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Heat- and Alkali-Induced Changes in the Conformation of Pepsinogen and Pepsin

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The conformational changes which occur when pepsinogen and pepsin are subjected to treatment with heat and alkali have been studied using (1) an immunologic method with antisera directed towards pepsinogen and pepsin, (2) potential enzyme activity of pepsinogen, and (3) tryptophan fluorescence of the proteins. The specificity of the antisera varied for the different conformational states of the proteins. Antipepsinogen was capable of distinguishing reversible and irreversible changes in the homologous antigen while antipepsin, directed toward pepsin denatured at *p*H 7, was capable of detecting an irreversibly denatured state of the zymogen in which the pepsin moiety was exposed. Antipepsin also could detect a more disorganized molecular state in pepsinogen and pepsin which was present when these proteins were subjected to elevated temperatures and alkali concentrations. The major structural transitions during alkali treatment, as measured by the three techniques, occurred at *p*H's between 9.4 and 10.2, confirming the results of other investigators that the ϵ-amino group of lysine or hydroxyl group of tyrosine may be involved in maintaining the structure of the zymogen.

The ability of antibodies to complex with globular proteins, since it is to some degree dependent upon the tertiary structure of the antigen as well as the integrity of the peptide sequence, provides a sensitive tool for the detection of structural changes produced in the antigen by the application of various chemical and physical treatments (Levine, 1962). The rabbit antisera directed against porcine pepsinogen and pepsin (denatured at *p*H 7.0) are capable of detecting structural alterations in their homologous antigens; in addition antipepsin is capable of detecting the "unmasking" of the pepsin moiety of pepsinogen (Van Vunakis *et al.*, 1963; Van Vunakis and Levine, 1963).

Herriott (1962) has reviewed the effects of some denaturing agents on pepsinogen and pepsin. This communication presents the results of investigations on the structural status of these proteins during treatment with heat or alkali. The techniques of quantitative complement fixation, estimation of potential peptic activity, and, as a physical parameter, the tryptophan fluorescence of the proteins have been utilized in an attempt to delineate the structural changes which occur during these modifications as well as to evaluate the discrimination of the various methods. Protein fluorescence was selected as the physical parameter for these studies because it has been shown to reflect major structural transitions in some proteins (Teale, 1960; Brand *et al.*, 1962; Gally and Edelman, 1962) and to vary in a nonlinear fashion when pepsinogen is subjected to increments of temperature and *p*H (Steiner and Edelhoch, 1962).[1] Considerable data on the structural modifications of pepsinogen and pepsin, utilizing the optical rotatory properties of the proteins as a physical constant, are available (Perlmann, 1959, 1963; Perlmann and Harrington, 1961). With these studies as a point of departure, the present investigation was undertaken.

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[1] J. A. Gally, G. E. Perlmann, and G. M. Edelman have also investigated the effect of denaturing agents on the fluorescence of pepsinogen. A preliminary report of some of their findings was made by Dr. Perlmann at the Symposium on Quantum Aspects of Polypeptides and Polynucleotides, and will appear in *Biopolymers 1* (1963, in press). A preprint of this manuscript was kindly made available to us by Dr. Perlmann.

[2] A strict comparison of our results with the optical rotation studies of Perlmann and Harrington (1961) and Perlmann (1963) is difficult since factors which influence this reaction, such as the concentration of pepsinogen and the duration of equilibration at each temperature, have varied between the two laboratories.

[3] The expression pH_f was introduced by White (1959) for the *p*H at which the fluorescence of a substance is quenched 50% by either acid or base addition.

Materials and Methods

Proteins.—Swine pepsinogen was prepared as previously described (Van Vunakis *et al.*, 1963); pepsin (Lot 661 twice crystallized from ethanol) was obtained from Worthington Biochemical Corporation.

Antisera.—The preparation, characterization, and immunologic properties of the antisera have been reported (Van Vunakis *et al.*, 1963; Van Vunakis and Levine, 1963).

Complement (C′) Fixation.—Reagents and procedures for the quantitative *C′* fixation technique are described by Wasserman and Levine (1961).

Fluorescence Measurements.—The Aminco-Bowman spectrofluorometer equipped with a 150-watt Osram high pressure xenon lamp and thermoregulated at 25° was used for most of the fluorescence measurements. Activation was at 286 mμ, and emission was monitored

at 348 mμ, the respective maxima for tryptophan fluorescence in these proteins.

Spectral measurements were made in the Zeiss spectrophotofluorometer at slit widths of 0.5 mm or less. Spectra were reproducible to ±1 mμ at their peaks.

Enzyme Activity Measurements.—The activation of pepsinogen was performed at *p*H 1.4 and 0° for 10 minutes. Pepsin controls were similarly treated. The hemoglobin digestion method of Anson (1938) was used for assay.

Heat Denaturation.—Into each of fifteen tubes was pipeted 0.5 ml of a solution of pepsinogen (1 mg/ml in 0.04 M phosphate buffer, *p*H 7.66 containing 0.05 M NaCl). The tubes were stoppered and placed in a thermoregulated water bath at 25°, and the temperature was raised by 5° increments. Each elevation of temperature required about 4 minutes at lower, up to 7 minutes at higher temperatures. After equilibrating for 10 minutes at each temperature, 4.5 ml of cold buffer was pipeted into each tube and aliquots were assayed immediately by the *C'* fixation procedure. Separate experiments, but under identical conditions, were performed for the estimation of fluorescence and potential peptic activity.

In the experiments on renaturation, pepsinogen (1 mg/ml) was heated at 65° or 70°; 0.5-ml aliquots were removed into 4.5 ml cold buffer at intervals and allowed to remain in the cold overnight. The same experimental conditions were used 24 hours later but these aliquots were assayed immediately for immunologic activity. *C'* fixation analyses were carried out simultaneously with those samples which had been withdrawn 24 hours previously.

