zero-gradient value of η_{sp}/c less than, but of the same order of magnitude as, the observed values at moderate concentrations at the lowest velocity gradients achieved. The length of such polymers would be sufficient to account qualitatively for the non-Newtonian viscosities observed.

The drop in apparent η_{sp}/c with dilution is then attributed to dissociation of the intermediate Φ itself, but as mentioned above the drop is exaggerated by the non-Newtonian character of the flow. A certain amount of dissociation is not inconsistent with the persistence of the fast peak in the ultracentrifuge when systems containing the intermediate polymer are diluted, since the value of the sedimentation constant is not very sensitive to length until the end-to-end degree of polymerization falls below five. The effect of dilution is being further investigated by light scattering.[25]

Thus the properties of the intermediate fibrinogen polymer, Φ, identified in urea and hexamethylene glycol solutions are consistent with a cross-section area double that of fibrinogen and a range of lengths of the order of ten times that of fibrinogen. When it is formed from fibrinogen and thrombin, its immediate precursor may well be a chemically altered fibrinogen such as the molecule identified in acidic clotting systems by Laki,[26] which may be symbolized F′. When it is formed from urea-dissolved fibrin, its immediate precursor is a fibrinogen-shaped fragment, symbolized F*, which Laki[26] has already surmised as possibly identical with F′. Probably F′ (and F*) has a molecular weight smaller than that of fibrinogen by 3 to 4%, having undergone the loss of one or more peptide units totalling this amount.[9] The number and location of these peptide units are no doubt reflected in the detailed geometry of formation of Φ. The observed transformations which interrelate these substances are summarized in Fig. 9.

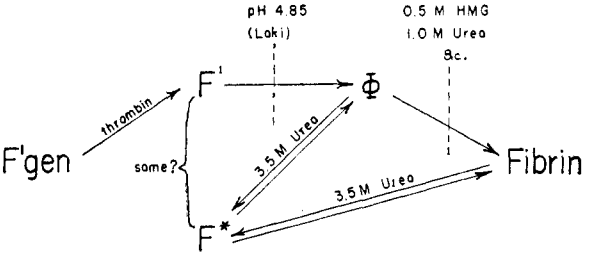

Fig. 9.—Relations among fibrinogen, fibrin and the intermediates Φ, F′ and F*.

Acknowledgments.—We are much indebted to the Minnesota Mining and Manufacturing Company for furnishing the glass spheres used in falling sphere viscosity measurements, and to Mr. W. Van Valkenburg and Mr. K. Gutfreund for help in some of the experiments.

MADISON 6, WISCONSIN RECEIVED OCTOBER 29, 1951

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[FROM THE DEPARTMENT OF CHEMISTRY, INDIANA UNIVERSITY, AND THE DEPARTMENT OF BIOLOGICAL CHEMISTRY, UNIVERSITY OF ISTANBUL]

The Reaction of Native and Denatured Ovalbumin with Congo Red[1]

BY FELIX HAUROWITZ, FRANK DIMOIA AND SHEVKET TEKMAN

Heat-treated ovalbumin and serum albumin combine with congo red to form complexes which remain red at *p*H 2, while mixtures of congo red with the unheated proteins have a purplish-blue color at *p*H 2. Since the absorption spectra of congo red in acid solutions of unheated and heat-treated albumins differ considerably, the kinetics of denaturation can be examined spectrophotometrically. The number R of red dye molecules bound per protein molecule is roughly proportional to c, the concentration of the blue dye molecules. After complete denaturation of the native proteins by heat, R increases by four in ovalbumin, and by two in serum albumin. Ovalbumin exposed to temperatures of 65 or 70° combines with less congo red than completely denatured ovalbumin exposed to 75°. The denaturation of ovalbumin by heat follows first-order kinetics with respect to protein concentration; the reaction order with respect to time is about 6.0 at 65°, 4.2 at 70° and 3.4 at 75°. It is concluded that the cleavage of hydrogen bonds and/or salt bridges, which presumably obeys first-order kinetics, is followed by the irreversible formation of new *intra*molecular cross-links between the peptide chains, and that this second phase of denaturation is a reaction of high order with respect to time.

Introduction

The color reactions of denatured proteins with nitroprusside,[2] phosphomolybdate[3,4] and diazobenzenesulfonate[4] are more intensive than those given by the same proteins in the native state. The low reactivity of the native proteins has been attributed to the inaccessibility of some of the groups in the closely folded peptide chains[5] and/or to their combination with other groups by hydrogen bonding.[6]

The present investigation was undertaken in order to find whether denaturation involves a similar increase in reactive cationic groups. Such an increase would alter profoundly the physical-chemical behavior of soluble proteins and particularly their electrokinetic potential.[7] Electrometric titration cannot give any information in this respect because all of the ionic groups of a protein are accessible to the small hydrogen ions. Previous investigations[8] had indicated that denaturation of proteins in-

(1) This work was supported by research grants from the U. S. Public Health Service, National Institutes of Health, and from the Graduate School of Indiana University. Some of the material was presented by Frank DiMoia in partial fulfillment of the requirements for a degree of Doctor of Philosophy at Indiana University. Earlier results were reported at the Meeting of the American Chemical Society in New York, September, 1947 (Abstracts, p. 29 C).

(2) A. C. Mirsky and M. L. Anson, *J. Physiol.*, **19**, 427 (1936).

(3) M. L. Anson, *ibid.*, **24**, 399 (1941).

(4) F. Haurowitz and S. Tekman, *Biochim. Biophys. Acta*, **1**, 484 (1947).

