[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. II. The Role of Tyrosyl Residues[1,2]

BY HOWARD B. BENSUSAN AND ANN SCANU

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Iodinated collagen was prepared by the treatment of solutions of collagen at *p*H 8.2 with a slight excess of iodine for short periods of time. The rate of fiber formation from solutions of iodinated collagen was very much greater than for the untreated collagen. The fibers formed appeared to be normal collagen fibers. Investigation of the product of iodination by several methods has shown only the formation of diiodotyrosyl residues. The activation energy for fiber formation falls by 50–57 kcal. after iodination. It was concluded that the ionization of the phenolic OH group of 8–10 tyrosyl residues occurs during the activation step for the formation of fibers from solutions of untreated collagen.

In order to elucidate the mechanism for the formation of fibers from solutions of collagen we have turned to a study of the effect of specific alterations of the constituent amino-acid side chains. In spite of the fact that no one has described a preparation of collagen analytically free of tyrosine, the presence of this amino acid has been thought to represent to a protein impurity. Investigators have used, as a criterion of purity of collagen preparations, the extent to which the tyrosine content can be reduced.

Despite this prejudice, we attempted to determine the effect of iodination of the tyrosyl residues on the rate of fiber formation. Contrary to our expectations, a pronounced effect on the rate resulted. The soluble collagen so treated clotted on warming in a fraction of the time that was required for the untreated control. The task of determining the nature of the effects of iodine resolved itself into two lines of investigation. The first was the determination of the product of iodination. The second was the elucidation of the mechanism for the increase in rate of fiber formation.

Materials and Methods

Preparation of Collagen Solutions.—The method of preparation of collagen solutions was slightly different from that which we reported previously.[3] After the extraction of soluble collagen from acetone-dried calf skin with cold 0.1 *M* HOAc, the *p*H was adjusted to *p*H 8.0–8.2 with 6 *N* NaOH and the solution was warmed at room temperature until the collagen had clotted. The collagen clot was centrifuged at 12,000 × *g*. The precipitate was redissolved in a volume of cold 0.1 *M* HOAc, equal to 0.75 of the volume of the original extract. This solution was then adjusted to *p*H 7.2 with 6.0 *N* NaOH and the collagen was precipitated in 18% NaCl as described previously. This precipitate was washed in several changes of acetone at 4°. The acetone was removed by decanting the excess, blotting the precipitate on filter paper and then allowing the remaining acetone to evaporate at room temperature. The solid was stored at −20° until used. Since these preparations contained a variable amount of salt, each one was tested for the proper ionic strength which would give optimum solution. Usually 1 g. of the preparation was dissolved in cold, 0.2 *M* NaCl by stirring overnight in a cold room. The insoluble material was removed by centrifugation and the supernatant solution was then dialyzed against the desired buffer.

Analysis of our preparation showed a value of 14.1% hydroxyproline, 0.6% tyrosine and 17.7% nitrogen.[4] These values are in excellent agreement with those obtained by Bowes, Elliott and Moss.[5]

Amino Acids.—Tyrosine, hydroxyproline, diiodotyrosine (DIT), monoiodotyrosine (MIT) and histidine were obtained from Nutritional Biochemicals Corporation and were used without further purification except when noted. Monoiodohistidine (MIH) and diiodohistidine (DIH) were prepared by the method of Brunings.[6]

Analytical Methods.—Protein concentration was determined either by Kjeldahl digestion followed by nesslerization or by refractometry using a Brice–Phoenix differential refractometer. A value for dn/dc of 1.92×10^{-4}/mg. protein/ml. at 546 mμ in tris buffer (0.2 *M* tris-(hydroxymethyl)-aminomethane, 0.1 *N* HCl, 0.05 *M* NaCl, *p*H 8.2) was found. This value is in good agreement with those reported in the literature for several types of collagen solutions.[7–9]

Hydroxyproline determinations were made using a modified Neuman and Logan procedure.[10,11]

The tyrosine content of our collagen preparation was determined in two ways. In the first method, the protein was converted to gelatin by heating at 100° in a waterbath, cooled and then centrifuged for 30 min. at 60,000 × *g*. The solution was made 0.1 *N* in NaOH and the optical density was read at 294 mμ in a Beckman DU spectrophotometer. A blank reading was obtained from an equivalent solution at *p*H 8. Protein determinations were made by nitrogen analysis. The tyrosine content was then determined using our value of 2,308/cm. for the molar extinction coefficient at 294 mμ for tyrosine in NaOH. We have determined by the method of Goodwin and Morton[12] that there was no detectable amount of tryptophan in our protein preparations. The second method served simply to check the spectrophotometric method as applied to whole protein solutions. A known amount of protein was hydrolysed in 8% $Ba(OH)_2$. Barium was removed as the carbonate and the tyrosine was isolated on a 50 cm. column of Dowex-1 in the acetate form as described by Hirs, Moore and Stein.[13] The isolated tyrosine was determined by reading the optical density at 294 mμ after making the solution 0.1 *N* in NaOH. The recovery of a known amount of tyrosine which had been added to an identical amount of protein served as a correction factor.