Alkali Denaturation.—To each of twelve tubes containing 1.0 mg of pepsinogen dissolved in 1.0 ml of 0.15 M NaCl adjusted to *p*H 7.0 with dilute NaOH was added 1 ml of solution of NaOH ranging in concentration from 1×10^{-5} M to 1.0 M. After 90 minutes of incubation at 25° an aliquot was removed from each tube, activated immediately in cold acid, and assayed for peptic activity. After 120 minutes of incubation at 25° one aliquot was delivered directly into cold Veronal buffer (*p*H 7.5) for the immunological assay and another into 9 volumes of cold, 0.2 M Tris buffer (*p*H 7.6) and read immediately in the fluorometer. There was no change in the fluorescence of these samples over a 24-hour period. The *p*H of the unbuffered solutions was noted, but because of their tendency to undergo a shift in *p*H toward neutrality with time the values obtained are only approximate. The alkaline denaturation of pepsin was carried out under similar conditions.

To determine the effect of direct titration of pepsinogen on its fluorescence, a solution containing 1 mg/ml pepsinogen in 0.1 M NaCl was titrated with 5 N NaOH, being stirred by bubbling N_2. Fluorescence and *p*H were measured after each addition of base. For back titration, 5 N HCl was used.

Results

Heat-induced Conformational Changes in Pepsinogen and Pepsin.—Pepsinogen equilibrated for 10 minutes at a succession of temperatures and assayed immediately for antigenic activity with antipepsinogen gives the series of *C'* fixation curves shown in Figure 1. These curves are superimposable for equilibration temperatures of 25°, 30°, 35°, and 40°. There then occurs a progressive shift at the curves toward the region of higher antigen concentration (i.e., more antigen is required to fix the same amount of *C'*) at

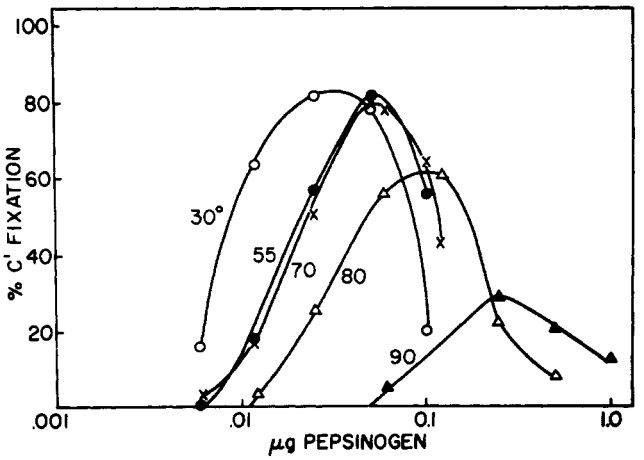

FIG. 1.—Fixation of C' by pepsinogen, equilibrated at various temperatures, with antipepsinogen (Ra 21-3; diluted 1/3300). Although they are not shown in the figure, identical curves were obtained at equilibration temperatures of 25°, 35°, 40°, and 45°.

45°, 50°, and 55°. The curves for pepsinogen heated at 55°, 60°, 65°, and 70° are, again, essentially superimposable. The curves obtained from samples of pepsinogen subjected to temperatures above 70° undergo a progressive shift toward the region of higher antigen concentration, but with an accompanying decrease in peak height until, at 100°, the pepsinogen no longer displays any reactivity with its homologous antiserum. Estimation of the amount of immunologically unaltered pepsinogen remaining at each temperature was made on the basis of the lateral displacement of the *C'* fixation peak. (Fig. 2A). This progressive displacement observed at equilibration temperatures of 45°, 50°, and 55° may have been caused by complete destruction of the antigenic competence in an increasing fraction of the molecular population (microheterogeneity) or the redistribution of all the intact antigenic determinants as a result of a conformational change. Since the modifications in pepsinogen induced at temperatures above 70° result in decreased maximal *C'* fixation, pepsinogen antigenicity cannot be estimated quantitatively by peak shift above this temperature, and is represented by a dotted line. Among the possible causes of decreased maximal *C'* fixation are the complete destruction of antigenic sites on some molecules or the production of a molecule which does not fix *C'* but competes with *C'*-fixing antigen for the antibody (Van Vunakis *et al.*, 1963).

The variation in the intensity of the tryptophan fluorescence of pepsinogen exposed to the same elevated temperatures (Fig. 2B) presents a profile somewhat similar to that obtained from the immunologic data. As noted in the optical rotation studies of Perlmann and Harrington (1961), there appears a temperature range ("plateau") over which no change is observed.[2]

Our findings on the effect of temperature upon the potential peptic activity of pepsinogen are illustrated in Figure 2C. The values obtained for potential enzymic activity depend upon the efficiency of quenching and the rapidity with which the samples are assayed. Considerable renaturation has already occurred after 4 minutes, although renaturation is not maximal for samples heated at 45° and 50° even after 1 hour. The "plateau," which is not clearly defined in the 1-minute sample, is discernible after 4 minutes and persists even at 24 hours, although over a less extensive temperature range.