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volves an increase in the ability to combine with the strongly basic protamines. In the present paper we report on the combination of heat treated and unheated ovalbumin and serum albumin with the large congo red anion. One could expect that an anion of this size would not be able to penetrate between the closely folded peptide grid and that it would therefore react only with easily accessible groups of the protein surface.

Experimental Methods

Solutions.—In most of the experiments the following three solutions were used: *Ovalbumin solution (I)* containing 6 mg. (1.33×10^{-7} mole) ovalbumin per ml. of a 0.1 molar sodium phosphate buffer solution *p*H 6.85; the ovalbumin was prepared and recrystallized according to Kekwick and Cannan.[9] *Serum albumin solution (Ia)* containing 6 mg. (0.88×10^{-7} mole) human serum albumin (Squibb) per ml. of the same solvent. *Congo red solution (II)* containing 1 mg. of the dye per ml. water. In most experiments 0.2 mg. dye (2.88×10^{-7} mole) was used. *0.53 N hydrochloric acid (III).*

Basic Experiment.—Mix 10 ml. of ovalbumin solution (I) or serum albumin solution (Ia) with 2 ml. of congo red solution (II). Transfer one-half of the mixture to another test-tube. Place one of the tubes for 3 minutes in a boiling water-bath. After cooling add 2.5 ml. of hydrochloric acid solution (III) to each of the tubes and mix. While the color of the solution containing unheated protein changes to purplish-blue, the solution containing heated protein remains red. If congo red paper is dipped into this red solution, the paper turns blue, demonstrating clearly the difference between free dye (blue) and dye bound to the denatured protein (red) at *p*H 2. If more acid is added, both solutions become blue. The same phenomena are observed when congo rubin is used instead of congo red.

General Procedure.—In all experiments on the kinetics of denaturation weighed Pyrex test-tubes 15 × 180 mm. containing 11.5 ml. of water were heated in a water-bath of the desired temperature, while tubes containing the ovalbumin solution (I) were heated to 54°. When the temperature of the water in the Pyrex tubes had reached that of the bath, one ml. of the warm ovalbumin solution (I) was added and the mixture stirred. Heating was continued for definite periods of time. Evaporation was minimized by loosely fitting glass stoppers. The tubes were rapidly cooled in an ice-bath, and precisely 2.0 ml. of the 0.01% congo red solution was added. Water was then added to bring the content of the tubes to 14.5 ml. The combination of congo red with denatured ovalbumin is a slow reaction requiring about 10 to 15 minutes for completion. We waited, therefore, 30 to 60 minutes before adding 0.5 ml. of the HCl solution (III) to the dye–protein mixture; the *p*H of the acidified solutions was 2.0 to 2.1. They were immediately transferred into Corex cells of 10 mm. depth, and their extinction

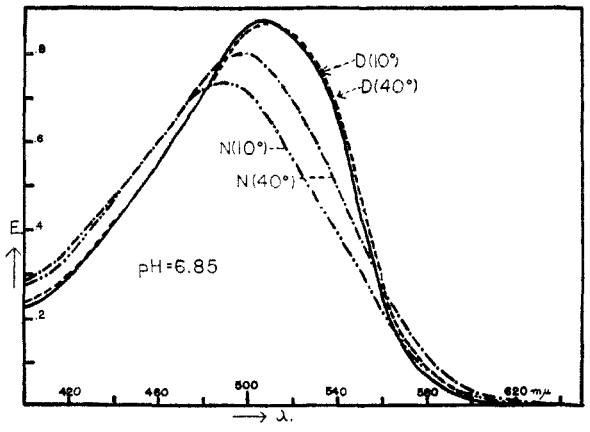


Fig. 1.—Effect of native (N) and denatured (D) ovalbumin on the absorption spectrum of congo red.

(9) R. A. Kekwick and R. K. Cannan, *Biochem. J.*, **30**, 227 (1936).

was measured with a Beckman DU spectrophotometer. Deviations from this General Procedure are indicated in the text.

Experimental Results

The Absorption Spectrum of Congo Red in the Presence of Unheated and Heat-Treated Ovalbumin.—Figure 1 shows the absorption spectrum of neutral solutions of congo red in the presence of native (N) and heat-treated, denatured (D) ovalbumin. Denaturation was achieved by 30 minutes heating at 90° before addition of the dye. The absorption spectrum of the dye in a protein-free buffer solution *p*H 6.85 (Fig. 2, Curve B) is identical with that displayed in the presence of native ovalbumin (Fig. 1, curve N). The denatured ovalbumin causes a slight increase in the absorption and a shift of the absorption maximum from 496 to 510 mμ (Fig. 1). Similar changes are brought about by raising the temperature from 10 to 40° (Fig. 1) or by using ethanol as a solvent for the dye (Fig. 2, curve E). All these changes are probably due to depolymerization of the dye micelles, and combination of the protein with dye monomers. Similar depolymerization of congo red by wool fibers,[10] of basic dyes by nucleic acids[11] and of pinacyanol by gelatine[12] has been reported by other authors.

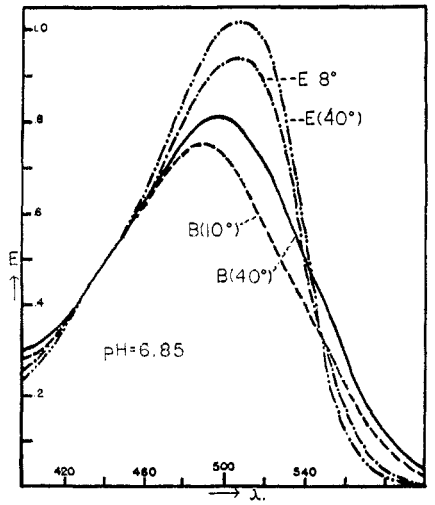


Fig. 2.—Temperature effect on the absorption spectrum of congo red dissolved in 95% ethanol (E) and in 0.0067 molar phosphate buffer *p*H 6.85 (B).

