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Cross-Linking of Bovine Fibrinogen with Formaldehyde

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The cross-linking of fibrinogen with formaldehyde has been investigated under a variety of conditions. The reaction proceeds in two steps. First, through an equilibrium reaction, a reactive hydroxymethyl-derivative is formed, which then polymerizes through a condensation reaction. The cross-link is formed between ε-amino and amide groups.

Cross-linking of globular, or fibrous proteins in the moist, semi-solid state has considerable technological importance and has been investigated extensively. Much less attention has been paid to the cross-linking of proteins in solution, with the resulting gel formation. In this paper some observations will be reported on the cross-linking of fibrinogen with formaldehyde in solution. Fibrinogen was chosen because of its gel-forming physiological function, and formaldehyde because of its great reactivity under extremely mild conditions. Some observations on this system have been presented already[1].

MATERIALS AND METHODS

Fibrinogen was purified from Armour's Bovine Fraction I by Laki's procedure[2]. Formaldehyde was Baker's Analyzed Reagent, 37 % solution, containing 10 % methanol as preservative. Gelation time was chosen as the point when flow of the reaction mixture in a test tube ceased. The opacity increase was followed in the Beckman DU-spectrophotometer at 600 mμ. The pH shift was recorded on a Leeds and Northrup Speedomax recorder coupled to a Vibron electrometer, Model 33 B.

Preparation of the formaldehyde-treated samples was performed in the following way. To 10 ml of 1 % fibrinogen in 0.3 M KCl, 1 ml of 1 M phosphate buffer of various pH and 1 ml of 10 % formaldehyde was added. At pH 6 no gelation occurred, at pH 7 and 8 a gel formed in 75 and 5 seconds, respectively. The mixtures were left at room temperature for 30 minutes and then dispersed in 5 volumes of ethanol. After centrifugation, the sediments were transferred into cellophane bags, dialyzed first against running distilled water for one day, and then against stationary water changed every day until no free formaldehyde could be detected in the outside solution with the chromotropic acid reagent (4–5 days)[3]. The protein was then suspended twice in ethanol, once in ether and finally dried in air and pulverized.

Estimation of free ε-amino groups was performed with the Sanger procedure[4] on the protein

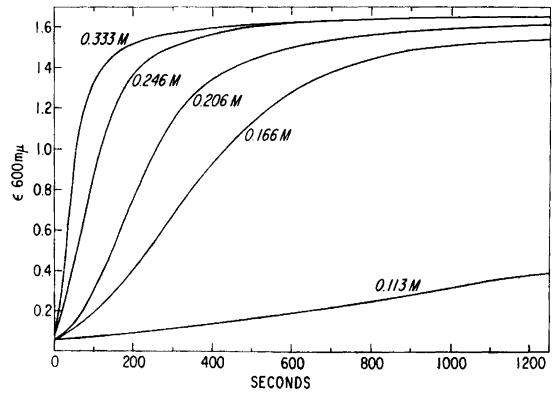

Fig. 1. Increase in optical density of fibrinogen solutions upon formaldehyde addition. Figures indicate formaldehyde molarities in each experiment. Fibrinogen 1.6 %.

powder suspended in water with $NaHCO_3$, or trimethylamine as buffer. The dinitrophenyl protein was hydrolyzed in a sealed vial with 6 N HCl for 18 hours at 115 °C. From the hydrolysate, the ether soluble DNP-amino acids were extracted; then the ε-DNP-lysine shaken out with n-butanol and estimated in the butanol solution at 360 mμ.

The amount of free formaldehyde in the preparation was determined with the chromotropic acid reagent[3], that of reversibly and irrevirsibly bound formaldehyde with the aid of ^{14}C-formaldehyde. The latter, of 99 % radiopurity, 3.8 mC per mM specific activity, was obtained from Isotopes Specialties Company, Burbank, California. In reaction mixtures as described above, a total of approximately 10 million c. p. m. was introduced. Counting was performed with the Packard Tri-Carb Scintillation counter. Reversibly bound formaldehyde was determined as described by Fraenkel-Conrat *et al.*[5]. The acidulated protein solution was distilled over twice in a dimedon solution and, after carrier formaldehyde was added, the precipitate was separated and counted. The total bound formaldehyde was estimated by counting in the scintillation counter, the protein dissolved in methanolic hyamin solution. Corrections for background counts and quenching were performed in the usual way.