The enzymic data (Fig. 2C) indicate that the renatur-

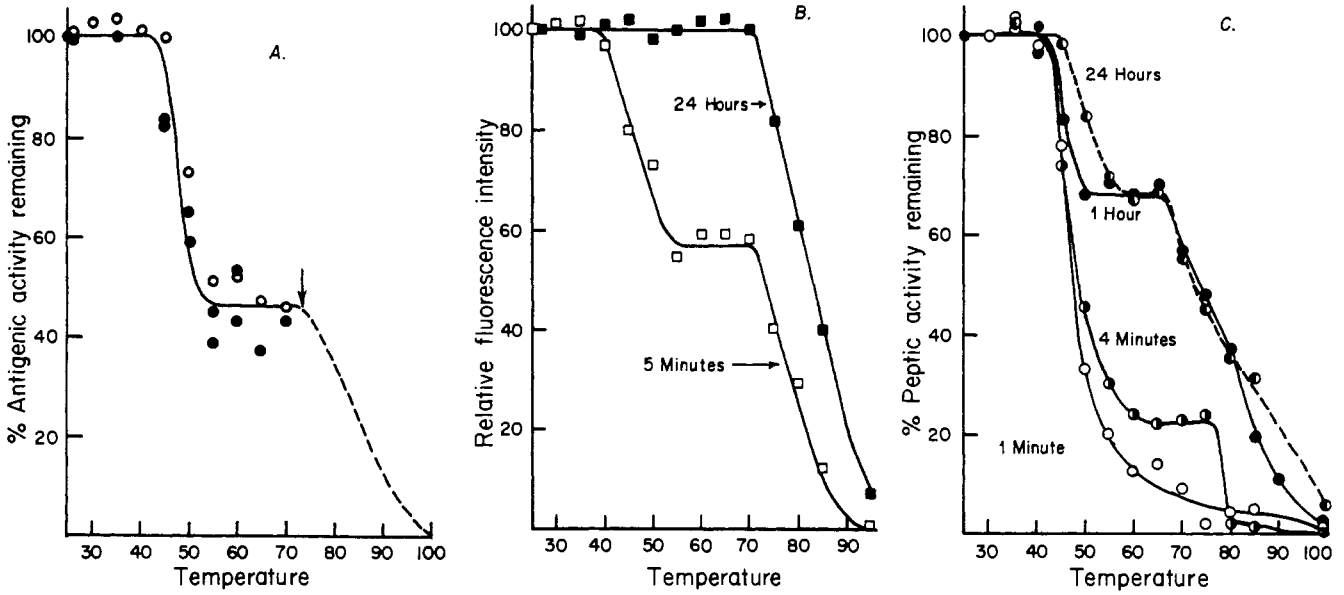

FIG. 2.—(A) Temperature profile of pepsinogen assayed with antipepsinogen (Ra 21-3; diluted 1/3300). Symbols represent values obtained in three different experiments. The per cent antigenic activity remaining was calculated from the lateral shift of the curves. The vertical arrow represents the point at which the peak height of the curves progressively decreased. Thus, estimation of remaining antigenic activity after this point could only be approximated. (B) Relative fluorescence intensity of pepsinogen equilibrated at various temperatures, diluted into buffer, and determined within 5 minutes (□) or after 24 hours at 0° (■). (C) Potential peptic activity of pepsinogen, heated at various temperatures and diluted into cold buffer. The samples were activated at *p*H 1.4 at 1 minute, 4 minutes, 1 hour, and 24 hours after dilution into buffer.

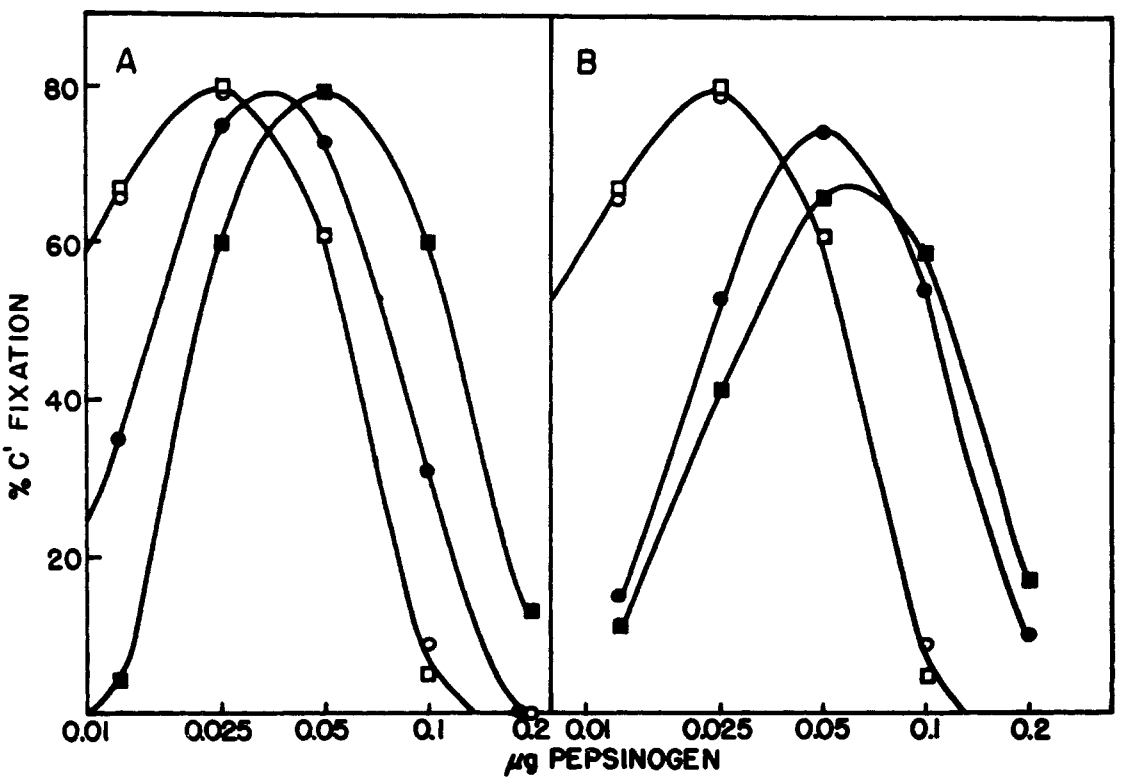

FIG. 3.—(A) C′ fixation curves, pepsinogen heated for 10 minutes at 65° and immediately assayed (■); pepsinogen similarly heated, diluted and allowed to stand 24 hours at 0° (●); pepsinogen control, assayed immediately (□); and pepsinogen control, diluted and allowed to stand 24 hours (○). (B) C′ fixation curves of pepsinogen and pepsinogen heated at 65° for 85 minutes. Symbols the same as Figure 3A.

ation of reversibly heat-denatured pepsinogen cannot be successfully quenched by dilution into cold buffer. Apparently, there exists in samples of pepsinogen heated at temperatures greater than 45° an equilibrium between the native and denatured forms of the zymogen (denaturation being designated by the absence of potential peptic activity). At higher temperatures, restoration of enzyme activity is never complete; i.e., some molecules are in an irreversibly denatured state. There would seem to be none of the irreversibly denatured form present at 45°, and only a small amount of the reversibly denatured form by the time 100° is reached.

