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Pepsinogen and Pepsin: Further Immunochemical Studies of the Conformational Changes Involved in the Formation of Porcine and Human Pepsins from Their Zymogens*

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Received January 6, 1964

Qualitative-immunodiffusion and quantitative-precipitation studies show that porcine pepsinogen (Pg) and pepsin (P), and human gastric zymogen [Z], pepsin [P], and gastricsin [G], have common antigenic determinant groups. The degree of their reaction with antiporcine pepsinogen antibody is in the order Pg > P > [Z] > [P] ≫ [G]. By precipitation of these systems with horse antirabbit globulin antibodies this decrease is shown to be caused by a loss in the number of determinant groups rather than a change in binding constants of the groups. This implies that, except perhaps in the catalytic region, the conversion of the pepsinogen molecule to pepsin involves no major conformational alteration. Although the molecular weight of [P] is less than that of [G] it is immunochemically more closely related to the human zymogen. This indicates that if [P] and [G] are both derived from [Z], the [P] is derived independently rather than sequentially through [G].

A number of investigations have been reported recently in connection with efforts to elucidate the conformational relation between pepsin and its precursor, pepsinogen (Van Vunakis *et al.*, 1963; Arnon and Perlmann, 1963; Schlamowitz *et al.*, 1963). These studies employed immunochemical as well as physicochemical methods. The value of the immunochemical approach to this problem stems from the fact that, because the zymogen and enzyme are polyvalent antigens, the detection of conformational changes is not limited to the region of the biochemically measurable catalytic site of the molecule.

The purpose of this report is to provide additional information regarding the number of combining sites of pepsinogen and pepsin for antipepsinogen antibodies, and to show that their binding constants have not been affected by the pepsinogen-to-pepsin conversion. Since binding data reflect conformational similarity or difference they are particularly relevant to an understanding of the extent of conformational changes that accompany the conversion.

This study also includes a comparison of the structural relation of human and porcine gastric zymogens and enzymes as deduced from immunochemical comparisons. Further, information is presented which bears on the question of whether the two proteases, human pepsin [P] and gastricsin [G], are derived sequentially or via independent pathways from their common precursor, the human zymogen [Z].[1]

* This research was supported in part by Institutional Grant 1-GS-125, and by a grant (AM-07174-01) from the National Institutes of Health, U. S. Public Health Service.

MATERIALS

Porcine Pepsinogen, Pepsin.—The pepsin used was a three-times-crystallized preparation (Pentex Co., Inc., lot D3709) obtained by fractionation with ethanol according to Northrop (1946).

Pepsinogen, prepared by a modification of the DEAE-cellulose chromatographic method of Ryle (1960), was obtained from Worthington Biochemical Corp. (lot 6007-10). It contained about 2% insoluble material that was removed before use.

These materials are the same as those that were characterized previously (Schlamowitz *et al.*, 1963).

Human Zymogen, Pepsin, Gastricsin.—Samples of the human zymogen, pepsin, and gastricsin were obtained from Dr. J. Tang. Their designations, molecular weights, and estimated purities are as given in Table I. The purity of these samples is defined in terms of their specific activities relative to the maxi-

[1] The following notations will be used: porcine pepsinogen, Pg; porcine pepsin, P; human gastric zymogen, [Z]; human pepsin, [P]; human gastricsin, [G].

TABLE I
PROPERTIES OF HUMAN ZYMOGEN, PEPSIN, AND GASTRICSIN

Substance	Sample Designation	Mol Wt[a]	Purity (%)
Zymogen	[Z]	36,000	50
Pepsin	$[P]_2$	30,500	88
	$[P]_3$		88–92
Gastricsin	$[G]_2$	35,000	62
	$[G]_3$		75

[a] Dr. Jordan Tang, Oklahoma Medical Research Institute, private communication.

mum activities obtained for chromatographically purified zymogen, pepsin, and crystalline gastricsin.[2]

Rabbit Antiporcine Pepsinogen Serum.—Antiserum 3113, previously described (Schlamowitz *et al.*, 1963), had been lyophilized and was reconstituted with 0.01 M sodium phosphate buffer, *p*H 5.8, just before use for these experiments. For the immunodiffusion studies concentrates of the globulin fraction from the normal serum and the antiserum were prepared using a slight modification of the method of Kekwick (1940) employing three precipitations at 16%, 14%, and 14% Na_2SO_4. For all other experiments the reconstituted antiserum was used without fractionation.