Diiodotyrosine and MIT samples were isolated using a 10 cm. Dowex-1 column in the acetate form referred to above. The commercial samples of DIT contained 5% MIT as an impurity and the sample of MIT contained 5%

(1) The research reported in this paper was supported 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 Elizabeth Severance Prentiss Foundation.

(2) The abbreviations used in this paper are as follows: Monoiodotyrosine, M1T; diiodotyrosine, DIT; monoiodohistidine, MIH; diiodohistidine, DIH; tris-(hydroxymethyl)-aminomethane, "tris."

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

(4) The nitrogen and iodine content were determined by the Clark Microanalytical Laboratories, Urbana, Illinois.

(5) J. H. Bowes, R. G. Elliott and J. A. Moss, "Nature and Structure of Collagen," ed. by J. T. Randall, Academic Press, Inc., New York, N. Y., 1953, pp. 199–207.

(6) K. J. Brunings, THIS JOURNAL, **69**, 205 (1947).

(7) H. Boedtker and P. Doty, *ibid.*, **78**, 4267 (1956).

(8) P. M. Gallop, *Arch. Biochem. Biophys.*, **54**, 486 (1955).

(9) M. B. M'Ewen and M. I. Pratt, "Nature and Structure of Collagen," ed. by J. T. Randall, Academic Press, Inc., New York, N. Y., 1953, p. 159.

(10) The modification was developed by Dr. L. H. Frank and will be published.

(11) R. E. Neuman and M. A. Logan, *J. Biol. Chem.*, **184**, 299 (1950).

(12) T. W. Goodwin and R. A. Morton, *Biochem. J.*, **40**, 628 (1946).

(13) C. H. W. Hirs, S. Moore and W. H. Stein, THIS JOURNAL, **76**, 6063 (1954).

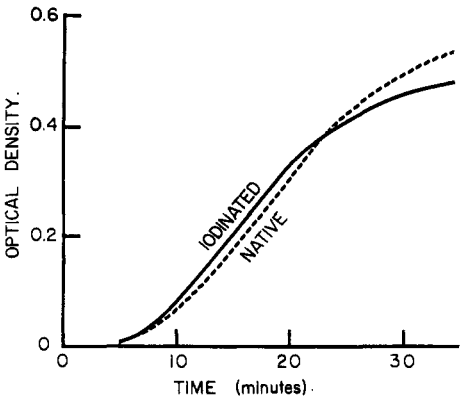

Fig. 1.—Typical curves of optical density *vs.* time for native and iodinated collagen during the determination of rate. The curves are not identical in size as the conditions were different. The experimental points were not included since there were no deviations from a smooth curve as the scale is drawn.

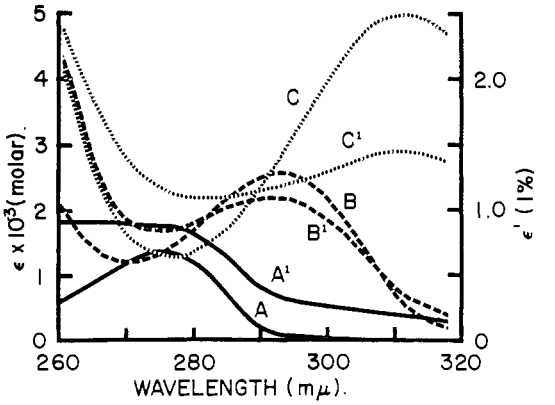

Fig. 2.—The absorption spectra of tyrosine, DIT, native collagen and iodinated collagen. The molar extinction coefficient is given for the amino acids and the 1% extinction coefficient is given for the proteins. The letters labeling the curves represent tyrosine at *p*H 8 (A), native collagen at *p*H 8 (A'), tyrosine in 0.1 *N* NaOH (B), native collagen in 0.1 *N* NaOH (B'), DIT at *p*H 8 (C) and iodinated collagen at *p*H 8 (C').

DIT. The molar extinction coefficients in 0.1 *N* NaOH of 5,570/cm. for DIT at 310 mμ and of 3,930/cm. for MIT at 305 mμ were determined from these purified preparations. The DIT content of our iodinated collagen samples was determined spectrophotometrically at 310 mμ in the same manner as described for tyrosine. Since the extinction coefficient for tyrosine in 0.1 *N* NaOH at this wave length is only about 70, the error introduced by its presence in the iodinated samples would be small. A blank correction at 310 mμ was made using an equivalent amount of uniodinated protein at *p*H 8. The molar extinction coefficient of tyrosine at this *p*H and wave length is negligible. This blank corrects for non-specific absorption.