What then is the form of the protein being measured

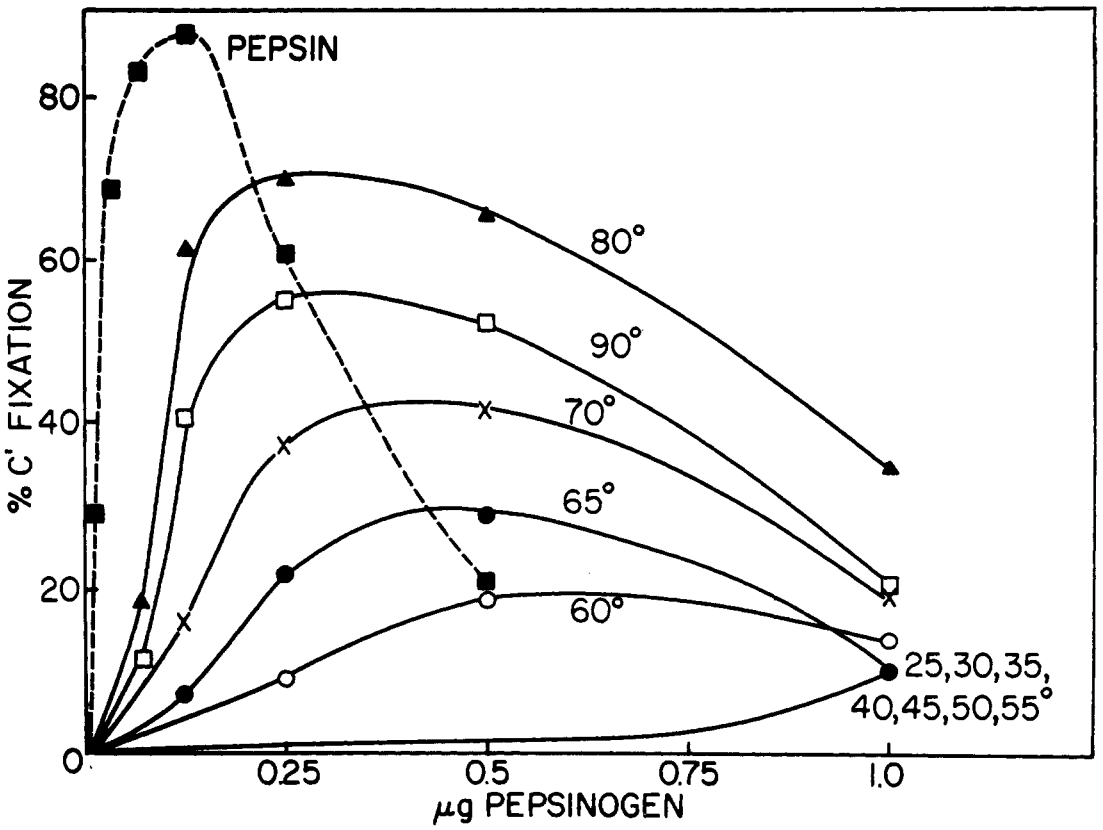

FIG. 4.—C′ fixation curves of pepsinogen incubated at various temperatures and assayed with antipepsin (Ra-249A diluted 1/800).

when we assay the antigenicity and fluorescence of heat-treated samples? Samples exposed to temperatures of 55°–70° and allowed to renature 24 hours fluoresce fully as efficiently as samples of native pepsinogen, although they possess significantly diminished enzymic activities. The complete return of fluorescence in the absence of full enzymic and antigenic (Fig. 3A) activities under identical renaturation conditions may reflect incorrect refolding such as was observed with lactate dehydrogenases denatured with urea and "renatured" by removal of urea (Levine, 1962). It is also possible that certain portions of pepsinogen were able to refold so as to align the tryptophan residues in a configuration similar to the original, whereas other segments were permanently disrupted so as to alter reactivity of the zymogen with antipepsinogen and prevent its proper hydrolysis during activation.

The ability of antipepsinogen to discriminate between the native, reversibly denatured, and irreversibly denatured forms of pepsinogen is demonstrated in Figure 3A, in which are depicted the C′ fixation curves of samples of pepsinogen heated for 10 minutes at 65°, quenched into cold buffer, and brought rapidly into contact with the antibody solution (the making of the necessary dilutions requires approximately 4 minutes), or quenched and kept at 5° for 24 hours before being exposed to antibody. If it is assumed that renaturation is maximal in 24 hours, then the middle curve of Figure 3A represents the antigenic competence of a solution containing only renatured (not necessarily native) and irreversibly denatured pepsinogen. The curve furthest to the right in Figure 3A derived from the sample of pepsinogen assayed immediately after quenching is less susceptible to analysis. This sample possessed 46% of the antigenicity of the control and undoubtedly represents a situation in which all three forms of the protein are present.

It is difficult to say at what point in the renaturation process sufficient immunologic interaction occurs to prevent further progression towards the native state. Presumably, once antigen and antibody have formed a complex, there would be great steric hindrance to

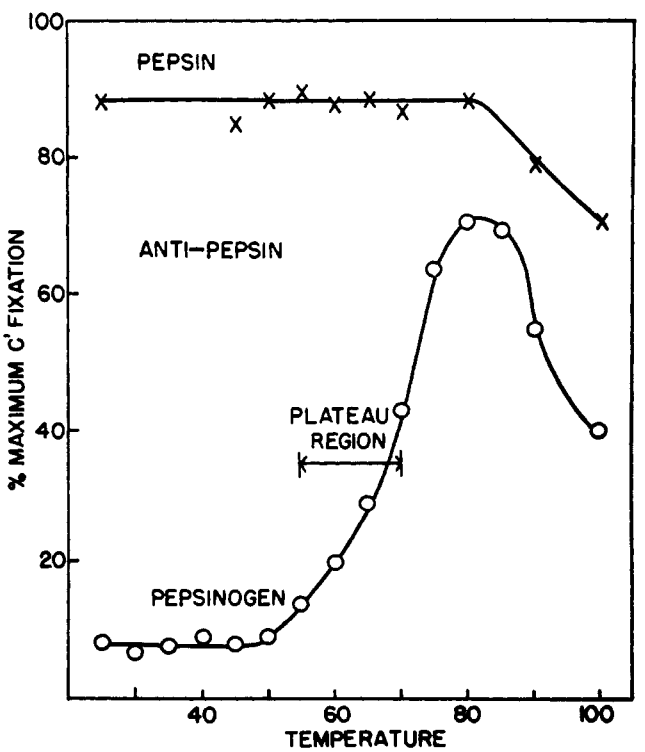

FIG. 5.—Temperature profiles of pepsinogen and pepsin assayed with antipepsin. Plateau region taken from Figure 2A.