Horse Antirabbit Globulin Antibodies.—The preparation of horse antirabbit globulin serum has been described (Schlamowitz, 1958). In the present study the globulin fraction of the serum (obtained by fractionation with Na_2SO_4 as described for the rabbit sera) was used.

Phosphate-saline Buffer.—Sodium phosphate buffer, 0.01 M, *p*H 5.8, was made up to ionic strength 0.15 with sodium chloride.

METHODS

Immunodiffusion.—Immunodiffusion was carried out at room temperature in 2% Difco-Noble agar gels, about 2.2 mm thick. The wells (2 mm in diameter, 5 mm apart) were filled with approximately 7 μl of solution and diffusion was allowed to proceed in a moist chamber for about 20 hours.

Phosphate-saline buffer was used for preparing the agar gels as well as for making up solutions of the antigens. All antigens were used in molar equivalent concentrations, i.e., corrected for molecular weight and per cent purity. The globulin solutions were adjusted to *p*H 5.8 before use.

Characterization of Antigens by Direct Quantitative Constant-Antibody Precipitation.—Precipitation studies were run on each antigen in a series of small tubes, each tube containing 0.2 ml of rabbit serum and 0.2 ml of antigen solution (containing increasing amounts from 2.5 μg to 100 μg of pepsin or the molar equivalent amounts of each of the other antigens). The solutions were adjusted to *p*H 5.8 before mixing and 0.6 ml *p*H 5.8 phosphate-saline buffer was added to make the total volume 1.0 ml. Mixtures were set for 5 minutes at 37°, 1 hour at room temperature, and 44 hours at 4°, after which precipitates were centrifuged for 30 minutes at 1900 × *g* at 4°. Portions of the clear supernatant solutions were assayed for remaining proteolytic activity at *p*H 1.7.

Determination of Bound Antigen by Quantitative Constant-Antibody Precipitation in the Presence of Horse Antibody.—In these studies horse antirabbit globulin antibody was added to insure complete precipitation of all antigen bound to the rabbit antibodies. Precipitation tubes were set up, each containing 0.2 ml of rabbit serum and 0.2 ml of antigen solution (containing increasing amounts of antigen, up to the equivalent of 200 μg pepsin in some cases). These were allowed to react 5 minutes at 37° and 1 hour at room temperature before 0.6 ml of horse antibody solution was added. This amount of horse antibody solution had previously been established to be capable of precipitating all the γ-globulin present in 0.2 ml rabbit serum. Controls with normal serum in place of rabbit antiserum were set up to establish the specificity of the coprecipitation.

After the mixtures were set for 5 minutes at 37°, 1 hour at room temperature, and 44 hours at 4°, the precipitates were centrifuged at 1900 × *g* for 30 minutes at 4°. The supernatant fluids were assayed for remaining proteolytic activity.

Determination of Proteolytic Activity.—The enzymes and activated zymogens were assayed at *p*H 1.7 with buffered (0.03 M glycine) hemoglobin (1.67%) solutions. Under these conditions pepsinogen and human zymogen are activated instantaneously. The digestions were run at 37° in 3 ml total volume for periods of time ranging from 12 minutes to 2 hours to allow for equal enzymatic reaction in the controls. Reaction mixtures were deproteinized with 5 ml of a 5% solution of trichloroacetic acid and the absorbancy of the filtrates at 280 mμ was measured. All values were corrected for absorbance of trichloroacetic acid filtrates of hemoglobin, rabbit serum, and horse antibody.

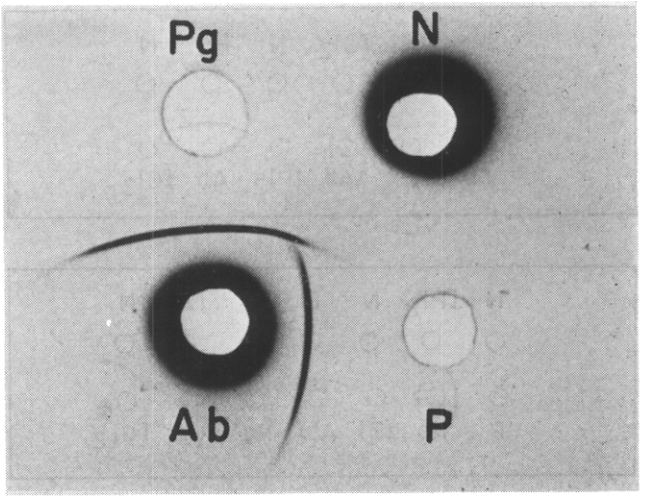


FIG. 1.—Immunodiffusion of porcine pepsinogen, Pg, and pepsin, P, against antiporcine pepsinogen antibody globulin, Ab, and normal globulin, N.

