per cent pepsin, 96 per cent pepsinogen; ▲, 100 per cent pepsinogen.

cause precipitation. Besides possessing negligible amounts of helix (23), pepsin is unusual in that it possesses few basic residues and contains high quantities of carboxylic, hydroxy, and non-polar amino acids. These characteristics of the pepsin molecule may be responsible for this anomalous behavior.

The C′ fixation curve describing the pepsin-antipepsin system is shown in Fig. 4A. The pepsin-inhibitor complex reacts somewhat less than does pepsin

itself. Alteration in the structure of pepsin during complex formation and/or the masking of antigenically reactive groups by the inhibitor are possible explanations for the observed difference. Pepsinogen does not react directly but functions as an inhibitor in the pepsin-antipepsin reaction (Fig. 4B).

Conversion of Pepsinogen to Pepsin

Pepsinogen, when activated autocatalytically to form pepsin at pH 4.6, loses its ability to react with antipepsinogen by direct C′ fixation. As the conver-

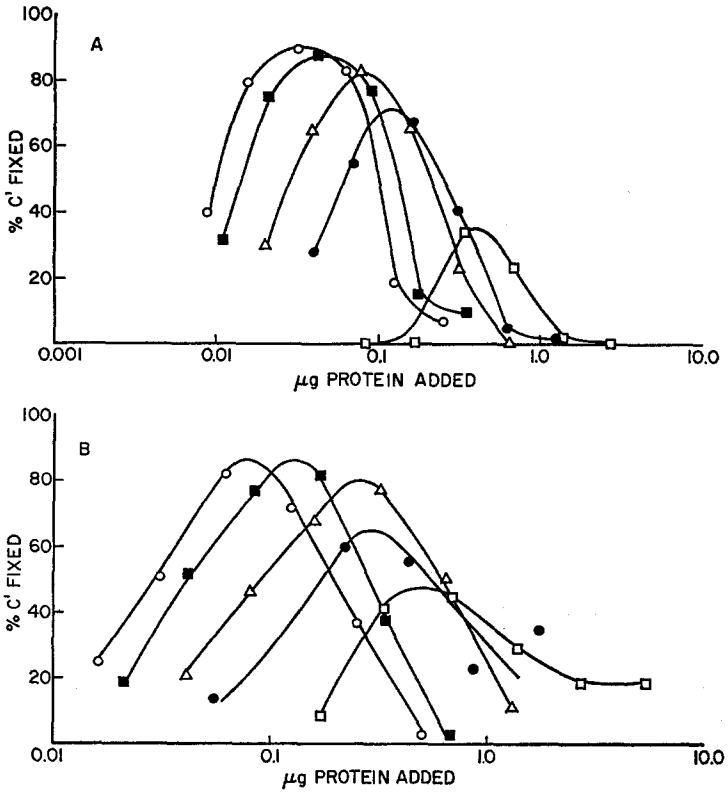


FIGURE 6. A. Fixation of C′ by pepsinogen with antipepsinogen (Ra 21-3) diluted 1:3300. To a constant amount of pepsinogen (0.25 μg/ml) was added varying amounts of pepsin. The two proteins were diluted simultaneously. ○, 100 per cent pepsinogen, 0 pepsin; ■, 71 per cent pepsinogen, 29 per cent pepsin; △, 39 per cent pepsinogen, 61 per cent pepsin; ●, 20 per cent pepsinogen, 80 per cent pepsin; □, 9 per cent pepsinogen, 91 per cent pepsin.

B. Fixation of C′ by pepsin with antipepsin (Ra 250A-8) diluted 1:800. To a constant amount of pepsin (0.50 μg/ml) was added varying amounts of pepsinogen. The two proteins were diluted simultaneously. ○, 100 per cent pepsin, 0 per cent pepsinogen; ■, 74 per cent pepsin, 26 per cent pepsinogen; △, 38 per cent pepsin, 62 per cent pepsinogen; ●, 29 per cent pepsin, 71 per cent pepsinogen; □, 9 per cent pepsin, 91 per cent pepsinogen.

sion proceeds, there is a progressive increase in the amount of added protein needed to give maximal fixation as shown by a lateral displacement of the peak (Fig. 5A). This is indicative of a loss of antigenic activity. There is also a decrease in the height of peak fixation after 50 per cent of the pepsinogen has been converted to pepsin. With antipepsin, however, a progressively smaller quantity of the conversion mixture is needed to give peak fixation and an increase in peak height is observed as the activation proceeds (Fig. 5B).