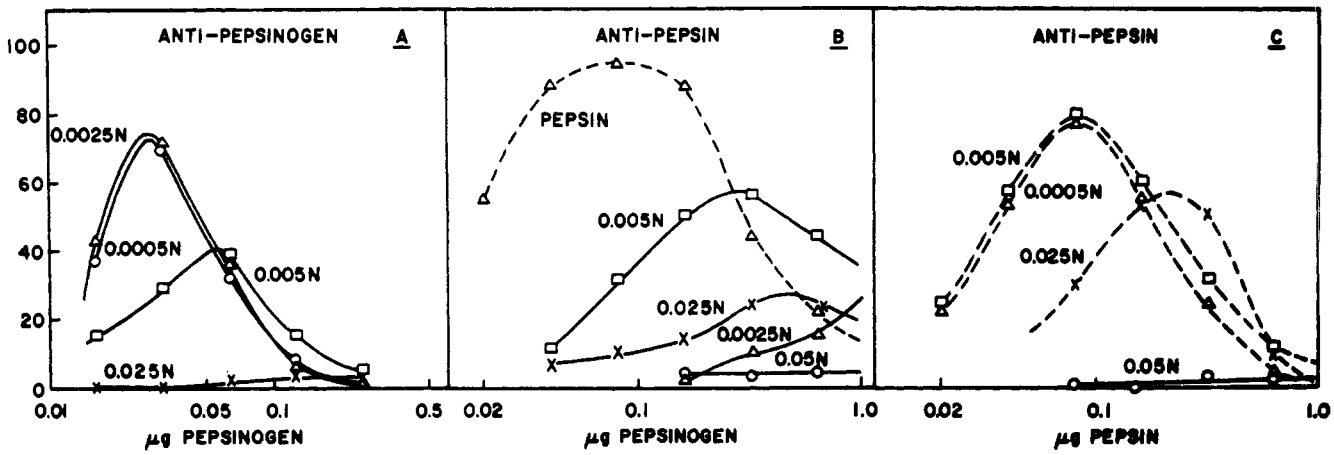

FIG. 6.—(A) Treatment of pepsinogen with alkali for 2 hours at 25°, and assayed with antipepsinogen (Ra 21-3 diluted 1/3300). (B) Pepsinogen samples (same as Fig. 2A) assayed with antipepsin (Ra 249A, diluted 1/800). (C) Treatment of pepsin with alkali and assayed with antipepsin (Ra 250, diluted 1/600).

further configurational changes, but the possibility that a totally unreactive molecule may renature to the point of interaction while in the immunologic assay mixture cannot be discounted. Exposure of a pepsinogen solution to 65° for 80 minutes, followed by immediate and delayed assay, produced the C′ fixation curves shown in Figure 3B. Here maximal C′ fixation is seen to have diminished, possibly because of the production of a molecule antigenically incomplete, which could react with antibody but could not fix C′. If this is so, such a species is capable of some reversion to a molecule which could fix C′, since assay of the renatured sample results in increased peak fixation as well as a shift back toward the region of lesser concentration.

The need for multiple methods in assessing the conformational status of a complex protein is demonstrated by the next set of experiments. Pepsinogen was heated as described previously, and diluted into cold buffer, and added rapidly to antipepsin. The series of C′ fixation curves shown in Figure 4 was obtained. A graphic presentation of the peak C′ fixation of these samples (Fig. 5) reveals that conformational changes are being detected in the plateau region, most reasonably interpreted as resulting from the "unmasking" of previously hidden antigenic sites. The other parameters had indicated the existence of a relatively stable molecular situation in this temperature range (Fig. 2). At 90° the reactivity of the pepsinogen with antipepsin begins to decline. This decline is probably a reflection of the structural instability of the previously exposed pepsin moiety at this temperature. Corroborative evidence of such a process is available from an experiment in which pepsin was similarly heated at *p*H 7.5. The antigenicity of pepsin with the homologous antiserum remains intact until a temperature of approximately 90° is reached. From this point, peak C′ fixation diminishes progressively as shown in Figure 5.

Unlike antipepsinogen (Fig. 3A), antipepsin does not discriminate among the native, reversibly, and irreversibly denatured states in pepsinogen. The pepsinogen solutions were heated 16 or 60 minutes at 70°, diluted into cold buffer, and allowed to remain at 0° overnight prior to assay with antipepsin. The same experiment was repeated 24 hours later and the samples were assayed immediately. There was no change in the C′ fixation curves of the samples which had been assayed immediately or after 24 hours at "renaturing" conditions.

Treatment of Pepsinogen and Pepsin with Alkali.—The C′ fixation curves plotted in Figure 6A show the effect of various concentrations of NaOH upon the antigenicity of pepsinogen. No antigenic changes are detectable with concentrations of NaOH less than 0.0025 N. Sodium hydroxide (0.005 N) causes considerable diminution, and 0.025 N the complete abolition, of the antigenicity detectable by antipepsinogen. When the same samples are assayed with antipepsin, treatment with increased concentrations of alkali results first in an emergence, then in a regression of pepsinlike antigenicity (Fig. 6B) similar to that observed when pepsinogen was heated (Fig. 5). Such diphasic behavior was also observable with time at a single concentration of NaOH; exposure of pepsinogen to 0.05 N NaOH resulted in an increase in C′ fixation with antipepsin over the first 30 minutes, followed by a progressive decline (H. I. Lehrer, unpublished observations). That the decrease of reactivity of pepsinogen with antipepsin which occurs subsequent to the initial unmasking is probably related to destruction of the exposed pepsin fragment of the zymogen may be inferred from the results of the experiment shown in Figure 6C. It can be seen that the antigenic competence of pepsin itself, as well as that of the "exposed" pepsin fragment of pepsinogen (Fig. 6B), is altered by 2 hours in 0.025 N NaOH and completely destroyed by 0.05 N NaOH. These findings indicate that the susceptibility of these two forms of pepsin to disorientation by alkali is of the same order.