RESULTS

Immunodiffusion of Porcine Pepsinogen, Pepsin and Human Zymogen, Pepsin and Gastricsin.—In Figure 1 is seen the immunochemical reaction of porcine pepsinogen (Pg) and pepsin (P) with antipepsinogen antibodies. Both antigens gave strong lines of precipitation. However, the spur is indicative of the partial-identity nature of the pepsin reaction, a consequence of the loss of some antigenic determinants.

In Figure 2 are the results of immunodiffusion of all the test antigens arranged in a manner that was designed to permit detection of all cross-reacting possibilities. The antigens were tested at molar-equivalent concentrations under conditions where the zymogens and enzymes are stable.

Human zymogen [Z] reacts with antiporcine pepsinogen antibodies less strongly than does porcine pepsin,

[2] Dr. Jordan Tang, Oklahoma Medical Research Institute, private communication.

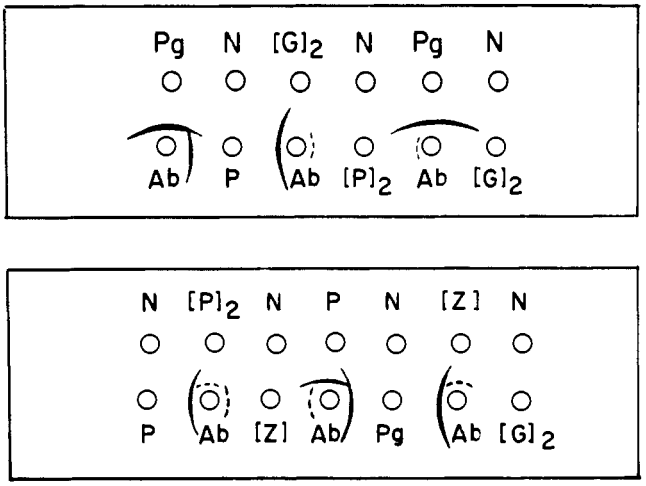


FIG. 2.—Representative sketch of immunodiffusion reactions of porcine and human gastric zymogens and enzymes, Pg, [Z], P, $[P]_2$, $[G]_2$, against antiporcine pepsinogen antibody globulin, Ab, and normal globulin, N. All test antigens are used in molar-equivalent amounts. The broken lines (-----) indicate weak precipitation lines.

showing the existence of even fewer common cross-reacting determinants. Two preparations of human pepsin, $[P]_2$ and $[P]_3$, showed discernible though very weak reactions. No detectable reaction was seen with one sample of human gastricsin, $[G]_2$, and only an extremely weak reaction was seen with the other sample, $[G]_3$. In no case were artifactual precipitation lines formed against normal globulin.

Constant-Antibody Quantitative Precipitation Reactions of Porcine Pepsinogen, Pepsin and Human Zymogen, Pepsin and Gastricsin.—Precipitation data shown in Figure 3 permit a quantitative evaluation of the immunodiffusion results described. Porcine pepsinogen, the homologous antigen, shows the greatest reaction with its antibody of any of the test antigens. The extensive region of complete precipitation and the state of antibody excess observed at low antigen concentrations are indicative of the large number of antibody species directed against its determinant groups. Of the remaining test antigens porcine pepsin shows the next greatest quantitative configurational similarity to pepsinogen. The number of reacting antibody species is less than for pepsinogen, as evidenced by the narrower range of complete precipitation and the earlier attainment of the state of antigen excess. However, this number is still relatively large since here, too, a state of antibody excess is readily attained.

Both human zymogen and human pepsin have antigenic determinants that simulate those of the porcine antigens in that they, too, are able to react with antibodies against the porcine antigen. However, as Figure 3 shows, the number of such groups is even less and a state of antigen excess is more readily achieved due to the correspondingly smaller number of utilizable antibody species. As was the case for the porcine antigens, the number of determinants in the human zymogen is greater than that in the human pepsin. The sample of gastricsin, $[G]_2$, which gave no observable reaction in the immunodiffusion studies, also failed to precipitate under conditions where human zymogen and pepsin precipitated extensively.

