INTERACTION OF EGG ALBUMIN AND PEPсин*

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(Received for publication, July 7, 1952)

The interaction between enzymes and their substrates to form intermediate complexes has long been of interest. We have found that when pepsin and egg albumin are mixed in solution at about pH 4 a precipitate forms. The influence of protein and of ion concentrations on the amount of precipitate so formed has been studied. It has been found that the precipitate dissolves at higher ionic concentrations and this has given us the opportunity of applying the light-scattering technique to solutions of the dissolved complex between egg albumin and pepsin. From this study we have been able to calculate the association constant of the two proteins as well as to make a rough estimate of the initial rate of digestion of egg albumin by pepsin at pH 4.0.

Methods

Crystalline egg albumin was prepared from fresh hen's eggs by the method of Kekwick and Cannan (1). It was exhaustively dialyzed against distilled water and the concentration determined by dry weight. Crystalline pepsin was prepared by the alcohol method of Northrop (2). An additional lot of crystalline pepsin was obtained from Armour and Company and used without further purification. The concentration of pepsin was determined by the micro-Kjeldahl method.

The complex was precipitated as follows: 2 ml. of a pepsin solution containing 4.8 mg. of pepsin (0.70 mg. of total protein nitrogen) were added to 16 ml. of egg albumin solution of the desired concentration. The resulting solution was brought to the desired pH by the addition of dilute sulfuric acid or of dilute hydrochloric acid and made up to 20 ml. with water. There was no difference in the quantity of the precipitate if pepsin solution was added before or after adjustment of the pH. Precipitation of the complexes occurred immediately, but the solutions were allowed to stand 1 hour at room temperature before being filtered. The total nitrogen of 5 ml. aliquots of the filtrates was determined by the micro-Kjeldahl method.

* From a thesis submitted by D. S. Yasnoff to the Graduate School of Northwestern University in partial fulfilment of the requirement for the degree of Doctor of Philosophy, June, 1952.
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The light-scattering photometer (Phoenix Precision Instrument Company) is designed to measure absolute turbidity by the "working standard method." In this method, the turbidity, \( \tau \), is related to the galvanometer deflection, \( G \), obtained at angles of 90° and of 0° with respect to the incident beam, according to the equation

\[
\tau = \frac{RG_{90}}{G_0}
\]

(1)

The proportionality constant, \( R \), was determined from Equation 22 of Brice et al. (3) by information provided by the manufacturers.

The molecular weight, \( M \), is given by the relation

\[
\frac{HC}{\tau} = \frac{1}{M} + 2BC
\]

(2)

where \( C \) is the solute concentration in gm. per ml., \( B \) is an interaction constant, and \( H \) is a constant for a particular system and is given by

\[
H = \frac{32\pi^2 n_0^2}{3N\lambda} \left( \frac{n - n_0}{C} \right)^2
\]

(3)

where \( N \) is Avogadro's number, \( n \) and \( n_0 \) the indices of refraction of solution and solvent, respectively, and \( \lambda \) the wave-length of light in air. As is evident from Equation 2, extrapolation of the function \( HC/\tau \) to infinite dilution yields the reciprocal of the molecular weight, which is a weight average value.

Solutions of known concentrations of protein and buffer were filtered directly into scrupulously clean square glass cells through Berkefeld or Selas filters. A dipping refractometer was used to determine the refractive index increments of the various protein solutions. The buffer used in the light-scattering work consisted of 0.1 M sodium-acetate buffer plus 0.05 M sodium chloride to yield an ionic strength of 0.15 and pH 4.0. Under these conditions, the egg albumin-pepsin complexes are soluble.

For egg albumin alone, \( H \) has the value of \( 0.954 \times 10^{-5} \) cm.² per gm.², for pepsin alone \( 1.050 \times 10^{-5} \) cm.² per gm.², and for equimolecular mixtures of egg albumin and pepsin \( H \) is equal to \( 0.924 \times 10^{-5} \) cm.² per gm.². These values were calculated from specific refractive index increments obtained from the best straight line relating refractive indices and protein concentrations for every concentration of solution used. The wave-length of the scattered light was 4370 Å.