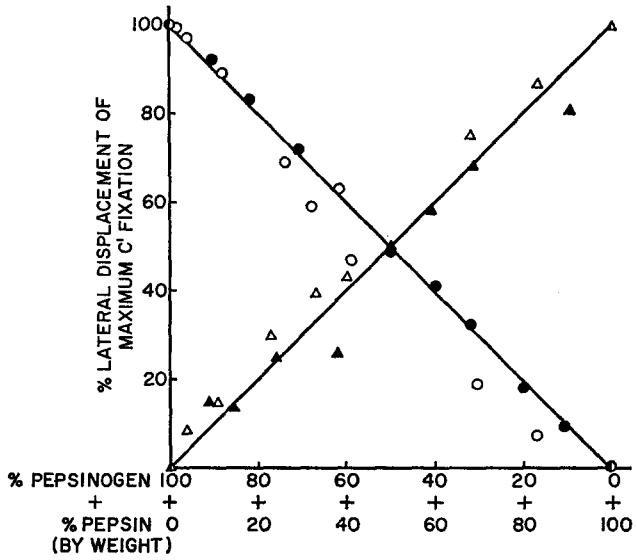


FIGURE 7. Per cent lateral displacement of maximum C′ fixation plotted against varying proportions of pepsinogen and pepsin. In the model system, the amounts of pepsin and pepsinogen were determined by weight. In the activation mixture, pepsin was determined by milk clot assay (8*a*) and pepsinogen was determined by difference. Model system, ●, and activation mixture, ○, with antipepsinogen; model system, ▲, and activation mixture, △, with antipepsin.

The results could be interpreted in two ways. First, a cross-reacting antigen; that is, one that is qualitatively different from the homologous antigen may have been formed. However, the presence of both an inhibitor and the intact homologous antigen in varying proportions might be responsible for the observed results. Since the products of the autocatalytic conversion are known it was possible to design experiments to test these alternatives. Varying quantities of pepsin were added to a constant amount of pepsinogen and the resulting mixture tested by C′ fixation with antipepsinogen (Fig. 6A). Similarly, pepsin was maintained constant and pepsinogen was varied and the mixture was assayed with antipepsin (Fig. 6B). The data are plotted as a function of the amount of protein (varying percentages of pepsin and pepsinogen) added.

The per cent lateral displacement at maximum C′ fixation varies propor-

tionally with the per cent pepsinogen and pepsin when measured by anti-pepsinogen and antipepsin (Fig. 7). In the model system, the amount of pepsin and pepsinogen was determined by weight of the purified proteins. In the autocatalytic conversion mixture, the per cent pepsin was determined by enzymatic activity and pepsinogen by difference.

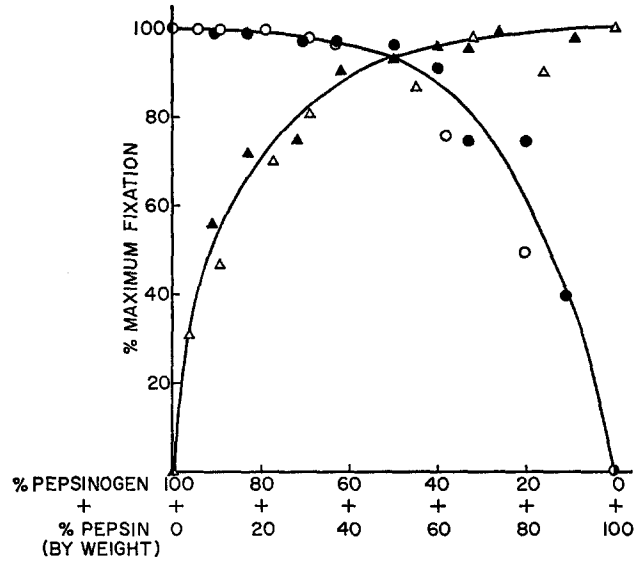


FIGURE 8. Per cent maximum fixation plotted against varying proportions of pepsinogen and pepsin. Samples and identifying symbols are the same as those described in Fig. 7.

If the per cent maximum peak fixation in the autocatalytic conversion and model systems is plotted against the per cent pepsinogen plus pepsin, one obtains the curves shown in Fig. 8. With both antisera, a significant decrease in peak height is obtained when the ratio of homologous to heterologous antigen approaches 1:1. The curves drawn are for the model system but the results obtained from the autocatalytic conversion fit these data (Fig. 8).

DISCUSSION

Physical and chemical studies (10, 11, 16, 28, 29) support the concept that pepsinogen is a single polypeptide chain, with a compact conformation. Rotatory dispersion measurements indicate that pepsinogen possesses some helix (24) while pepsin, even though it constitutes seven-eighths of the zymogen, possesses little if any helix (23). When pepsin is denatured at neutral pH, further structural changes detectable by several physical techniques occur (6, 10).

Antibodies to pepsinogen and denatured pepsin can detect the specific con-

formation of their homologous antigens. The antipepsin prepared by Seastone and Herriott (26) reacted with pepsinogen but precipitation was not obtained between pepsin and antipepsinogen. On the other hand, Lobachevskaya (15) found that pepsin reacted with antipepsinogen and the antibodies elicited by pepsinogen reacted with pepsin. Arnon and Perlmann (3) using the sensitive passive cutaneous anaphylaxis technique also observed cross-reactions between pepsin and antipepsinogen indicating common antigenic determinants in pepsin and pepsinogen. Some of the contradictory observations (15, 26) are probably attributable to the strength of the antisera employed and the means of assay.

