rate constant, K, with a proportionality constant which is independent of temperature. The determination of the slope of the straight-line portion of the curves is equivalent to the determination of the rate at a particular o.d. on the straight line. It is, therefore, justifiable to determine activation energies by this method.

If, for the sake of simplicity, we assume that the o.d. bears a constant relation to fiber volume throughout the course of the reaction equation 1 may be written as

$$dD/dt = K'D^{1/3}(D_f - D) \qquad (2)$$

Plots of the integral of equation 2 and our experimental data are shown in Fig. 7. The fit indicates that the simplifying assumption is at least a reasonable approximation.

Setting the derivative of equation 2 equal to zero and solving for D gives the o.d. at the time of maximum rate, which is equal to 0.4 D_f. Taking the midpoint of the linear portion of our curves as an approximation of that value corresponding to the o.d. at maximum rate, we have found by repeated determinations under varying conditions a value of $(0.37 \pm 0.02)D_f$. Determinations of the o.d. at the time of maximum rate serve as a routine check on the constancy of the final o.d. when the reaction is stopped prior to the final stage.

[CONTRIBUTION FROM THE BENJAMIN ROSE HOSPITAL AND THE DEPARTMENT OF BIOCHEMISTRY, WESTERN RESERVE UNIVERSITY SCHOOL OF MEDICINE, CLEVELAND, OHIO]

Fiber Formation from Solutions of Collagen. III. Some Effects of Environment on the Rate of Fiber Formation[1]

BY HOWARD B. BENSUSAN

RECEIVED OCTOBER 24, 1959

The effect of alcohols on the rate of fiber formation from solutions of collagen at a *p*H greater than 7 has been investigated. At low concentrations of alcohols the rate is decreased. The extent of this decrease is correlated with the activity coefficient of the alcohol. At higher concentrations of alcohol the rate is increased beyond that of the control in the absence of alcohol. This increase is correlated with the decrease in the dielectric constant of the medium. Increasing the alcohol concentration also causes a decrease in the energy of activation of the reaction. Increasing the ionic strength of the medium decreases the rate of fiber formation. This decrease in rate is correlated with the decreased activity of the charged groups of the protein.

The *in vitro* formation of fibers from solutions of collagen constitutes an interesting system for the study of protein interactions. The polymerization appears to be under the influence of a well-ordered mechanism in that the end product is a fiber having a major repeat spacing of 600–700 Å. and several intermediate spacings which are similar to those of the native fiber.[2] We have been concerned in this Laboratory with the elucidation of some of the factors involved in this transformation.[3,4]

The use of alcohols in this study has a distinct advantage. The soluble alcohols occur in an ordered series whose physical and chemical properties are well catalogued.

Materials and Methods

The methods of protein preparation and analyses are the same in this study as those described in the preceding paper.[4] The method for the determination of rate of fiber formation was essentially the same. The alcohol was included in the preincubation mixture of water and tris-(hydroxymethyl)-aminomethane ("tris") buffer prior to the addition of the protein in tris buffer. The increase in optical density at 290 mμ during fiber formation was then recorded in a Beckman DU spectrophotometer. Again, the rate was expressed as increase in optical density per minute (× 1000).

Results

The Effect of Various Alcohols on the Rate.—The effect of a wide range of concentrations of five different alcohols is shown in Fig. 1. It will be seen that at low concentrations the alcohols tended to inhibit fiber formation. As the concentration of the alcohols increased there was a rapid acceleration of the rate.

Electron micrographs of the clots formed in the presence of ethanol concentrations which covered the entire range used showed the presence of normal collagen fibrils in all cases. At the very highest concentrations of ethanol there appeared to be some amorphous material present.

In Fig. 2 are plotted the results of an experiment performed to amplify the effect of low concentrations of alcohol on the inhibition of fiber formation. The maximum degree of inhibition differed with the alcohol used. The order of increasing extent of maximum inhibition was methyl < ethyl < *i*-propyl < *t*-butyl < *n*-propyl alcohol.