The least square line relating \( HC/\tau \) and \( C \) yielded a molecular weight for egg albumin of 45,200, with a probable error of 1700. This value compares favorably with those reported in the literature. The corresponding value for pepsin was 37,600, with a probable error of 4400, which is also consistent with the values reported in the literature for this protein.
Results

Fig. 1 shows the amount of total nitrogen in the precipitate as a function of the amount of egg albumin present at several pH values, the pH being adjusted with sulfuric acid and the amount of pepsin being held constant (0.70 mg. of total pepsin nitrogen).

Fig. 2 shows the effect of the variation of pH in the presence of various salts at ionic strengths of $4 \times 10^{-4}$ on the amount of precipitated complex formed. Also contrasted is the influence of hydrochloric acid and of sulfuric acid alone; 2.54 mg. of egg albumin and 4.8 mg. of pepsin were used.

No free sulfhydryl could be detected in the precipitated complex with the sodium nitroprusside test as modified by Shinohara and Kilpatrick (4) and the pepsin retained its enzymatic activity. We are, therefore, not dealing with precipitation of denatured protein.

It was found that the turbidity of the dissolved complex formed from an equimolecular mixture of egg albumin and pepsin decreased linearly with time up to 5 hours. Such turbidity plots are shown in Fig. 3 for various total protein concentrations. The slopes of these lines were measured as well as their intercepts at zero time. The $HC/\tau$ values obtained by extrapolation to zero time have been plotted against total protein concentration and are shown in Fig. 4. The curve is extrapolated to zero protein concentration to an $HC/\tau$ value corresponding to the weight average molecular weight of an equimolecular mixture of pepsin and egg albumin without interaction.
The logarithms of the slopes of the lines in Fig. 3 were plotted against the logarithms of the initial egg albumin concentrations to obtain a slope of 2. This plot is shown in Fig. 5. The equation for this line is

\[
\log \left( \frac{dr}{dt} \times 10^4 \right) = \log 0.036 \times 2 \log [E_0] \\
\]

where \([E_0]\) is the molar egg albumin concentration.

Eliminating logarithms from Equation 4, we have

\[
\frac{dr}{dt} = 0.036 \times 10^{12} [E_0]^2 \]

Since \([E_0] = [P_0]\), Equation 5 can be written

\[
\frac{dr}{dt} = 0.036 \times 10^{12} [E_0][P_0] \]
Fig. 3. Light-scattering turbidity as a function of time for equimolecular mixtures of egg albumin and pepsin; pH 4.0, ionic strength 0.15.

Fig. 4. Reduced intensity-concentration for equimolecular mixtures of egg albumin and pepsin; pH 4.0, ionic strength 0.15 at zero time.
As shown in Fig. 1, the maximum precipitation of the egg albumin-pepsin complex is reached at approximately equal molar concentrations of these two proteins. With the addition of more egg albumin and constant amount of pepsin, less and less precipitate forms, until at about 29 mg. of egg albumin total nitrogen and at pH 4 no precipitate at all occurs.

![Graph showing the logarithm of initial rate of decrease of turbidity of equimolecular mixtures of egg albumin and pepsin, pH 4.0, ionic strength 0.15, and room temperature, with time as a function of the logarithm of initial egg albumin or of initial pepsin molar concentration.]

(This is not shown in Fig. 1.) It has been found that the precipitate is also soluble in large excess of pepsin at pH 4.

As can be seen from Fig. 2, the amount of precipitate formed is very much dependent on the pH, and this dependence is modified by the presence of dilute salts. It is probable that electrostatic interaction is of great importance in the formation of the precipitated complexes between egg albumin and pepsin. From the titration curves of egg albumin (5) and of pepsin (6), it is evident that the net negative charge on the pepsin molecule is very nearly equivalent to the net positive charge on the egg
albumin molecule in the pH range 3.6 to 4. From these considerations and from the results shown in Fig. 1, it is probable that in the maximum precipitation zone the precipitated complex consists of equal molar ratios of egg albumin and of pepsin joined together by electrostatic interaction. The non-specific nature of this interaction is also indicated by the great rapidity with which the precipitate forms.