Estimation of the Concentration and Binding Strength of Combining Sites of Porcine and Human Gastric Zymogens and Enzymes.—The quantitative precipitation curves described above distinguish the several antigen-antibody systems and reflect the decrease in the number of common antigenic sites in porcine pepsin

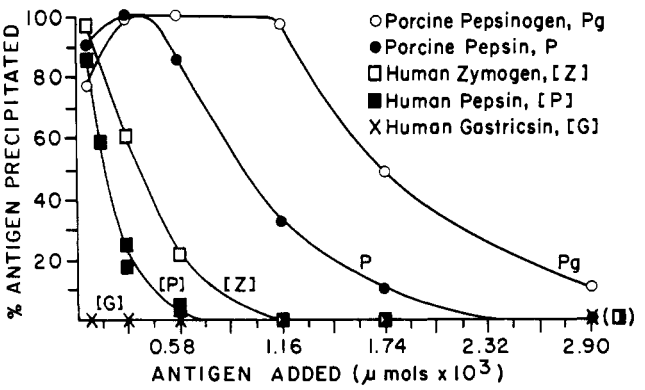


FIG. 3.—Constant-antibody precipitation of 0.072–2.9 μmoles porcine and human gastric zymogens and enzymes by 0.20 ml of antiporcine pepsinogen serum at *p*H 5.8.

and human zymogen and enzyme relative to porcine pepsinogen. However, they are not able to quantitate the *two* factors that characterize an antigen-antibody system, namely, the number of combining groups on the antigen and the binding constants of these groups. The last mentioned factor is of special significance in assessing whether the conversion of pepsinogen to pepsin involves changes limited to the region that becomes the catalytic site, or whether it entails conformational reorientation of the entire molecule. Both situations could result in a decrease in the number of determinant groups, but probably only in the latter would the binding constants of the remaining determinant groups be altered.

Both factors may be evaluated from the study of a constant-antibody variable-antigen system provided that the concentrations of free and bound antigen sites at equilibrium can be determined. For the reaction of an antigen containing independent binding sites and an antibody containing independent sites with equivalent binding strengths (Samuels, 1963; Klotz, 1954) the equation of the general form applies.[3] Here

$$\frac{x}{E - x} = -Kx + KAb$$

E is the total concentration of antigen sites, x is the concentration of bound antigen sites, and Ab is the total concentration of antibody sites.

The ratio of bound to free antigen sites is a linear function of the concentration of bound antigen sites and a plot of $x/(E - x)$ versus x yields a straight line with negative slope, $-K$, where K is the intrinsic equilibrium-binding constant. The intercept on the abscissa is a measure of the concentration of antibody sites capable of combining with antigenic determinants. Therefore, it is also the total concentration of antigen sites that are bound by the available antibody, at infinite concentration of antigen.

Since the intercept on the abscissa measures the concentration of antibody sites capable of binding with antigen, a deletion of antigen sites, such as occurs

[3] The assumption that mass action considerations apply to antigen (also hapten)-antibody systems is implicit. It is the underlying basis for this and a number of other systems which have been investigated to obtain information about the number and strength of binding sites (Karush, 1956, 1957; Berson and Yalow, 1959; Pressman *et al.*, 1961; Grossberg *et al.*, 1962; Samuels, 1963). The term "independent," used to describe the sites on the antigen and antibody, implies, as a good approximation, that the sites react in a fashion uninfluenced by the presence of other sites on the molecule, i.e., as though they were univalent antigens and antibodies.

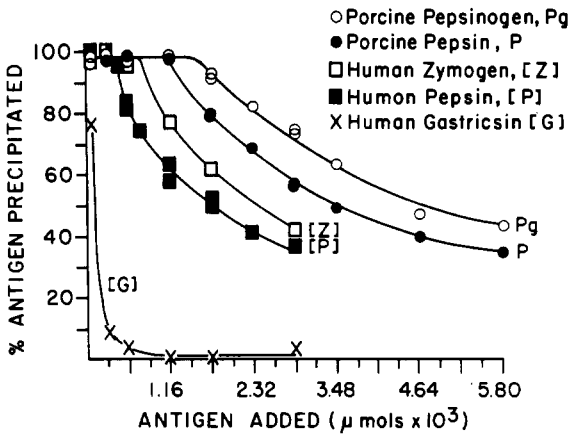


FIG. 4.—Constant-antibody reaction of 0.072–5.8 μmoles porcine and human gastric zymogens and enzymes with 0.20 ml of antiporcine pepsinogen serum at *p*H 5.8, followed by precipitation with horse antirabbit globulin antibody.