Figure 7 summarizes the immunologic, enzymic, and fluorescence data obtained during alkali treatment of pepsinogen and pepsin. The *pK*'s for the transitions all fall between *p*H 9 and *p*H 10, those for pepsin lying slightly higher than those for pepsinogen. Points representing immunologic activity were plotted from the decreasing peak C′ fixation of the various samples as compared to that of the controls and are indicative rather than quantitative. In this case, fluorescence seems to be a reasonably accurate indicator of the structural status of the proteins. When a pepsinogen solution was titrated with NaOH and the fluorescence measured after each addition, a pH_f of 9.9 was found.[3] Since hydroxyl ions themselves interfere with fluorescence only at *p*H's above 12 (White, 1959), it is probable that a pH_f of about 9.9 represents the *pK* for the ionization of some group in the protein involved directly with the fluorescence efficiency of tryptophan (White, 1959) or indirectly via its function in maintaining the conformation of the protein. The two functional groups in proteins which possess *pK*'s in this range are the

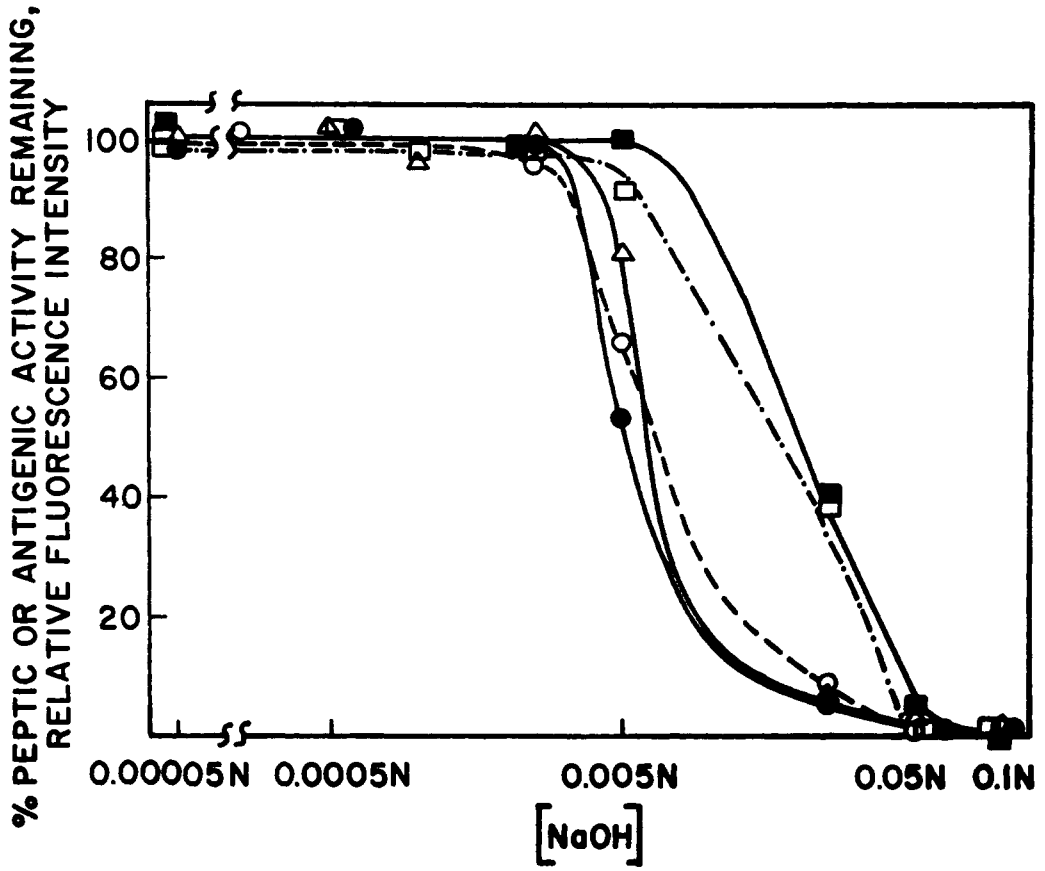

FIG. 7.—The effect of alkali on pepsinogen and pepsin. Immunologic activity of pepsinogen (○) and pepsin (□). Relative fluorescence of pepsinogen (●) and pepsin (■). Potential peptic activity of pepsinogen (△).

hydroxyl group of tyrosine (pK = 9.8–10.4) and the ε-amino group of lysine (pK = 9.4–10.6) (Alberty, 1958). Tyrosine fluorescence is not detectable by ordinary techniques in proteins containing tryptophan, although it has been shown to be present (Weber, 1961). Since the anionic form of tyrosine is not fluorescent, it is conceivable that energy transfer between tryptophan and the anionic form of tyrosine might result in a quenching of tryptophan fluorescence. However, energy transfer between tryptophan and tyrosine has been shown not to occur, whether the tyrosine is in the ionized or un-ionized state (Teale, 1961). Thus, if this pH_f reflects the titration of a tyrosine hydroxyl group, it is probable that it is the function of this group in maintaining protein conformation (e.g., by hydrogen bonding), which is being perturbed by titration.