The nature of the complex is still further illuminated by the light-scattering studies. It is evident from Fig. 4 that $HC/\tau$ is not a linear function of the total protein concentration. Indeed, since the two proteins react to form complexes, linearity is not to be expected. At higher total protein concentrations, undoubtedly complexes of the type $(EP)_n$ and perhaps also $(E_P)_n$ will form. As the total protein concentration approaches zero, the only complex in significant quantity will be the monomeric, equimolecular complex $(EP)$, provided the forces leading to the formation of the higher complexes are of the same sort as give rise to the initial complex $(EP)$. This assumption appears reasonable and permits us to continue our analysis. With this assumption, we can write the association constant for the $(EP)$ constant as

$$K = \frac{[EP]}{[E][P]} \quad (7)$$

The total concentration in gm. per ml. of all the protein species as the concentration approaches zero will then be


where $[E]$, $[P]$, and $[EP]$ are expressed in moles per ml. and $M_E$, $M_P$, and $M_{EP}$ are the molecular weights of egg albumin, of pepsin, and of the first egg albumin-pepsin complex. The weight average molecular weight of the protein species is then


Combining Equations 7, 8, and 9 and using the condition that $[E]$ is equal to $[P]$, we obtain

$$K = \frac{2M_NM_P(2M_EM_P - M_{EP}(M_{EP} - M))}{CM_{EP}(M_{EP} - M)^2} \quad (10)$$

Differentiating Equation 10 with respect to the variables $M$ and $C$, we obtain

$$\frac{d}{dc} \left( \frac{1}{M} \right) = -\frac{1}{M^2} \left[ \frac{K(M_{EP} - M)^2}{2M_EM_P + 2KC(M_{EP} - M)} \right] \quad (11)$$

The initial slope of the curve in Fig. 4 is the initial rate of change of
HC/\tau$ with concentration and reflects the rate of change of the reciprocal of the weight average molecular weight with concentration. This initial slope is estimated to be $-0.43 \times 10^{-2}$ and the weight average molecular weight approaches 41,400 as the protein concentration approaches zero. The molecular weight of the first complex, $M_{EP}$, is the sum of the molecular weights of egg albumin and of pepsin, or 82,800. By substituting these values in Equation 11 and setting $C$ equals zero and solving for $K$, the association constant of the first complex has the value $1.46 \times 10^{7}$ ml. per mole. Bull and Currie (7), from a conventional kinetic study of the rate of digestion of egg albumin by pepsin over the pH range 1.3 to 2.8 and by the application of a modified Michaelis-Menten treatment, found an association constant of $0.14 \times 10^{7}$ ml. per mole. The difference between the present result and that of Bull and Currie may arise from a partial failure of theory, or it is possible that the association constant between egg albumin and pepsin at pH 4 is significantly larger than it is in the neighborhood of pH 2.

The molar interaction constant of a mixture of two high molecular weight components is given by Stockmayer and Stanley (8) as

$$B_{ij} = \frac{B (M_i + M_j)^2 \epsilon_i \epsilon_j}{4 M_i M_j \epsilon_i \epsilon_j}$$ (12)

when the two components are mixed in an equimolar ratio. $B$ is the constant defined in Equation 2; $M_i$ and $M_j$ are the two molecular weights, and $\epsilon_i$ and $\epsilon_j$ are the two index of refraction increments. $\epsilon_{ij}$ is the index of refraction increment of the mixture of the two components. The interaction constant of egg albumin with itself turns out to be $0.91 \times 10^{-4}$ ml. mole per gm.$^2$ and $-0.62 \times 10^{-4}$ ml. mole per gm.$^2$ for pepsin alone. The theoretical value of $B_{ij}$ for an equimolecular mixture of egg albumin and pepsin (if they did not associate) would be $-0.70 \times 10^{-4}$ ml. mole per gm.$^2$. The actual value of $B_{ij}$ for the equimolecular mixture of these two proteins is $-25.67 \times 10^{-4}$ ml. mole per gm.$^2$, reflecting the large interactive forces between these proteins. The relation between the interaction constant, $B_{ij}$, and the association constant, $K$, turns out to be

$$-B_{ij} = \frac{1}{16} \frac{M_{EP}^2 \epsilon_{ij}}{M^2 M^2 \epsilon_i \epsilon_j}$$ (13)