Figure 3 shows the results obtained from determinations of rate at higher concentrations of the alcohols. The slope of the lines drawn through these points is a measure of the rate of acceleration with respect to alcohol concentration. Figure 4 is a plot of the slopes of these lines as a function of the dielectric increment[5] of the various alcohols used. There appears to be a good correlation between the rate increase per mole of alcohol and the negative dielectric increment. We determined the effect of increasing the dielectric constant of the medium by adding glycine at concentrations which had no observable effect on the rate in the absence of alcohol. Figure 5 shows the results of such a determination. Consistent with our expectation, glycine decreased the acceleration of high concentrations of ethanol. However, we have found that glycine does not change the extent of inhibition produced at lower concentrations of alcohols. This observation is further illustrated in Fig. 5 by the fact that the lines are not parallel as would be expected if the dielectric effect were operating alone. Since glycine had no influence at lower concentrations of alcohol and an increasing effect at higher concentrations of alcohol, it is apparent that the possible expression of

(1) This study was aided by Grant No. A-1825 from the National Institute of Arthritis and Metabolic Diseases of the United States Public Health Service and by a grant from the Elisabeth Severance Prentiss Foundation.

(2) J. T. Randall, F. Booth, R. E. Burge, S. Fitton Jackson and F. C. Kelly, "Symposia of the Society for Experimental Biology, No. IX," Academic Press, Inc., New York, N. Y., 1955, pp., 127–147.

(3) H. B. Bensusan and B. L. Hoyt, THIS JOURNAL, **80**, 719 (1958).

(4) H. B. Bensusan and A. Scanu, *ibid.*, **82**, 4990 (1960).

(5) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, p. 144.

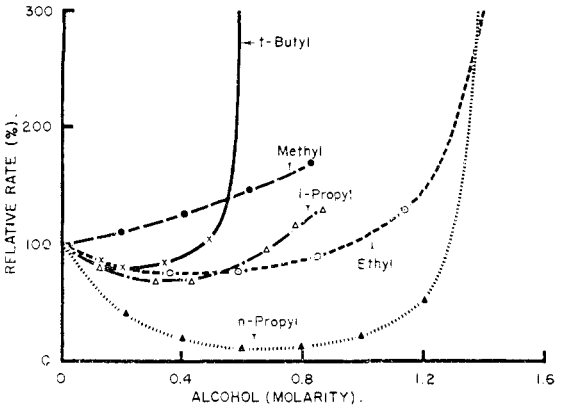

Fig. 1.—The effect of different concentrations of alcohols on the rate of fiber formation. The basic environment contained tris buffer, *p*H 8.2, at an ionic strength of 0.067 plus 0.033 *M* NaCl. The temperature was 25° and the final protein concentration was 0.013%. The extension of the *t*-butyl, ethyl and *n*-propyl alcohol curves to greater than 300% indicates that at these molarities the rate was too fast to determine.

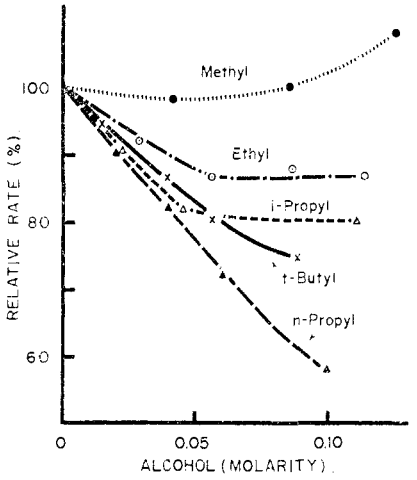

Fig. 2.—The effect of lower concentrations of alcohols. The basic environment contained tris buffer, *p*H 8.2, at an ionic strength of 0.07 plus 0.037 *M* NaCl. The protein concentration was 0.01%. The temperature was 25°.

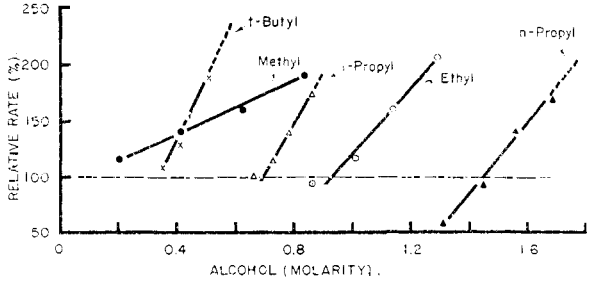

Fig. 3.—The effect of higher concentrations of alcohols. The basic environment was tris buffer, *p*H 8.2, at an ionic strength of 0.087 plus 0.043 *M* NaCl. The protein concentration was 0.02%. The temperature was 25°.

the dielectric effect may occur to a greater extent as the alcohol concentration is increased.