RESULTS AND DISCUSSION

The polymerization process follows a sigmoidal curve as shown in Fig. 1. Gelation occurs in approximately twice the time necessary to attain half of the maximal opacity. At the gel point, approximately 80 % of the protein is involved in the network, and can be removed by high-speed centrifugation. Also, the opacity at the gel point is about 80 % of the final value. The reciprocal of the gel time, or the reciprocal of the time necessary to reach half of the final optical density can be used as a convenient measure of the rate of polymerization. In Fig 2, the logarithm of these quantities is plotted against the logarithm of formaldehyde concentration. The reaction mixtures had an ionic-strength of 0.6 and were buffered with 0.2 μ phosphate buffer to pH 7.19, so that the pH drift during the reaction amounted to less than 0.1 units.

Two experiments were performed, one with 0.8 % and the other with 1.6 % fibrinogen. The formaldehyde was in over 30-fold excess over the free amino groups even at its lowest concentration. The half-maximal opacity times are multiplied by two in order to bring the two sets of measurements together. The

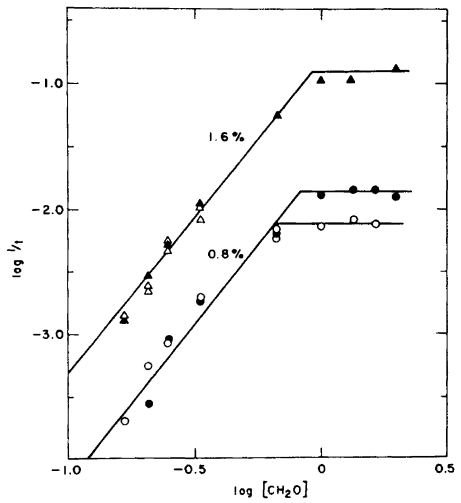

Fig. 2. Log reciprocal reaction time (gel time – solid symbols; 2 × half maximal optical density time – open symbols) plotted against log formaldehyde concentration. The two curves were obtained at the two fibrinogen concentrations indicated.

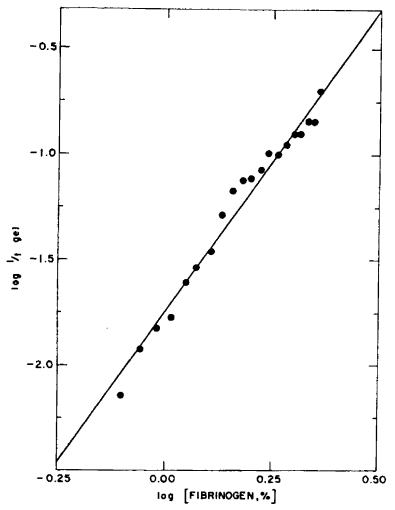

Fig. 3. Log reciprocal gel time plotted against log fibrinogen concentration. pH 7.19, μ 0.6, formaldehyde 0.5 M.

points fall reasonably close to a straight line up to a formaldehyde concentration of approximately 1 M. Above this, the rate seems to be independent of formaldehyde concentration. The slope of both lines, *i. e.*, the order of reaction with respect to formaldehyde, is 2.5 and their separation corresponds to a 7.25 fold increase in rate upon doubling the fibrinogen concentration. A more extended investigation of the dependence of rate on fibrinogen concentration is shown on Fig. 3. From the slope of the line, the reaction appears to be of 2.8 order with respect to fibrinogen in agreement with the value which can be calculated from the previous experiment. The empirical rate equation is therefore, $dx/dt = k' [F]^{2.8} \times [CH_2O]^{2.5}$, where k' is the apparent rate constant, [F] is the concentration of free fibrinogen and $[CH_2O]$ that of free formaldehyde.

The rate of reaction is also very sensitive to ionic strength. Below an ionic-strength of 0.1, polymerization does not occur, above this, the rate increases proportionally with the square-root of the ionic strength (Fig. 4). Such a relationship is expected for the interaction of charged macromolecules.

Formaldehyde reacts in one way or another with nearly all the reactive side-chains of a protein molecule[6]. Undoubtedly, the most important of these reactions is that with the free amino-groups because of its very fast rate and also the large number of groups involved in it. One, or at high formaldehyde concentrations, two moles can combine with one amino group giving a reactive mono- or di-hydroxymethyl derivative. In the concentration range of importance in this study only the monohydroxymethyl compound needs to be considered. The reaction appears to be completely reversible and involves a shift of the p*K* of the amino

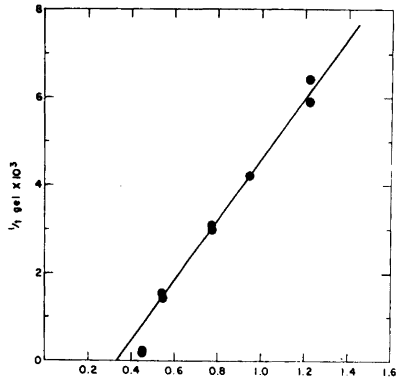

Fig. 4. Reciprocal gel time plotted against square root of ionic-strength. pH 7.19, fibrinogen 0.8 %, formaldehyde 0.33 M.