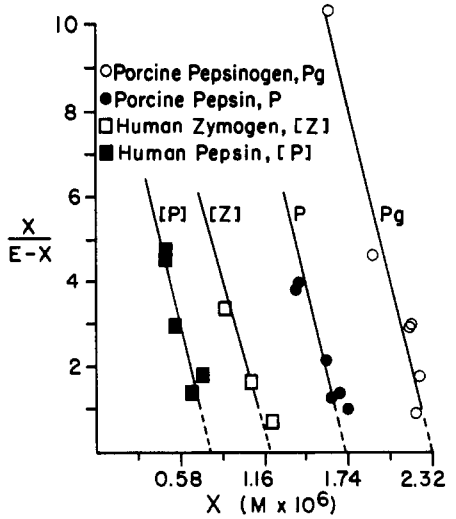


FIG. 5.—Plot of bound/free versus bound antigen (porcine and human gastric zymogens and enzymes). Data derived from Fig. 4.

when pepsinogen is converted to pepsin, will be reflected by a shift of this intercept to lower values. Further, if the remaining sites were configurationally altered so as to affect their binding strengths there would also be a change in the slopes of the lines.

Experimentally, the zymogens and enzymes, in varying concentrations, were allowed to react with a constant amount of antibody. After equilibration, horse antirabbit γ-globulin antibody was added in an amount sufficient to completely precipitate all antiporcine pepsinogen antibody, both free and bound.[4] The percent antigen bound is calculated from analysis of the remaining free unprecipitated zymogen or enzyme. A plot of the percentage of zymogen or enzyme bound versus the amount added is shown in Figure 4. The order of ability of antigen to react with antibody is seen again to be Pg > P > [Z] > [P]. The same was true in the direct precipitation study (Fig. 3) where insoluble, but not soluble, antibody-antigen complexes are measured. Gastricsin reacted very weakly and much differently from the other four antigens.

The plots of $x/(E - x)$ versus x for the strongly binding antibody species[5] of the four main systems are shown in Figure 5. The concentrations of bound antigen sites, x, are expressed as moles/liter.[6] The data are reasonably fitted by straight lines, supporting the assumption of independently acting sites. The relative concentration of antibody (also antigen) sites bound at infinite antigen concentration, i.e., at the x intercept, is 4:3:2.1:1.4 for Pg-P-[Z]-[P]. The slopes of the lines were the same for all the systems.

It was previously reported (Schlamowitz *et al.*, 1963) that the antigen valence of porcine pepsinogen is 4 and that conversion to pepsin results in a decrease of recognizable determinant groups to 3. Consistent with this are the observations of the present study (Fig. 5) that only three-fourths of the antibody sites reactive with pepsinogen was found to react with pepsin. It is reasonable to assume that this results from the loss (in terms of availability for binding) of the species of antibody directed against the determinant group of pepsinogen which is missing in pepsin.

From the fact that the x intercept was given by the binding of 2.31×10^{-6} M pepsinogen, we conclude that the corresponding total antibody site concentration was also 2.31×10^{-6} M. For the pepsin system, where only three-fourths of these available antibody-binding sites could be utilized, the value of its abscissa intercept is $^3/_4 \times 2.31 \times 10^{-6}$ M, 1.71×10^{-6} M. The binding constants for both these systems, calculated from the slopes of the lines, are 1.4×10^7 M^{-1}. The calculations of the binding constants for the human zymogen and pepsin systems yielded similar values. The data for all four test-antigens are presented in Table II.

The values of the binding constants, 1.4×10^7 M^{-1}, for these systems are in the range reported for the insulin-antiinsulin systems, 10^7–10^{10} M^{-1} (Berson and Yalow, 1959).

