nitro derivatives showed that the triphenyltetrazolium chloride is most subject to photoreduction in sunlight. A solution of the latter compound was definitely pink after 10 min in direct sunlight. At the end of 1 hr, the iodo derivative was barely pink, whereas the other two compounds showed no pink coloration.

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Use of Dyestuffs for Determining the Activity of Proteolytic Enzymes

Benjamin Carroll[1]

Newark Colleges of Rutgers University, Newark, and Department of Chemistry, Columbia University, New York

Numerous investigators (*1*) have shown that native albumin has an affinity for simple dye anions, whether the protein is on the acid or alkaline side of its isoelectric point. This is usually indicated by a spectral change when the protein is added to an aqueous solution of the dye. Pepsin, as well as other proteolytic enzymes, shows practically no combining capacity for these dyes. This fact suggests a new method of determining activity of proteolytic enzymes which may offer advantages.

Klotz (*2*) has shown that common denaturants, such as sodium hydroxide and heat, cause bovine serum albumin to lose its binding ability for anionic dyes. We have found that the action of proteolytic enzymes upon this native protein produces the same effect. Thus a change in the structure of the albumin molecule due to denaturation or fission of its peptide linkages can be detected in the presence of an enzyme. Quantitative activity measurements may be made by estimating the concentration of the native protein in the presence of the proteolytic enzyme as a function of the time, using an empirical curve as is usually done in spectrophotometric analysis. It should be emphasized that this procedure alone will not indicate the state of the protein that has been acted upon by the enzyme. The method is based on measuring the quantity of native protein remaining in solution, whereas the usual activity determination is based on an analysis of the products of hydrolysis.

Fig. 1 shows the effect of a 0.1% pepsin solution upon a 0.2% bovine albumin[2] solution at pH 5.4 in the presence of a constant concentration of the dye, orange I. The extinction is plotted as a function of the wavelength at various time intervals. The cuvettes containing the reaction mixture were kept in the housing of the Beckman quartz spectrophotometer throughout the experiment. No attempt was made to regulate the temperature of the solution, which was about 37° ± 2° C. It will be seen that addition of bovine albumin to the dye causes a depression of about 20% in the absorption maximum, which is in keeping with the data reported by Klotz. Upon addition of crystallized pepsin, the optical density of the reaction mixture in the presence of the dye approaches that for the dye alone.

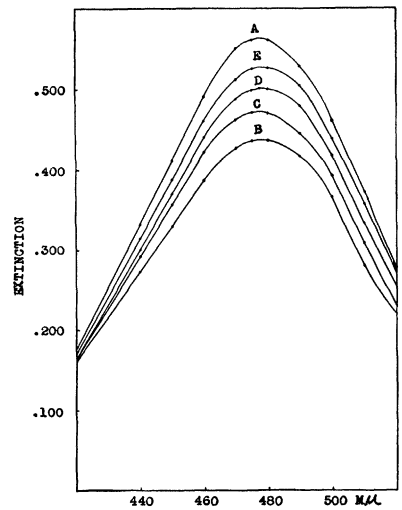

FIG. 1. Effect of 0.1% pepsin on 0.2% albumin in 2.0×10^{-5}M orange I at pH 5.4 (phosphate buffer). A—Dye alone. B—Dye + albumin. C—Dye + albumin + pepsin after 25 min. D—Same as C after 62 min. E—Same as C after 195 min.

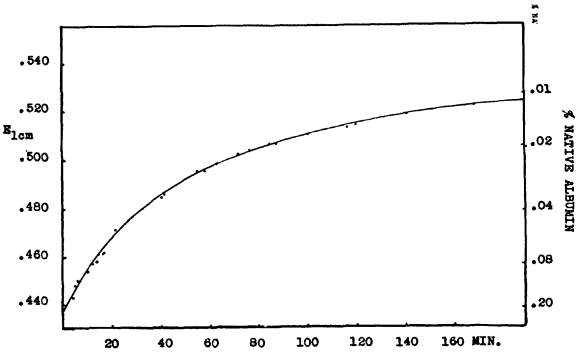

FIG. 2. Optical density at 475 mμ for orange I (2.0×10^{-5}M) in presence of 0.2% albumin and 0.1% pepsin at pH 5.4.