From Equation 6, the initial rate of decrease of turbidity with time is proportional to the first power of the initial egg albumin and of the initial pepsin concentrations. Solving Equation 2 for $\tau$, the turbidity, we have

$$\tau = \frac{HCM}{1 + 2BCM}$$ (14)
Differentiating Equation 14 with respect to \( \tau \) and \( M \) and dividing by \( dt \), there results, for a given protein concentration,

\[
\frac{d\tau}{dt} = HC \left[ \frac{1}{(1 + 2BCM)^2} \right] \frac{dM}{dt}
\]  

(15)

The digestion reaction mechanism may be described as

\[
E + P \rightleftharpoons (EP) \rightarrow P + \sum n_i A_i
\]

where \((EP)\) represents the intermediate complexes, \( A_i \) the peptides formed by digestion, and \( n_i \) the number of moles of \( A_i \) formed. The weight average molecular weight of the mixture of peptides, complexes, and proteins becomes

\[
M = \left[ \sum M_{\text{EP}} \right] + [P]M_P^3 + [(EP)]M_{EP}^3 + \sum [A_i]M_{A_i}^3
\]  

(16)

Taking the derivative of \( M \), in respect to time, yields

\[
\frac{CdM}{dt} = M_{EP}^2 \frac{d[E]}{dt} + M_P^3 \frac{d[P]}{dt} + M_{EP}^3 \frac{d[(EP)]}{dt} + \sum M_{A_i}^3 \frac{d[A_i]}{dt}
\]  

(17)

We have no present knowledge of how \([EP]\), \([P]\), or \([A_i]\) changes with time. We can make the tentative assumption that the change of the weight average molecular weight of the reaction mixture is due principally to the change in the amount of egg albumin resulting from peptic digestion. On the basis of this simplification, Equation 17 becomes

\[
\frac{dM}{dt} = \frac{M_{EP}^2 \frac{d[E]}{dt}}{C} \]

(18)

Substituting Equation 18 in Equation 15, there results

\[
\frac{d\tau}{dt} = H \left[ \frac{1}{(1 + 2BCM)^2} \right] M_{EP}^2 \frac{d[E]}{dt}
\]  

(19)

Substituting Equation 19 in Equation 6 and taking the limiting value as time approaches zero give

\[
\frac{d[E]}{dt} \frac{0.036 \times 10^{[E]_0[P]}}{H \left[ \frac{1}{(1 + 2BCM)^2} \right] M_{EP}^2}
\]  

(20)

By using an average value of \(1/(1 + 2BCM)^2\) of 1.14 and substituting the known values in Equation 20, we find the initial velocity of the reaction at unit molar concentrations of pepsin and of egg albumin to be 1.68 \( \times 10^6 \) ml. per mole hour or 0.47 liter per mole second. This value is to be compared with the value of 2.0 liters per mole second predicted by the use of the kinetic equation of Bull and Currie (7) extrapolated to pH 4.0.
SUMMARY

1. It has been shown that egg albumin and pepsin when mixed in the pH range 3.1 to 4.2 form a precipitate which is not denatured protein.

2. The amount of precipitate depends on the ratio of the concentrations of the two proteins, the optimum ratio corresponding approximately to a 1:1 molar ratio. The amount of precipitate also depends upon the pH and the salt concentration, the precipitate dissolving at higher salt concentrations.

3. The light-scattering properties of the soluble egg albumin and pepsin complex at pH 4.0 and at an ionic strength of 0.15 have been studied.

4. The association constant of the monomeric complex between egg albumin and pepsin has been calculated to be $1.46 \times 10^7$ ml. per mole at pH 4.0.

5. The velocity constant of the digestion of egg albumin by pepsin has been estimated on the basis of tentative assumptions from light-scattering measurements to be about 0.47 liter per mole second at pH 4.

This investigation was supported in part by a research grant from the National Institutes of Health, United States Public Health Service. It is also a pleasure to acknowledge the financial support of Swift and Company, which provided a fellowship for D. S. Yasnoff.

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