TABLE II

BINDING CHARACTERISTICS OF GASTRIC ZYMOGENS AND ENZYMES OF PORCINE AND HUMAN ORIGIN

Substance	Antigen Sites: Relative Concentration	Antigen Sites: Concentration (M × 10⁶)	Binding Constants (1/M × 10⁻⁷)
Porcine pepsinogen	4.00	2.31	1.4
Porcine pepsin	2.96	1.71	1.4
Human zymogen	2.07	1.20	1.2
Human pepsin	1.37	0.79	1.4

[4] The assumption is made that the equilibrium which is established between the antigen and the rabbit antiporcine pepsinogen antibody is not disturbed by the subsequent reaction of this rabbit antibody with the horse antibody. Similarly, antibodies with binding constants greater than 1.4×10^7 M^{-1} cannot be excluded. The presence of such antibody species would be concealed in the portions of the curves in Figure 4, where 100% precipitation of antigen occurs.

[5] Weakly binding antibody sites would be revealed at high concentrations of free antigen. At these concentrations the error in x is very large. This region of the antigen binding curve was not investigated in detail for lack of sufficient amounts of antigen and antibody. The presence of weakly binding antibody sites is thus not excluded.

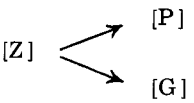
[6] In its reactions with antibody molecules of a particular group specificity each antigen molecule is considered to have among its many sites only one of the corresponding specificity. Therefore the concentration of these sites of an antigen is numerically equal to the molar concentration of antigen. These molar concentrations are the values plotted in Figure 5.

Discussion

The formation of pepsin from pepsinogen is known to occur via autocatalytic proteolysis which removes approximately 20% of the pepsinogen molecule. The present, as well as the earlier, immunochemical data (Schlamowitz *et al.*, 1963) indicate that the conversion is attended by a loss of about one-fourth of the antigenic-determinant groups of pepsinogen and that there is no significant change in the binding constants of the remaining determinants. Had a generalized, significant conformational alteration of determinants occurred the slopes of the two systems would not be the same (Fig. 5). It is unlikely that the three remaining determinants on pepsin are all in one small region of the protein's surface, by virtue of the large size (mw 160,000) of the antibody molecules which must be simultaneously accommodated. In discussing the findings concerning these groups we are therefore, in fact, dealing with a major portion of the molecular achitecture of pepsin. It is therefore suggested that that part of the pepsinogen molecule which becomes pepsin incurs little or no conformational alteration during the conversion. The small difference in the specific optical rotation for pepsinogen and pepsin (Herriott, 1938; Perlmann and Harrington, 1961; Schlamowitz *et al.*, 1963) also support this conclusion.[7]

The present investigation shows both qualitatively and quantitatively that human zymogen and pepsin are capable of cross-reacting immunochemically with antibodies prepared against porcine pepsinogen. A qualitative demonstration of species cross-reactivity of the porcine and human zymogens has been reported (Van Vunakis and Levine, 1963). The weak cross reactions seen in immunodiffusion (Fig. 2) and direct precipitation (Fig. 3) may now be attributed (Fig. 5 and Table II) to the presence of a small number of cross-reacting groups. These groups, too, have binding constants similar to those of the groups in porcine pepsinogen. This suggests that complete characterization of porcine and human zymogens and enzymes will reveal areas of chemical and conformational similarity, if not identity, in the molecules of the two species, over and above any similarities of their catalytic regions. From Figure 5 and Table II it also is seen that, as with the porcine system, the formation of human pepsin from its zymogen is attended principally by a change in the number of antigenic determinants, with little alteration of the remaining structure.

The fact that the molecular weight of gastricsin (35,000) is intermediate between the zymogen (36,000) and human pepsin (30,500) would suggest that it is an intermediate in the formation of human pepsin from its zymogen, e.g., [Z] → [G] → [P]. However, the marked difference in its immunochemical reactions compared with human zymogen and pepsin indicates that if indeed the zymogen is the precursor of the two proteases, they arise independently, e.g.,



corroborating the evidence obtained from the kinetic data of Tang and Tang (1963).

[7] Deductions from rotatory dispersion studies (Perlmann, 1963) indicate a very sizable loss of helical content, from 25% in pepsinogen to 0% in pepsin; but this may be confined to the region involved in the 20% loss of structure.

Acknowledgment

The authors wish to express appreciation to Dr. Jordan Tang, Oklahoma Medical Research Institute, for the samples of human zymogen, gastricsin, and pepsin used in these studies.

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