The values of the optical density of the reaction mixture at 475 mμ are plotted as a function of the time in Fig. 2. The values for the concentration scale are on

[1] The interest and suggestions of Prof. A. F. Thomas are gratefully acknowledged.

[2] Crystallized bovine albumin and crystallized pepsin (porcine mucosa) were used in preparation of solutions. These were purchased from the Armour Laboratories, Chicago, Illinois.

the right of Fig. 2. These were obtained from an empirical curve where the optical density was observed at a constant concentration of dye at pH 5.4, while varying the concentration of bovine albumin. The course of the reaction is shown on a semilogarithmic scale in Fig. 3.

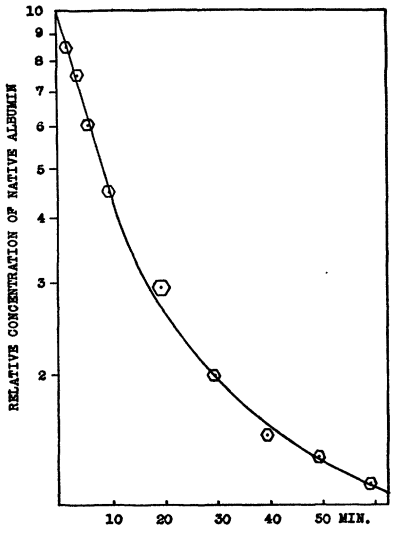

FIG. 3. Fraction of native albumin in presence of 0.1% pepsin at pH 5.4.

It is interesting to note that the half-life of the reaction is about 8 min and that it has gone to 90% completion in 80 min.

The reason for having the concentration of pepsin as high as 0.1%, and the pH at 5.4 instead of the optimum value of pH 2, was to gather information on the nature of enzyme-substrate interaction. Nevertheless, the method is generally the same if conditions of pH and initial concentration of enzyme and substrate are altered. Using the procedure outlined, no particular difficulty was encountered in studying activity of pepsin on albumin at pH 2 when enzyme concentration was one part per billion.

The kinetics of the pepsin-albumin system were studied in considerable detail in 0.01*N* HCl. It has been found that bovine albumin can be kept for several weeks in 0.01*N* HCl without appreciably affecting its binding capacity for simple dye anions. Kinetic experiments involving the variation of the initial concentration of enzyme and substrate showed that these factors had about the same effect as has been established with the usual techniques (*3*) using the formal titration method. An important difference in the activity when determined by the dye method instead of classical methods is that the linear effect of enzyme concentration on the reaction rate can be observed not only at the beginning of the experiment, but even when 50% or more of the native protein has been removed.

Several objections to this method may be raised. One is the possible inaccessibility of substrate to enzyme during the course of the reaction in the presence of the dye. This problem may be removed by simply adding a constant quantity of dye to a given aliquot of the dye-free reaction mixture at various time intervals. This procedure may remove another experimental difficulty, provided the added dye solution is properly buffered to stop the reaction. Thus it is possible to avoid the assumption that the time interval for the albumin and dye to reach equilibrium is inappreciable.

One of the reviewers has criticized this method, stating that "since it is not known what structural changes in the albumin molecule are sufficient to destroy its ability to yield a spectral shift, it is impossible to interpret these kinetic results in terms of denaturation or rate of hydrolysis." Although this may be the state of our knowledge at present, analytical methods do exist which make it possible to determine the extent of both processes and thus interpret the spectral changes quite accurately. A study of the effects of temperature using the dye method indicates an activation energy of about 19,000 calories. This would strongly suggest that the spectral change is due to the breaking of peptide linkages.

Expressing the optical density in terms of concentration of native protein may be an oversimplification. It would be more exact to express results in terms of bound dye anions or available sites on the substrate. The point of complete denaturation (or hydrolysis) and the absence of binding sites on the substrate may not be exactly equivalent. Furthermore, all binding sites on the substrate may not be alike. Until this problem is solved, the use of an empirical curve to obtain concentration values for the substrate appears to be most convenient.

The use of dyes as indicators for following the course of a proteolytic reaction may offer the advantages of increased sensitivity and ease of adaptation of the method to microprocedures. During the course of some work in searching for long range forces (Rothen type [*4*]) in solutions, it became necessary to determine the effect of pepsin on bovine albumin, which had an initial concentration of 1 ppm. The dye method was found suitable for handling this problem.

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