Protein-free solutions of congo red become turbid on acidification. Curve B (Fig. 3) shows the approximate absorption spectrum of such a solution measured immediately after acidification to *p*H 2.0, before any turbidity was visible. More stable blue solutions are obtained in the presence of 0.04% human serum albumin; such solutions are purplish-blue at *p*H 2 and become pure blue on acidification with HCl to *p*H 0.0 (Fig. 3, curve SA). Mixtures of the dye with ovalbumin become turbid at this high acidity.

While the acid blue solutions B and SA (Fig. 3) have absorption maxima at about 560 and 580 mμ, the acid solution containing *denatured* ovalbumin (Curve DA) is red and shows the typical absorption spectrum of the neutral dye with a maximum at 510 mμ. This is in agreement with earlier observations of Pauli.[13]

The mixture of congo red with *unheated* ovalbumin has a purplish-blue color at *p*H 2. Its absorption maximum at 510 mμ (Fig. 3, curve NA) shows that some of the dye is present in the red form, although most of it is found in the blue form. The spectrum remains practically unchanged for 15 to 30 minutes after acidification of the dye–protein mixture. If the mixture is exposed to *p*H 2 for many hours or days, some of the blue form of the dye is slowly converted into the red form.

The Protein Error of Congo Red.—Four ml. of ovalbumin solution (I), 55.2 ml. of water and 0.8 ml. of undiluted congo

(10) E. Valko, in Alexander, *Colloid Chem.*, **6**, 594 (1946).
(11) L. Michaelis, *J. Phys. Colloid Chem.*, **54**, 1 (1950).
(12) I. M. Klotz, *Chem. Revs.*, **41**, 373 (1947).
(13) W. Pauli and L. Singer, *Biochem. Z.*, **244**, 76 (1932).

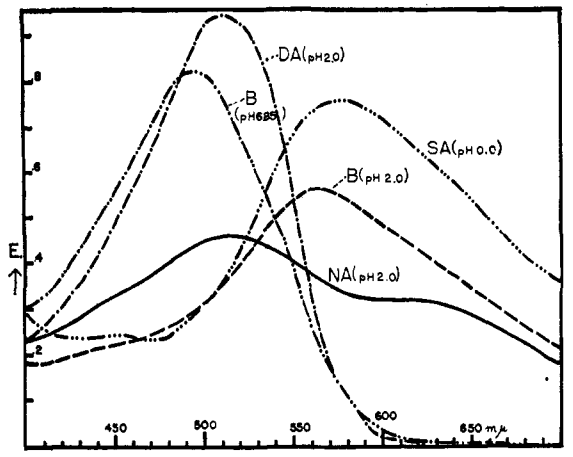


Fig. 3.—Absorption spectra of congo red in the presence of unheated ovalbumin (NA), heat-treated ovalbumin (DA), human serum albumin (SA), and in protein-free buffer solutions (B).

red solution (II) were mixed and kept in a boiling water-bath for 5 minutes. After cooling, hydrochloric acid was added dropwise while the solution was stirred. At various times, *p*H was determined with a glass electrode. At the same times, the extinction was measured at 650 mμ on a small sample of the mixture (Fig. 4, curve D). Titration was continued to a *p*H of 0.3, where the solution became turbid. Similar experiments were run with native ovalbumin (Curve N) and with a protein-free solution of the dye (Fig. 4, curve C). In the absence of protein the dye solution became turbid at *p*H 4.5, so that measurement at lower *p*H values was impossible.

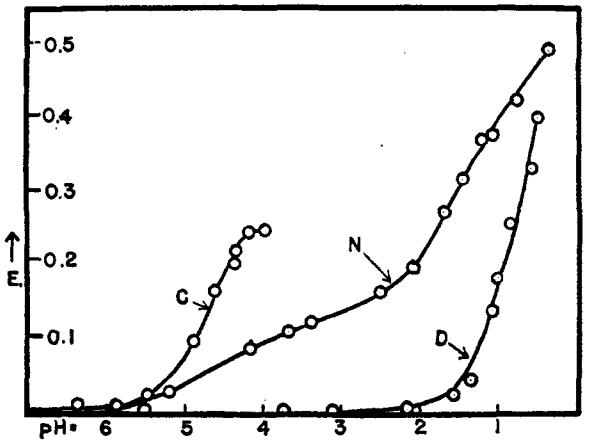


Fig. 4.—Extinction of congo red solutions at 650 mμ in the presence of unheated ovalbumin (N), denatured ovalbumin (D), and in protein-free solution (C).

Determination of the Amount of Congo Red Bound by Ovalbumin and Serum Albumin.—Each of 6 test-tubes containing 6 mg. of ovalbumin or human serum albumin was kept for one hour at 80° and 90°, respectively. After addition of the dye and acidification, as described under "General Procedure," the extinction, E_D, was determined at 650 mμ. The extinction of the solution containing unheated protein, E_N, was measured in a parallel experiment. E_R in Table I is the extinction of the pure red form of the dye in 0.04% ovalbumin. It is identical with the extinction in 0.04% serum albumin or in the protein-free buffer solution (see Fig. 2). The extinction of the blue form, E_B, was measured in 1.5 *N* hydrochloric acid containing 0.04% serum albumin.