Since pepsinogen is converted to pepsin below pH 6.0, it was not possible to carry out any titrations below this pH. However, the autocatalytic conversion of the zymogen could be followed enzymatically (Herriott, 1938b), immunologically (Van Vunakis *et al.*, 1963), and by determining changes in relative fluorescence. The rate of loss of fluoresence during activation of pepsinogen at pH 3.7 corresponds precisely to the rate of emergence of active enzyme until approximately 90% of the zymogen has been converted. At this time, a secondary decay of fluorescence occurs, perhaps related to the initiation of autodigestion of the pepsin. The depression of fluorescence up to this point is on the order of 20% of the maximum depression seen under any denaturing influence used by us (heat, alkali, urea). Since all six tryptophan residues of pepsinogen reside in the pepsin segment, quenching of fluoresence might reflect the removal of a direct effect of the basic segment upon the tryptophan(s) or a significant structural alteration of pepsin occurring during its transition from a component of the zymogen to the active enzyme.

DISCUSSION

A highly schematic representation of pepsinogen is shown in Figure 8A (after Herriott, 1962). This molecule is essentially a composite of two profoundly dissimilar moieties: an exceedingly acidic fragment (pepsin) with an isoelectric point less than pH 1.0, and an overwhelmingly basic segment (consisting of the inhibitor and miscellaneous peptides) such that the isoelectric point of the intact molecule is at pH 3.7. The basic segment (constituting approximately $^1/_6$ of the molecule) is probably attached to pepsin by a single peptide bond susceptible to cleavage by pepsin at acid pH. Six tryptophan and three cystine residues

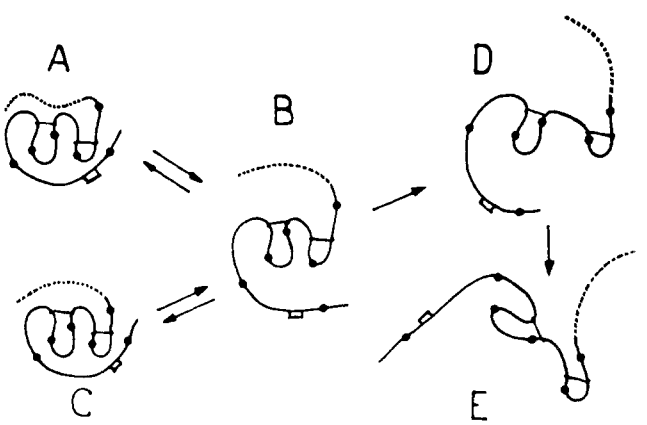

FIG. 8.—Possible conformational states of pepsinogen

are common to both pepsinogen and pepsin; the tryptophans have been randomly placed in the model, and, although the true position of the disulfides is not known, they are located so as to reflect the changes in the stability and activity of the molecule which are elicited by their sequential reduction. Some of the bonds and forces that may function in maintaining the configuration of the enzyme and zymogen have been considered (Bovey and Yanari, 1960; Herriott, 1962; Perlmann, 1963). Measurements of optical rotation indicate that pepsin is almost devoid of helix, while pepsinogen does possess some helical structure, but the assignment of helix to a definite segment of the precursor cannot be made with certainty (Perlmann and Harrington, 1961; Perlmann, 1963).

Figure 8 also depicts possible conformational states of the pepsinogen molecule induced by our experimental conditions. Pepsinogen, upon heat treatment at temperatures from 45° to 55°, undergoes changes in conformation reflected equally well by decreased tryptophan fluorescence, potential enzymic activity, and antigenic competence with the homologous antibody. If pepsinogen so treated is diluted into buffer and allowed to renature at 0°, it recovers the complete capacity to fluoresce and significant amounts of its antigenic and potential enzymic activities. Since interaction with antipepsin begins to emerge only at 55°, it can also be concluded that conformational changes induced at the lower temperatures are not accompanied by the unmasking of the pepsin moiety in a form susceptible to combination with that antibody. At temperatures between 45° and 55° only limited intramolecular changes appear to be occurring (Fig. 8B), since reversal to the native (Fig. 8A) or a slightly altered renatured molecule is possible. Such a molecule (Fig. 8C) may possess full fluorescence capacity with some loss in the antigenic and potential enzymic properties.

As the pepsinogen solution is subjected to temperatures in the range of 55°–70°, pepsin antigenicity emerges while fluorescence and pepsinogen antigenicity are relatively stable. Antipepsin, however, could not detect the structural changes which accompany the renaturation of pepsinogen; it reacted only with irreversibly denatured pepsinogen. Since antipepsin is directed toward pepsin denatured at the *p*H of the blood stream, it may be capable of reacting only with the pepsin moiety which is unmasked and denatured at the *p*H of the buffer (*p*H 7.6). A possible representation of such an irreversibly denatured molecule is depicted in Figure 8D. The emergence of this molecule cannot be estimated quantitatively because peak-fixation is progressively changing and the varying intermediate states have not been separated from one another (Van Vunakis *et al.*, 1963).

When pepsinogen is subjected to temperatures above 80°, the antigenicity with antipepsin ceases to emerge and then begins to disappear. Over the same temperature range the C′ fixation curves with the antipepsinogen start to fix less C′ at peak, indicating destruction of antigenic sites on the molecule. Likewise, the fluorescence and potential enzymic activities are rapidly lost and the restoration of these activities under renaturing conditions diminished progressively. The irreversibly unmasked molecules (Fig. 8D) are then pictured as undergoing a transition to an even more disordered form (Fig. 8E) which is incapable of reacting with either antibody, is devoid of potential enzyme activity, and possesses a minimal tryptophan fluorescence. Stage 8E is depicted as a random coil whose structure is limited only by the disulfide bridges.