The Effect of Alcohols on Optimum *p*H.—In Fig. 6 are shown the rates of fiber formation at

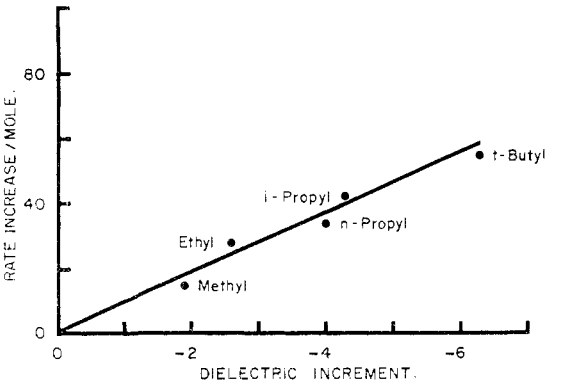

Fig. 4.—The correlation between rate of acceleration of fiber formation and the dielectric increment of the alcohol.

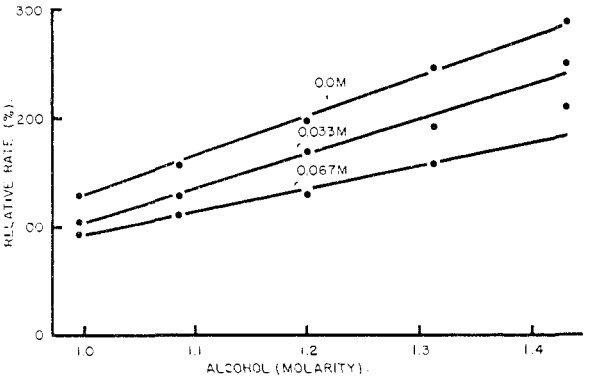

Fig. 5.—The effect of glycine on the rate in the presence of varying concentrations of ethanol. The experimental conditions were the same as given in the caption for Fig. 3.

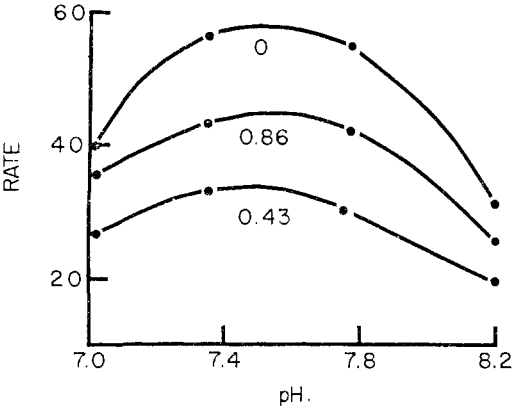

Fig. 6.—The effect of alcohol and *p*H on the rate of fiber formation. The ionic strength was kept constant at 0.115 in tris-HCl. The protein concentration was 0.03%. The temperature was 28°.

differing values of *p*H in the presence and absence of alcohols. There is no pronounced effect of the alcohols on the optimum *p*H.

The Effect of Alcohols on the Energy of Activation.—The results in Fig. 7 show that the energy of activation for the formation of fibers is greatly decreased in the presence of alcohols. The decrease is proportional to the alcohol concentration.

The Effect of Alcohols on Iodinated Collagen.—By iodinating the soluble collagen we can affect the ionization of the phenolic group of tyrosine.

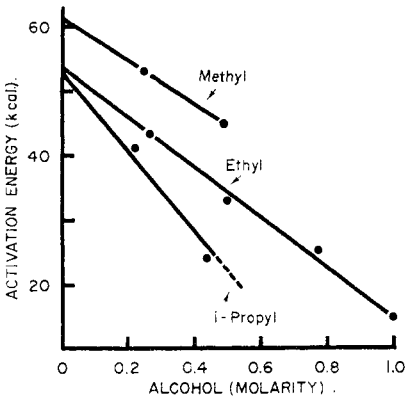

Fig. 7.—The effect of alcohols on the energy of activation. The ionic environment and protein concentration were the same as given in the caption for Fig. 3. The temperature range was 26–31°. Although not indicated by experimental points, the energies of activation at zero alcohol concentration are those which were determined experimentally.

Therefore, the effect of alcohols on the ionized and un-ionized form of this group could be determined by comparing their effect on the native and iodinated protein. The results of such an experiment are shown in Table I. It is evident that there was no appreciable difference in the relative rates.