Although the extinctions E_R and E_B are not strictly proportional to the amounts of dye added, an approximate calculation of the amount of dye bound in the red form is possible if the following simplifying assumptions are made:

TABLE I

EXTINCTION OF PROTEIN–CONGO RED MIXTURES

W (mg. dye added)	0.100	0.200	0.400	0.800	1.200	1.600
E_B	.388	.600	1.22	2.56	4.00	5.36
E_R	.002	.004	0.008	0.011	0.020	0.032
E_D (ovalbumin)	.004	.028	.192	0.63	1.24	1.88
E_N (ovalbumin)	.144	.302	.622	1.23	1.84	2.46
E_D (serum albumin)	.075	.210	.447	0.95	1.63	2.12
E_N (serum albumin)	.107	.254	.517	1.05	1.76	2.30

(a) The intensity of light-scattering due to protein is the same in all solutions since all of them contain the same amount of protein; (b) the deviation from Lambert-Beer's law is the same for solutions containing the same amount of dye, *i.e.*, for identical values of W (Table I). If W_R and W_B are the amounts in mg. of red and blue dye, respectively, the amount of dye present in the blue form is $W_B = W - W_R$, and $W_R = W(E_B - E)/(E_B - E_R)$, where E is E_D or E_N of Table I. Plotting of R, the ratio of moles red dye bound per mole protein, against C, the molar concentration of the blue dye, gives the curves shown in Fig. 5.

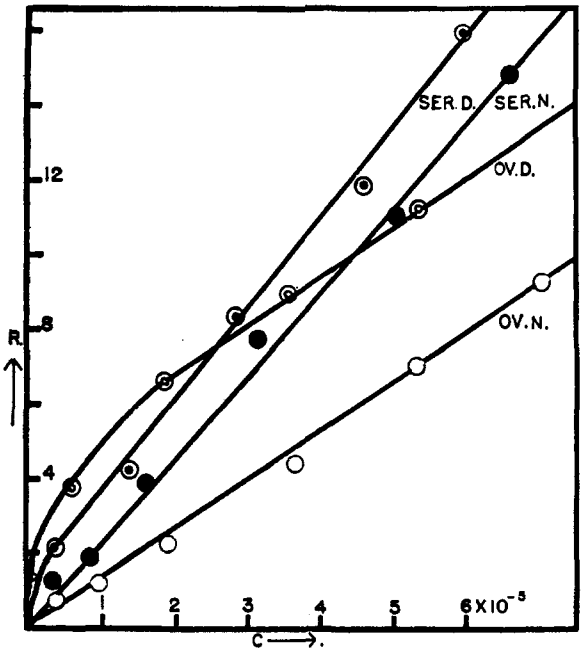


Fig. 5.—Molecules of red dye per protein molecule at *p*H 2.1; R = number of red dye molecules per protein molecule; C = molar concentration of blue dye; Ser = human serum albumin; Ov = ovalbumin; N = unheated; D = heat-treated.

Denaturation of Ovalbumin at Different Temperatures.—The experimental conditions were those described in the General Procedure. The progressive change in the absorption spectrum of ovalbumin–congo red mixtures exposed to 75° for various lengths of time is shown in Fig. 6. The figure demonstrates that the absorption spectrum of the completely denatured solution ($t \doteq 60$ minutes), after acidification to *p*H 2, is similar to that of a neutral solution containing the dye and unheated ovalbumin (curve CR). Wave length 650 mμ, where the mixture of the dye with completely denatured ovalbumin has a very low extinction, was used for the determination of native and denatured ovalbumin. The photometric measurement is made easy by the solubility of the dye–ovalbumin complex; the denatured solutions are free of turbidity. In this respect our method differs essentially from methods which are based on the insolubility of the denatured protein.

The calculation of the amount of denatured ovalbumin is based on the curves shown in Fig. 7. The curves 1, 2 and 3 give the extinction at 650 mμ of acidified solutions of congo red in the presence of increasing amounts of ovalbumin kept at 88° for 60 minutes. In the experiments 1 and 2, native

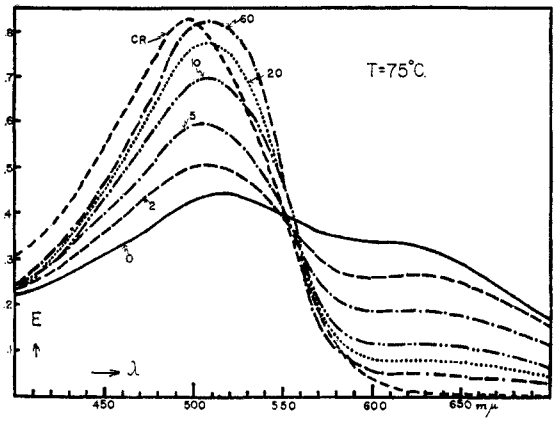


Fig. 6.—Absorption spectra of ovalbumin solutions heated for various lengths of time, mixed with congo red and acidified to *p*H 2.1. The length of the heat-treatment is denoted in minutes. CR = absorption spectrum of the neutral mixture of congo red with native ovalbumin at *p*H 6.85.

ovalbumin was added after the heat-treatment to bring the total ovalbumin to 6 mg. No native ovalbumin was added in 3. In experiment 2 the dye (0.2 mg.) was added *before* the heat-treatment, whereas in 1 and 3 it was added to the heat-treated and then cooled protein solutions. Evidently more of the red dye is bound under the conditions of experiment 2. In experiment 3, the extinction at zero concentration of the denatured ovalbumin was not measured because of turbidity of the solution. Repeated measurements proved that the inflections of the curves 1 and 2 were real. They are due to the fact that the unheated ovalbumin is also able to combine with small amounts of the dye in the red form as shown by Figs. 3 and 5. The curves 1 and 2 were used to calculate milligrams of denatured ovalbumin from the extinction. This procedure does not involve any assumption on the free or bound state of the dye, or on the nature of the reaction taking place during the heat-treatment.