Herriott (1938, 1962) has shown that alkali denaturation of pepsinogen is accompanied by the removal of two protons, the *pK* of the donating groups being approximately 9.4, and suggested that this *pK* represents the titration of either the ε-amino group of lysine and/or the phenolic hydroxyl of tyrosine. Titration of pepsinogen with NaOH produced a pH_f of 9.9. This quenching of fluorescence was initially reversible, but delayed back-titration resulted in progressively less restitution of fluorescence. Tryptophan itself undergoes fluorescence changes with elevated *p*H (White, 1959). That the quenching of fluorescence can be due to conformational changes in the proteins and not solely to direct perturbation of the chromophores by hydroxyl ions was demonstrated by determining fluorescence at neutrality after 2 hours' exposure to elevated *p*H's, at which time irreversible structural changes have occurred. The range of *p*H over which the tryptophan fluorescence becomes markedly diminished and pepsinlike antigenicity emerges (*p*H 9.4–10.2) is within the range in which the titration of the ε-amino group of lysine and/or the phenolic hydroxyl group of tyrosine may be expected to occur. That only the ε-amino group of lysine may be crucial in maintaining the structure of pepsinogen is indicated by Perlmann's results (1963). The ability of polylysine and the lysine-rich pepsin inhibitor to form complexes with pepsin which effectively prevent its activity (Katchalski *et al.*, 1954; Herriott, 1962) and the ability of the inhibitor to protect pepsin from alkaline denaturation (Schlamowitz *et al.*, 1963) suggest that in the intact pepsinogen molecule interaction between the functional groups of lysine and acidic groups in the pepsin fragment is a reasonable possibility.

One obvious conclusion from this study is that a physical method (in this case protein fluorescence) depending upon the characteristic behavior of a protein constituent which may be perturbed by factors not affecting the over-all structure or may fail to be perturbed by transformations in another part of the molecule or, similarly, enzymic activity, which may depend upon the conformation in a limited locale, cannot give a comprehensive picture of all conformational changes. A single antibody may also be incapable of detecting all significant changes in a modified antigen. The ability of antipepsin to detect unmasking of the pepsin moiety of pepsinogen provides a valuable supplement to the detection of conformational changes in modified pepsin and pepsinogen by a direct reaction with the homologous antibody. The emergence of pepsin antigenicity in the zymogen subsequent to treatment with heat and alkali indicates the unmasking of additional sites available for reaction with this antibody. The reversal of this trend at higher temperatures and alkali concentrations probably reflects a structural disorganization of the previously unmasked pepsin moiety, since pepsin itself undergoes antigenic deterioration at similar temperatures and alkali concentrations. The use of these three techniques to study the effect of urea on these proteins and the role played by the disulfide bridges will be discussed in a subsequent paper.

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Immunochemical Studies of Hemoglobin and Myoglobin and Their Globin Moieties

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Rabbit antisera to porcine hemoglobin and horse muscle myoglobin have been characterized. Antihemoglobin was identified by immunodiffusion analysis and constant specific immunologic activity during chromatography. Similar techniques were used to identify the antigen-antibody system being measured by C′ fixation as myoglobin-antimyoglobin. Both antihemoglobin and antimyoglobin detected specific conformational differences between the heme proteins and their globin moieties. Addition of heme to the globin moieties restored full serologic capacity to the heme proteins. Restoration of specific antigenic competence by addition of heme to globin permitted measurement of heme transfer from heterologous hemoglobin to the globin moieties of porcine hemoglobin and horse muscle myoglobin.

Precipitating antibody to hemoglobin was first conclusively demonstrated by Heidelberger and Landsteiner in 1923. In subsequent years reports confirming both the antigenicity and the narrow species specificity of the hemoglobins have appeared (Hektoen and Boor, 1931; Johnson and Bradley, 1935). The study of globin, the apoprotein of hemoglobin, received little immunochemical characterization because, until recently, isolated globins were heterogeneous (Gralen, 1939; Moore and Reiner, 1944; Havinga and Itano, 1953) and their reconstitution products, on addition of heme, were different from native hemoglobins (Gralen, 1939). Rossi-Fanelli *et al.* (1958) have recently described the preparation of homogeneous human globin preparations and have reconstituted native human hemoglobin, as measured by physical variables, by addition of stoichiometric amounts of heme.

The present report is concerned with the immunochemical characterization of hemoglobin and myoglobin and their globin moieties. Our objective was to examine the possibility that conformational changes occurring during conversion of hemoglobin could be detected by antibodies to the heme proteins, and to use this sensitive method to measure transfer of the hemes to their respective globin moieties.

MATERIALS AND METHODS

Antigens: Porcine Hemoglobin.—Either crystalline porcine hemoglobin (purchased from Pentex Corp.) or porcine hemoglobin twice crystallized from fresh red blood cells by the method of Drabkin (1946) was used. The hemoglobin was crystallized from both 2.8 M PO_4, *p*H 6.8, and 65% saturated $(NH_4)_2SO_4$ adjusted to *p*H 6.8. The crystals were washed and suspended in either strong salt solution at 4°. Under these conditions the hemoglobin was maintained as oxyhemoglobin for 2–3 months as judged by periodic spectral determinations. Methemoglobin was prepared from oxyhemoglobin by treatment with ferricyanide, followed by dialysis as described by Austin and Drabkin (1935).

Porcine globin was prepared only from freshly crystallized hemoglobin and the method of Rossi-Fanelli *et al.* (1958) was used. All experiments with globin were performed within 48.0 hours of its preparation. Porcine globin has properties similar to human globin and will be described under Results. All preparations used had less than 1 mole% unsplit heme as judged by spectral analysis.

Horse Muscle Myoglobin.—Initially horse heart myoglobin, twice crystallized (purchased from Pentex Corp.), was used for immunization. This material was found subsequently to contain 3% hemoglobin, an unidentified nonheme protein, and also the chromatographically separable myoglobins described by Akeson & Theorell (1960). The immune sera prepared from this antigen was heterogeneous and will be described in detail under Results.

Crystalline horse muscle myoglobin was also prepared by a modification of Bowen's method (1948). This material was recrystallized three times and was uniformly crystalline microscopically. The dissolved crystals were dialyzed exhaustively against 0.005 M PO_4, *p*H 6.4, and chromatographed on carboxymethylcellulose previously equilibrated with the same buffer. Five peaks were separated by this procedure and identified spectroscopically and ultracentrifugally as myoglobin. The major fraction, which constituted about 80% of the myoglobin, eluted sharply between *p*H 6.9 and 7.2. It was reapplied to carboxymethylcellulose equilibrated with 0.02 M PO_4, *p*H 6.4.

Under these conditions a major fraction separated as a sharp band and was preceded by a faster component. The resin was gently blown from the column by compressed air, cut out, and eluted with 0.2 M Na_2HPO_4

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