TABLE I

THE EFFECT OF ALCOHOLS ON THE RATE OF FIBER FORMATION FROM SOLUTIONS OF NATIVE AND IODINATED COLLAGEN[b]

Alcohol	Alcohol concn., *M*	Native (relative rate[a])	Iodinated (relative rate[a])
Ethyl	0	100	100
	0.143	156	145
	0.286	219	208
n-Propyl	0	100	100
	0.100	96	90
	0.200	123	106
i-Propyl	0	100	100
	0.109	170	158
	0.218	259	259

[a] The rate in the absence of alcohol was taken as 100. [b] The medium for the native collagen contained tris buffer (ionic strength, 0.052) and 0.018 *M* NaCl. The medium for the iodinated protein contained tris buffer (ionic strength, 0.094) and 0.031 *M* NaCl. The *p*H was 8.0 and the temperature was 10.2° in both cases. Iodination was carried out for 2 min.

The Effect of Salts on the Rate of Fiber Formation.—In our previous study[3] we demonstrated that increasing the ionic strength of the medium decreased the rate of fiber formation. The extent of this decrease depended on the type of salt used. We have since noted that a plot of log rate *vs.* ionic strength gives a straight line, the slope of which is negative and dependent upon the salt used. A few of these plots are given in Fig. 8.

Gross and Kirk,[6] on the other hand, showed an acceleration with increasing concentration of added salts, especially KCNS. The basic difference between our systems is the fact that they used phosphate buffers at an ionic strength of 0.15–0.4 while we employed tris buffers at an ionic strength near 0.1. We have been able to verify their results using 0.03 and 0.06 *M* NaCNS in the presence of phosphate (*p*H 7.6, at an ionic strength of 0.27). However, we found that in the presence of lower phosphate concentrations (ionic strength, 0.03) the results are quite different, as seen in Table II. Under these conditions only the lowest concentration of NaCNS accelerated the reaction.

(6) J. Gross and D. Kirk, *J. Biol. Chem.*, **233**, 335 (1958).

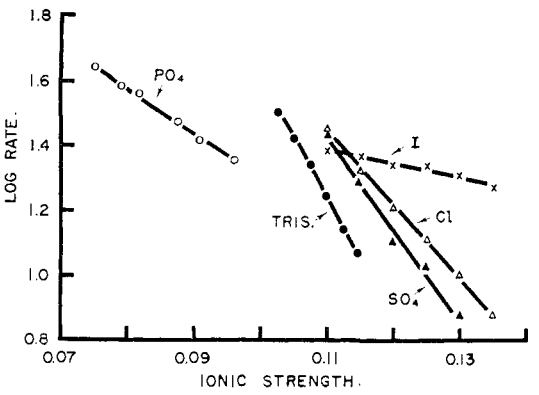

Fig. 8.—The effect of ionic strength on the rate of fiber formation. The *p*H of the phosphate buffer was 7.2. The basic environment was 0.1 *M* NaCl. The *p*H of the tris buffer was 8.2. No other salt was present. The basic environment for the sodium salts of the three cations was the same as in the caption of Fig. 2.

TABLE II

THE EFFECT OF SALTS ON THE RATE OF FIBER FORMATION IN THE PRESENCE OF LOW CONCENTRATIONS OF PHOSPHATE BUFFER[a]

Salt added	Salt concn., *M*	Rate	Relative rate, %
NaCNS	0	18.4	100
	0.03	24.4	132
	.06	17.8	97
	.08	14.7	80
	.10	11.8	64
	.12	7.1	39
NaBr	0	16.5	100
	0.02	12.4	75
	0.03	8.1	49

[a] The basic environment was phosphate buffer, *p*H 7.6, 0.03 ionic strength, plus 0.13 *M* NaCl.

Discussion

We are not dealing with a simple system. There are many effects that might be expected by the addition of alcohols. With the information at hand, we are able to infer which of these effects play a dominant role.

It is possible that low concentrations of alcohols inhibit by hydrogen-bonding of the alcohol with the protein. The order of increasing electronegativity around the oxygen atom is methyl < ethyl < *n*-propyl < *i*-propyl < *t*-butyl.[7] Although the order is similar to the order which shows increasing inhibition (methyl < ethyl < *i*-propyl < *t*-butyl < *n*-propyl), there is one obvious deviation which suggests that hydrogen bonding may not play a major role in the inhibition of fiber formation. An alternative explanation for the inhibition would be on the basis of an interaction between the aliphatic portion of the alcohol molecule and the non-polar side

(7) M. J. S. Dewar, "The Electronic Theory of Organic Chemistry," Oxford University Press, Amen House, London, 1949, pp. 51–53.

chains of the protein. The order of increasing attraction would be expected to follow the order of increasing activity coefficient of the alcohols as determined by vapor phase measurements of aqueous solutions. The expected order of attraction exactly follows the order of increasing inhibition.[8] Such an interpretation is attractive since it may explain in part the lowering of the energy of activation for fiber formation in the presence of alcohols. Considering the thermodynamic quantities for non-polar interactions,[9] the degree of binding of alcohol to protein would be expected to be greater with an increase in temperature.