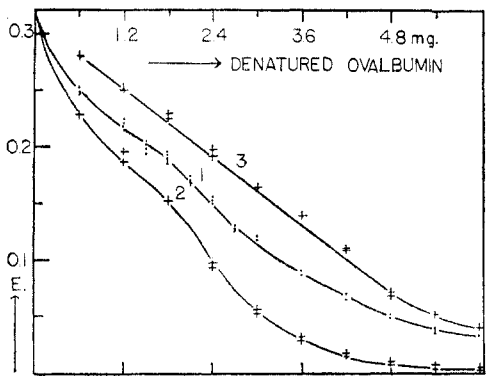


Fig. 7.—Effect of various amounts of denatured ovalbumin on the extinction at 650 mμ of congo red at *p*H 2. In 3 no native ovalbumin was added. In 2 the dye was added before the heat-treatment.

The results of a typical experiment are shown in Fig. 8, where the extinction at 650 mμ is plotted against the time of heating. The dye was added after the heating so that the conditions were the same as in curve 1 of Fig. 7. Denaturation at 75° was complete since the same final extinction, *E*, resulted when ovalbumin solutions were exposed to temperatures of 80 or 90°. The extinction, *E*, of solutions exposed for several hours to 75° remained also unchanged when these solutions were subsequently kept at 37.5 or 53° for 2 or 4 hours. This proves that denaturation of ovalbumin is *irreversible* under our experimental conditions.

In the experiments represented by the curves 1a and 1b of Fig. 9, 1 ml. of ovalbumin solution (I) was mixed with 12.5 ml. and 2.0 ml. of water, respectively, and exposed to a temperature of 67.5°. After cooling, the volume of 1b was brought to 13.5 ml., and both solutions were treated as described in General Procedure. In the experiments represented by the curves 2a and 2b (Fig. 9) the dye was added before the heat-treatment; here the velocity of denaturation,

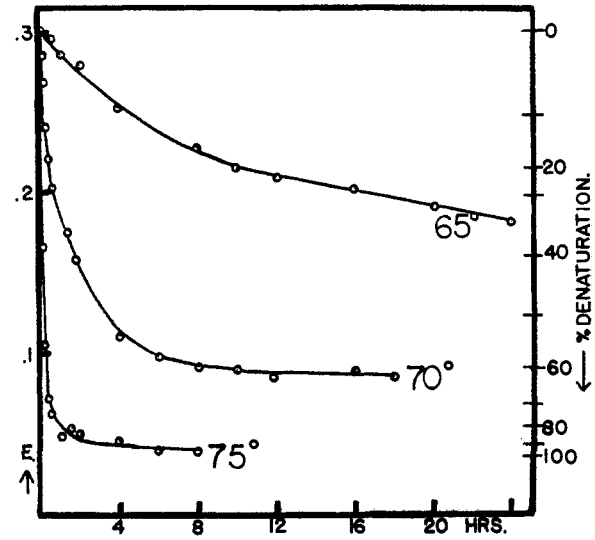


Fig. 8.—Extinction at 650 mμ of congo red mixed with ovalbumin exposed to different temperatures for various lengths of time.

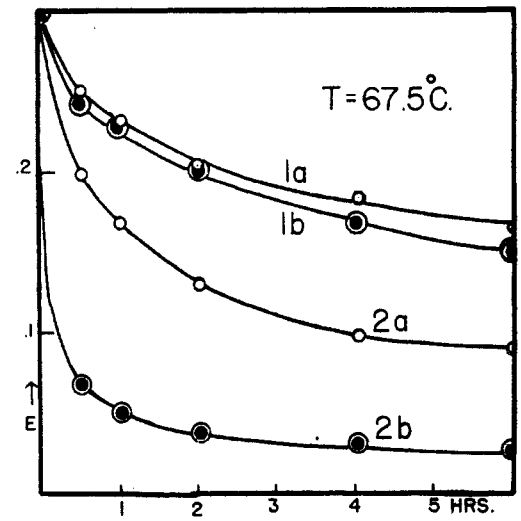


Fig. 9.—Effect of protein concentration and of added congo red on the velocity of ovalbumin denaturation by heat. In 1a and 1b the dye was added after the heat-treatment, in 2a and 2b before heating. The protein concentration during exposure to heat was 0.044% in 1a and 2a, and 0.02% in 1b and 2b; *E* = extinction at 650 mμ.

in contrast to experiment 1a and 1b, depends on the concentration of the protein, and is higher than in the absence of dye. This is also shown by Fig. 10 where the dye was added to the ovalbumin solutions before the exposure to heat. The experimental conditions were here the same as in curve 2 of Fig. 7.