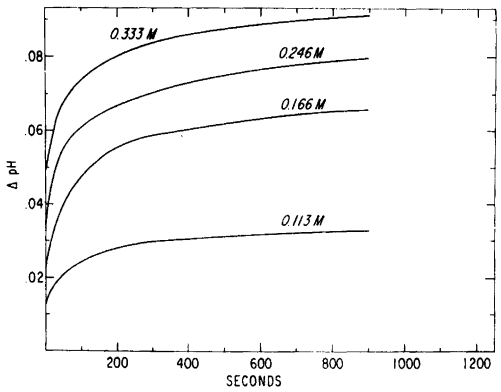

Fig. 5. pH shift on formaldehyde addition. Figures indicate molarities of formaldehyde in each experiment. pH 7.19, μ 0.6, fibrinogen 1.6 %.

group of some 3 units in an acidic direction. Thus, at the proper pH, the reaction can be easily followed by the amount of H^+ liberated, or the pH-shift. Since there are very few data of this nature on proteins, the kinetics of the reaction of formaldehyde with the amino groups of fibrinogen was investigated with the pH shift technique. Reaction with other groups is either too slow, or involves uncharged molecules and thus remains undetected by this method. The pH of the reaction was 7.19 and the ionic strength 0.6, with sufficient phosphate buffer added to limit the pH change to below 0.1 units. Figure 5 shows the recordings of pH shifts with 1.6 % fibrinogen on adding formaldehyde to the indicated molarities. It appears that the rates are not very much affected by the three-fold variation in formaldehyde concentration, but the equilibrium position the reaction finally reaches is very much so.

Comparison of Figs. 1 and 5 reveals that the polymerization process involves two steps. The first is the reversible binding of formaldehyde to the amino group. This leads to an equilibrium which is established fast relative to the polymerization. Thus, as an approximation, the concentration of molecules with bound formaldehyde can be expressed in terms of an association constant K and the concentrations of free fibrinogen and formaldehyde. In the second step, the polymerization of these molecules proceeds as a condensation reaction; consequently this step will be of second order with respect to the fibrinogen-formaldehyde compound. Combining the two steps and denoting by k the bimolecular reaction constant results in: $dx/dt = k \cdot K^2 [F]^2 [CH_2O]^2$. The experimentally found relationship has somewhat higher exponents, but shows nearly equal order with respect to both fibrinogen and formaldehyde, as required by the theory. The difference in the exponents may be explained by the fact that the theoretical equation does not take into account the side reactions which rapidly inactivate the reactive molecules. The effect of these will be more pronounced when the rate of polymerization is slow, than when it is fast, and may

Table 1. Free ε-amino groups and irreversibly bound formaldehyde, expressed as moles per 10^5 g in native and formaldehyde treated fibrinogen.

Material	Free ε-amino groups	Irreversibly bound formaldehyde	Remarks
Native fibrinogen	48.7 ± 1.9		No gel
Treated with CH_2O at pH 6	48.7 ± 0.7	2.7 ± 1.2	No gel
Treated with CH_2O at pH 7	41.2 ± 0.3		Gel
Treated with CH_2O at pH 8	37.3 ± 1.4	11.1 ± 1.8	Gel
Difference: Native – treated at pH 8	11.4 ± 3.3	11.1 ± 1.8	

accentuate in this way the concentration dependence of the rate, making it appear of an order higher than the actual one.

The number and nature of cross-links introduced was inferred from determinations of free ε-amino groups and irreversibly bound formaldehyde. As shown in Table 1, the decrease in the number of free ε-amino groups equals that of the irreversibly bound formaldehyde molecules. The bridge, therefore, cannot be between two ε-amino groups, as proposed earlier[1], but between one amino group and some other group. Estimates of the free tyrosine and tryphophan groups showed no change upon treatment with formaldehyde, but there was a decrease in the number of amide groups. Therefore, the cross-link is probably between the ε-amino and an amide group. Similar suggestions have been made by several authors[7,8]. The number of cross-links introduced is fairly large, up to 38 per molecule of fibrinogen (this may be an overestimate, because some of the links might be intramolecular). The large number suggests an intimate contact between the molecules, which could be achieved only by side-by-side aggregation. Thick bundles may be formed in this way, which could explain the large increase in turbidity which accompanies the cross-linking.

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