It is reasonable to rule out the possibility that alcohols inhibit by increasing the pK of the phenolic group of the tyrosyl residues. As shown in Table I, the effect of alcohols on the iodinated collagen was the same as for the untreated protein.

We have already pointed out the correlation between the extent of acceleration and the dielectric increment of the alcohol. It appears that by lowering the dielectric constant of the medium the electrostatic interactions between protein molecules are facilitated. One would then expect to see the decrease in the energy of activation with increasing alcohol concentration which was observed. Conversely, an increase in the interactions between counterions and protein would be expected to increase the energy of activation. This was found on increasing the ionic strength.[4] With decreasing dielectric constant of the medium an increase in the pK of the free amino groups of the protein might be expected. This may be discounted as a major factor since ethanol does not appreciably alter the pH optimum for fiber formation.

Assuming that the rate of fiber formation is proportional to the activity of the ionizable groups on the soluble-collagen molecule a plot of the log rate *vs.* ionic strength should give a straight line with a negative slope as does the plot of log activity coefficient *vs.* ionic strength.[10] The fact that we obtained such a linear relationship supports the above hypothesis.

The apparent contradiction in the results obtained by Gross and Kirk[6] and by us can now be reasonably explained. It would be expected that the divalent $HPO_4^=$ would bind in a bridge-like arrangement between two protein molecules in solution. Such a bond would be expected to inhibit the rate of fiber formation beyond the simple decrease in activity of the charged groups.[11] The addition of a monovalent anion results in a competition with the $HPO_4^=$ for the positive charges thereby reducing the inhibitory effect of the bridge. It has previously been shown that the inhibition of fiber formation by monovalent anions is in the order $CNS^- < I^- < Br^- < Cl^-$.[3] If the univalent anion were (*i.e.*) CNS^- where the inhibitory effect is small the net effect would be a marked acceleration. The net effect of adding Cl^- would then be a less marked acceleration than with CNS^-.

Although one would expect that hydrogen-bond formation plays a role in fiber formation, we have yet to demonstrate its importance in our system. Since we deduced that the alcohols at low concentrations inhibit through non-polar interactions, it is reasonable to expect that non-polar groups of the protein could take part in intermolecular binding and, therefore, fiber formation. There are several lines of evidence to suggest that ionic interactions play a major role. The marked effect of salts, pH and the dielectric constant of the medium point to their importance. There is no reason to believe that collagen should be different from other proteins, for which similar effects of salt, pH and alcohols are common findings.

There are certain aspects of the formation of collagen fibers which are of particular interest. Not only are the fibers well-ordered structures, but the structure may be altered by changing the conditions of fiber formation.[12] We believe that the sensitivity of the collagen system and its ease of measurement make it ideal for the study of protein interactions.

Acknowledgments.—The author wishes to express his gratitude to Miss Barbara Hoyt for her excellent technical assistance, to Dr. Leonard Frank for his many valuable comments and to Dr. Melvin D. Schoenberg for the electron-microscopy involved in this work.

(8) J. A. V. Butler, *Trans. Faraday Soc.*, **33**, 229 (1937).

(9) W. Kauzmann, Abst. of Am. Chem. Soc., September, 1959.

(10) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, pp. 609–617.

(11) The collagen fiber has a major repeating unit of approximately 700 Å. The soluble monomer has a length about four times this value. The current explanation for the periodicity involves the arrangement of the monomers with the same ends pointing in one direction but with the ends staggered by specific fractions of their length.[12] In view of this and of the importance of ionic interactions, it is easy to see how the phosphate "bridges" would inhibit fiber formation. By binding the like-charged basic groups of two molecules, the phosphate would interfere with the alignment of oppositely charged groups, thus preventing the attainment of the preferred staggered arrangement. It would also contribute some steric hindrance.

(12) For a summary see F. O. Schmitt, *Proc. Am. Phil. Soc.*, **100**, 476 (1956).