Evidence that the antibodies are directed toward pepsinogen or pepsin was obtained from the following experiments: (*a*) The diffusion coefficients obtained when these proteins and antipepsinogen or antipepsin diffused and precipitated in agar from depots set at a 90° angle (1). (*b*) Inhibition of peptic activity by antipepsinogen or antipepsin at pH 5.5. (*c*) Complete recovery of enzyme activity from washed pepsinogen-antipepsinogen precipitates in the regions of antibody excess and equivalence. (*d*) Pepsin's protection from denaturation at neutral and alkaline pH when complexed with antipepsinogen.

Antibodies directed specifically toward pepsinogen or denatured pepsin were demonstrated by the greater C′ fixation with pepsinogen or pepsin respectively. Quantitative precipitin analyses with antipepsinogen at pH 5.5 also showed that conformational differences exist among pepsinogen, pepsin, and denatured pepsin. Pepsinogen precipitates more antibody nitrogen from antipepsinogen than does native or denatured pepsin. The latter, despite the shape changes it has undergone, still cross-reacts in this system.

Specific antibodies to pepsinogen and pepsin were used to measure the autocatalytic conversion of pepsinogen to pepsin. At pH 4.6, the decrease in pepsinogen concentration is accompanied by the concomitant and equal increase in pepsin activity and hydrolysis of peptide bonds (8 *b*). When this conversion was followed immunologically with the homologous antisera, the loss of precursor and the gain of enzyme could be calculated from the shift of the C′ fixation curves (Fig. 7). The change in height of such peaks represented the formation of an inhibitory molecule (in the assays with antipepsinogen) or the destruction of an inhibitory molecule (in the assays with antipepsin). Such results would normally, but incorrectly, have been interpreted as a qualitative change in the molecule. However, since the components of the autocatalytic conversion are known, suitable model systems could be set up varying the homologous antigen and its inhibitor. Any marked deviation from such curves would reflect the formation of a molecule which differs from pepsin qualitatively. The correlations obtained when the results of the autocatalytic conversion mixtures are compared with the model system suggest that little, if any, stable intermediate with differing immunological properties is formed.

The unmasking of pepsinogen to reveal the pepsin moiety also occurs in at least four additional ways which do not involve the concomitant hydrolysis of peptide bonds observed during the autocatalytic activation. If pepsinogen is treated with alkali, urea, heat, or photooxidized with methylene blue (12), the pepsin moiety of the zymogen is revealed. This conformational change is detected immunologically by an increased reactivity with antipepsin sera.[2] This technique also permits the detection of structural changes beyond the unmasking and in this way has certain advantages over some of the physical techniques. The conformational changes caused by various physical and chemical agents in the components of the pepsinogen system will be the subject of further communications.

At pH 7, where the C′ fixation assays were carried out, denatured pepsin proved to be a potent inhibitor of the pepsinogen-antipepsinogen system but the pepsin-inhibitor complex gave complete inhibition with a smaller mole ratio of material. In addition, pepsinogen precipitated more antibody than either native or denatured pepsin. Both observations suggest that antipepsinogen contains antibodies directed toward the inhibitor. The possibility exists, however, that the enhanced inhibition of the pepsin-inhibitor complex is caused by an altered conformation of pepsin resulting from complexing with the inhibitor. Since pepsinogen is a potent inhibitor of the pepsin-antipepsin system, it must possess some sites which are antigenically similar to pepsin. It is probable that such antigenic sites do not reside on a helical portion of the zymogen since antipepsin is directed against the denatured pepsin which appears to possess no helix and is even devoid of some of the ordered structure present in the native enzyme (6, 23).

Although both pepsinogen and pepsin are weak antigens, pepsinogen has yielded antisera at least tenfold more potent than pepsin in the fifteen rabbits that were immunized during the course of this investigation. This might be a reflection of the greater helical configuration of the zymogen molecule. In studies with RNase and performic-oxidized RNase, the native enzyme was found to be vastly more superior in eliciting the antibody response than was the molecule devoid of secondary and tertiary structure (4). However, the role of the helix in antibody production should be demonstrable by immunization with helical and non-helical polypeptides. Arnon and Perlmann (3) have enhanced the antigenicity of pepsinogen by attaching tyrosine peptides to the molecule.

[2] The use of antipepsin to detect either pepsin or "pepsin-like" structures offers a sensitive criterion for determining contamination or degradation in pepsinogen preparations. We have examined some commercial pepsinogen preparations and have found appreciable amounts of material which react with antipepsin. Even with our best preparations, some fixation was observed with pepsinogen at high concentrations. This reactivity prevented us from using greater amounts of pepsinogen to inhibit the pepsin-antipepsin reaction (Fig. 4B).

The authors are grateful to Miss Monique Delpeuch for technical assistance.
Contribution No. 184 from Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts.
Supported in part by grants from the National Science Foundation (G-20,029) and the National Institutes of Health (E-1940) and (E-2792).
Some of these results were presented at the 5th International Congress for Biochemistry, Moscow, 1961, and the 46th Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, 1962.
Received for publication, July 31, 1962.

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