Velocity constants for first order reaction were calculated by means of the equation $k = (2.3 \log(100/100 - w)/t)$, where w is the per cent. of denatured ovalbumin, and t the time in seconds. We find that k at 65° (Fig. 8) decreases from an initial value of 1.29×10^{-5} to 0.50×10^{-5} after 20 hours. At 70° k decreases within 12 hours from 1.26 to 0.21×10^{-4}, and at 75° within one hour from 1.7 to 0.54×10^{-3} sec.$^{-1}$; in the plateau k is negligibly small. An attempt was made to extrapolate the curves of Fig. 8 to infinity and to find

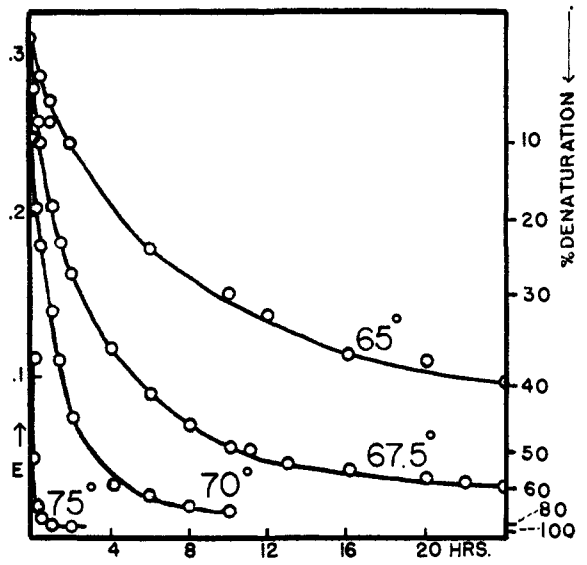


Fig. 10.—Extinction at 650 mμ of congo red–ovalbumin mixtures heated at different temperatures.

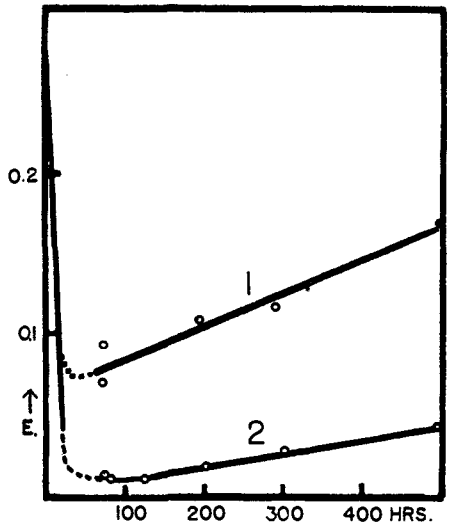


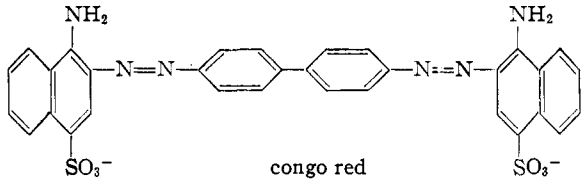
Fig. 11.—Extinction at 650 mμ of congo red solutions containing ovalbumin kept at 70° for various lengths of time. The dye was added in 1 after, in 2 before the heat-treatment.

whether they approach asymptotically a finite extinction, corresponding to a finite extent of denaturation. We are indebted to Drs. V. Hlavaty, L. L. Merritt, Jr., and J. C. Thacher, Jr., for their help in this matter. The mathematical treatment had to be abandoned when we found that heating over periods of several weeks is accompanied by a slow decomposition of the protein and a corresponding slow increase of the extinction at 650 mμ after acidification (Fig. 11). The experiment 1 (Fig. 11) was carried out as described in General Procedure, while in experiment 2 the dye was added before the heat-treatment. In samples of 1, taken at 288 and 504 hours, the non-protein nitrogen was 2.77 and 5.28 μg. per ml., respectively.

Discussion of the Results

Mechanism of the Combination of Protein with Congo Red.—The combination of proteins with anionic dyes has been investigated during recent years by Steinhardt,[14] Klotz,[15] Luck[16] and Karush[17] who found typical changes in the absorption spectra of the dyes on combination with proteins and determined the amount of bound dye by equilibrium dialysis. Since congo red does not penetrate semipermeable membranes, this method cannot be applied in our experiments. The behavior of congo red differs also in other points from that of the smaller dye anions: (1) congo red forms *soluble* complexes with the denatured ovalbumin and serum albumin and thus prevents their precipitation in acid solution. (2) While the affinity of serum albumin for methyl orange decreases on denaturation,[18,19] more congo red is kept in the red form at *p*H 2 by heat-treated than by unheated ovalbumin or serum albumin (Figs. 3 and 5). According to Fig. 5, the number R of red dye molecules bound per protein molecule at *p*H 2 is roughly proportional to C, the concentration of the dye present in the blue form. The linear correlation between R and C suggests that we are dealing here with an adsorption equilibrium where the protein molecules act as adsorbent, and the red and blue form of the dye as adsorbed and free solute, respectively. If R_N and R_D are the numbers of dye molecules kept at *p*H 2 in the red form by native and denatured ovalbumin, respectively, R_D is approximately equal to $R_N + 4$; for serum albumin R_D is approximately $R_N + 2$ (Fig. 5). (3) While the "protein error" of the anionic dyes investigated by Klotz[12,20] hardly exceeds one *p*H unit, the protein error of congo red amounts to 2 *p*H units in solutions of the unheated, and to 4 *p*H units in the solutions of heat-treated ovalbumin (Fig. 4).

The unique behavior of congo red and of the similar congo rubin is probably due to the size of these molecules and to the particular distribution of their positive and negative ionic groups within the rigid molecular structure. Steinhardt[21] has pointed to the fact that flatness of dye ions favors combination with proteins. The congo red anion is probably kept in a flat extended state by the mutual electrostatic repulsion of its two sulfonic acid groups.



congo red

The combination of anionic dyes with proteins is partly due to electrostatic attraction between negative groups of the dye and positive groups of the protein.[15] The higher combining power of the denatured albumins, as compared with unheated albumins, indicates that denaturation involves an increase in the reactive basic groups of the protein; this is in agreement with the higher reactivity of β-lactoglobulin with dinitrofluorobenzene after denaturation.[22]

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While there is no doubt that the sulfonic acid groups of the dye combine with basic groups of the protein, they can hardly be involved in the color change, since they are too remote from the long chain of 7 conjugated double bonds which causes the absorption of visible light. On acidification this chain combines with a proton, and the color changes to blue, owing to resonance between structures like $N^+H_2=C-C=N-N=C-C=C-C=C-C=C-C=N-NH-$ and $NH_2-C=C-N=N-C=C-C=C-C=C-C=C-N=N^+H-$.[23] The same color change occurs in congo rubin where one of the amino groups of congo red is replaced by a hydroxyl group. Since the color change from red to blue is prevented by combination of the dye with denatured ovalbumin or serum albumin molecules, we conclude that the chain of conjugated double bonds is somehow involved in the formation of the dye–protein complex. Possibly the aromatic structure of the dye combines with aromatic side chains of the protein. Similar conclusions were drawn on the basis of the combination of serum albumin with asymmetric azo dyes.[24]

The Kinetics of Ovalbumin Denaturation.— While protein denaturation in some cases, particularly in concentrated solutions, proceeds according to first order kinetics,[6,25] marked drifts in the first-order coefficients, indicating a higher order of reaction, have frequently been observed. Casey and Laidler[26] attribute similar phenomena in the inactivation of pepsin to a coöperative reaction involving the participation of several protein molecules.

The reaction order of the denaturation of ovalbumin by heat was determined from the curves shown in Figs. 8 and 10 by means of the Differential method.[27] If v is the velocity of denaturation at the concentration c of the native ovalbumin, the reaction order n is equal to the slope of the curves obtained by plotting log v *versus* log c. Since straight lines were obtained by this method, we conclude that the reaction order remains unchanged during denaturation. However, the reaction order of denaturation is different at different temperatures. For the three curves shown in Fig. 8 the reaction order, n, was 6.0, 4.2 and 3.4 at 65, 70 and 75°, respectively. For the four curves of Fig. 10 the following values of n were obtained: 6.5 at 65°, 6.0 at 67.5°, 3.05 at 70° and 1.85 at 75°. It is clear from these results that n is always higher than first-order, and that the reaction order decreases when the temperature is raised. Evidently, *the mechanism of denaturation at high temperatures is different from that of denaturation at low temperatures.* Although we find $n > 1$, the velocity of denaturation by heat, in the absence of dye, does *not* depend on the protein concentration as shown by the curves 1a and 1b in Fig. 9. We are confronted, therefore, with the fact that the reaction is first-order with respect to protein concentration, but of higher order with respect to time.

When we try to interpret this dualism of the reaction order, we have to take into account that the first phase of denaturation is the cleavage of salt bridges or hydrogen bonds. If we represent such bonds by AB, the first step of denaturation is represented by the reaction: $AB \rightarrow A + B$, where A and B are groups which after cleavage of AB may or may not undergo hydration. If denaturation consisted only of reactions of the type $AB \rightarrow A + B$, it would follow first-order kinetics. However, if the groups A_1 and B_1 combine irreversibly with similar groups B_2 and/or A_2 within the *same* protein molecule, the reaction $A_1B_1 \rightarrow A_1 + B_1$ is followed by $A_1 + B_1 + A_2B_2 \rightarrow A_1B_2 + A_2B_1$. This means that new intramolecular cross-links A_1B_2 and/or A_2B_1 are formed. It is clear that the velocity of this *intra*molecular reaction does not depend on the protein concentrations; it is, therefore, first-order with respect to protein concentration. If all groups of the types A and B were available for mutual combination, the formation of new bonds of the type AB would depend only on the number of groups A and B within the protein molecule, and would proceed strictly according to second order kinetics with respect to time. However, it is clear that the formation of a cross-link AB reduces the flexibility of adjacent parts of the peptide chain, and that other groups of the type A and B, present in the immobilized parts of the chain, become unavailable for further cross-link formation. Accordingly, the velocity of this reaction decreases rapidly and soon becomes zero, as shown by the plateaus in Fig. 8. These plateaus indicate that bonds AB of different strength are present in native ovalbumin, and that more of them are split at 75° than at 70° or at 65°. The decrease in the reaction order n at high temperatures is probably due to the larger number of groups A and B available for intramolecular irreversible bond formation. One can expect similar dual reaction order, first order with respect to the concentration, and higher order with respect to time, whenever irreversible *intra*molecular cross-linking occurs in macromolecules.

In addition to the *intra*molecular reactions, *inter*molecular reactions may take place when groups of the type A of one protein molecule combine with groups of the type B belonging to another protein molecule. Reactions of this type are responsible for the aggregation and flocculation of denatured protein molecules.[8] No flocculation being observed under the conditions of our experiments we have no reason to assume the formation of intermolecular bonds.

In the interpretation of our results it must be kept in mind that we measure the increase in dye binding groups during heat-treatment of the proteins, while other investigators measure the increase in reactive sulfhydryl groups,[2] phenol groups,[3,4] changes in viscosity[28] and optical rotation,[28] or the increase in the amount of insoluble protein.[6,25] Kauzmann, *et al.*,[28] have demonstrated that the velocity of these changes is not the same. Evidently there are numerous stages in the denaturation process, each

(23) We are indebted to Prof. H. Kuhn (Basle, Switzerland) for suggesting these structures. See also H. Kuhn, *J. Chem. Phys.*, **16**, 840 (1948).

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(26) E. J. Casey and K. J. Laidler, THIS JOURNAL, **73**, 1455 (1951).

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of them depending in a different manner on *p*H, concentration, temperature, mode of denaturation, and on other factors. It is not surprising, therefore, that different over-all orders of kinetics result when different properties of the protein are used as criteria of denaturation.

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Preparation of Secondary and Tertiary 2-Thiazolylamines

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Basically mono- or disubstituted derivatives of 2-aminothiazole were prepared by condensation of an appropriately substituted thiourea with dimethylchloroacetal. The reaction of 2-bromothiazole with either a primary or secondary amine was a less satisfactory method for the preparation of these compounds.

In a recent patent[2] there appeared a description of a method for the preparation, in low yield, of N,N-dimethyl-N′-benzyl-N′-(2-thiazolyl)-ethylenediamine (I). Since compounds of this type are of interest as chemotherapeutic agents, an investigation of some methods for the preparation of secondary and tertiary 2-thiazolylamines was initiated.

Sondern and Breivogel[2] prepared the diamine (I) by refluxing a mixture of 2-bromothiazole and N,N-dimethyl-N′-benzylethylenediamine in pyridine. Although we were unable to effect an improvement in their yield of this compound, a similar reaction (Method B) with 2-diethylaminoethylamine afforded a 60% yield of N,N-diethyl-N′-(2-thiazolyl)-ethylenediamine.[3] When the reaction was conducted in a high-boiling hydrocarbon rather than pyridine, yields fell off considerably. For instance, benzylamine and 2-bromothiazole in either cumene or xylene gave only a 9.5% yield of 2-benzylaminothiazole. Under the same conditions, dibenzylamine gave only tars from which none of the desired product could be isolated. This method suffers from the disadvantage that the elevated temperatures necessary for reaction are accompanied by extensive decomposition of the reaction mixture. The preparation of 2-bromothiazole is also rather lengthy and troublesome, especially when large amounts are required.[4]

A few 2-thiazolyl tertiary amines, containing substituents on either or both the nuclear carbons, have been prepared (in unstated yield) by condensing N,N-disubstituted thioureas with α-haloketones.[5a–e] Replacement of the latter by dimethyl chloroacetal (Method A) offered a route which was superior to Method B for the preparation of both N-mono- and N-disubstituted 2-aminothiazoles. The intermediate unsymmetrically disubstituted thioureas were prepared by treatment of the corresponding cyanamides with hydrogen sulfide in ammoniacal methanol.[6] N-Monosubstituted thioureas were obtained from isothiocyanates by reaction with aqueous ammonia.[7]

The reductive alkylation of 2-aminothiazole with benzaldehyde in formic acid gave intractable tars and none of the expected 2-benzylaminothiazole. Similar results were obtained by refluxing a solution of the pre-formed Schiff base (2-benzylideneaminothiazole) in formic acid.[8]

One of the products, N,N-diethyl-N′-benzyl-N′-(2-thiazolyl)-ethylenediamine, tested for antihistaminic activity on the isolated guinea pig ileum strip, showed 1.5% of the activity of Pyribenzamine.[9] This compound has recently been reported[10] as a solid melting at 121–122°. Since our product is a liquid at room temperature, a property shared by other compounds of this type,[11] and was prepared by a method which should yield compounds of unequivocal structure, it would appear that the procedure of Fox and Wenner,[10] involving a catalytic reductive alkylation, gave some other substance. N,N-Dimethyl-N′-benzyl-N′-(2-thiazolyl)-ethylenediamine (I), previously patented as an antihistaminic,[2] was 20% as active as Pyribenzamine.[12] Against acetylcholine, this compound (I) had 0.15% of the activity of atropine.[12]

(1) From a thesis submitted by Chester L. Parris to the Graduate Faculty of Brooklyn College, June, 1951, in partial fulfillment of the requirements for the Master of Arts degree.

(2) C. W. Sondern and P. J. Breivogel, U. S. Patent 2,440,703, May 4, 1948.

(3) One compound of this type, 2[(4-diethylamino-1-methyl-butyl)-amino]-thiazole, was prepared in 18% yield in similar fashion by J. N. Ashley and J. F. Grove, *J. Chem. Soc.*, 768 (1945).

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(6) O. Wallach, *Ber.*, **32**, 1872 (1899), prepared N,N-dibenzylthiourea, m.p. 139–140°, in this fashion. His dibenzylcyanamide, prepared from dibenzylamine and cyanogen bromide, was collected at 145–148° (10 mm.), m.p. 54°.

(7) A. W. Hofmann, *ibid.*, **1**, 201 (1868).

(8) I. A. Kaye and I. C. Kogon, *Rec. trav. chim.*, **71**, in press (1952).

(9) Pyribenzamine is the trade mark for N,N-dimethyl-N′-benzyl-N′-(2-pyridyl)-ethylenediamine monohydrochloride, Ciba Pharmaceutical Products, Inc.

(10) H. H. Fox and W. Wenner, *J. Org. Chem.*, **16**, 225 (1951).

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(12) This information on the pharmacological activities was kindly offered by Dr. Harold Blumberg and Mr. Eric Meyer of Endo Products, Inc.