Determination of the Rate of Fiber Formation.—The increase in turbidity produced by the formation of fibers from the clear collagen solutions formed the basis for our determinations of rate. The apparatus consisted of a Beckman DU spectrophotometer equipped with a double set of themospacers through which water from a constant temperature bath was circulated. Our Beckman spectrophotometer was equipped with a Brown recorder and an automatic sample changer which was set to change samples at intervals such that a complete cycle of readings of four cuvettes was obtained in one minute. In a typical experiment, a 2 ml. mixture of water and tris buffer (0.2 *M* tris, 0.1 *N* HCl, 0.05 *M* NaCl, *p*H 8.2) was made in a Wassermann tube and placed in the water-bath. The mixture was made in a ratio such that the desired ionic strength was obtained upon the addition of 1.0 ml. of the protein in the tris buffer. After allowing about 15 min. for temperature equilibration, 1.0 ml. of a convenient dilution of the protein in a Wassermann tube was made up at 4°. At zero time the two solutions were mixed by pouring back and forth four times. The mixture was then placed in the constant-temperature bath for four minutes to allow the temperature to equilibrate more rapidly. The solution was transferred to a Corex cuvette which had been preincubated by placing it in the instrument. Beginning at 5 min. the optical density at 290 mμ was read at one-minute intervals.

When the rate of fiber formation of the iodinated samples was being determined the general method was the same. Just prior to zero time 0.02–0.03 ml. of a 0.02 *N* I_2 solution in 0.3 *M* KI was added to the 1.0 ml. of protein using a microburet. After the indicated length of time the reaction was stopped by the addition of 0.1 ml. of a 0.01 *N* thiosulfate solution. Typical data on the rate of fiber formation for the native and the iodinated samples are plotted in Fig. 1.

The method of quantitating the rate was different than that previously reported.[3] We have subsequently found that a more reliable measure of the rate is obtained by using the slope of the apparently-linear portion of the curve. The rate is expressed as the increase in optical density per minute multiplied by 1000. Calculations of the reliability of this method from replicate determinations showed a standard deviation of 5%. A discussion of the kinetics of the reaction will be found in the Appendix.

Electron micrographs of the fibers formed from both the native and iodinated samples showed the presence of only typical collagen fibers with the usual 600–700 Å. spacings.

Paper Chromatography.—The iodinated protein was prepared by iodinating 20–25 ml. aliquots of a 0.10–0.15% solution of collagen in tris buffer (0.2 *M* tris, 0.1 *N* HCl, 0.05 *M* NaCl, *p*H 8.2) for varying lengths of time at 4°. The amount of iodine added (0.02 *N* I_2 in 0.3 *M* KI) was calculated to be about twice that required for the iodination of the tyrosyl residues. The reaction was stopped after the appropriate time by the addition of an excess of sodium thiosulfate. The solutions were allowed to clot at room temperature. The protein was collected by centrifugation and the dense clot was dialyzed against running tap water. The clots were hydrolyzed in 8% $Ba(OH)_2$ and the barium was removed as the carbonate. The solutions of amino acids were concentrated in a boiling-water bath. Ascending chromatograms were run in the usual manner. The solvents used were those given in the appropriate table. Tyrosine, histidine and their iodinated derivatives were detected by Pauly's reagent as described by Baldridge and Lewis.[14] Iodine-containing compounds were detected by the $CeSO_4$–$HAsO_2$ reagent as described by Bowden and Maclagan.[15]

Results

The Product of Iodination.—Figure 2 represents the absorption spectra of the untreated and iodinated collagen after their conversion to gelatin as compared with the absorption spectra of tyrosine and DIT. The untreated collagen at *p*H 8.2 shows the typical flat portion between 255 and 275 mμ.[3,16] At a *p*H close to 13 there is a peak at 294 mμ. This peak is identified with the ionization of the phenolic group of tyrosine. The shift on iodination to a maximum at 310 mμ is consistent with the formation of DIT.

Since the formation of MIH and DIH is known to occur during long periods of iodination,[17] we have endeavored to demonstrate their presence or ab-

(14) R. C. Baldridge and H. B. Lewis, *J. Biol. Chem.*, **202**, 169 (1953).

(15) C. H. Bowden and N. F. Maclagan, *Biochem. J.*, **56**, vii (1954).

(16) J. R. Loofbourow, B. S. Gould and I. W. Sizer, *Arch. Biochem.*, **22**, 406 (1949).

(17) H. Frankel-Conrat, *ibid.*, **27**, 109 (1950).

TABLE I

THE TWO-DIMENSIONAL CHROMATOGRAPHY OF THE BASE-HYDROLYZED IODINATED COLLAGEN

Sample[a]	Rf KI	MIH	DIH	MIT	DIT
Standard 1	.....	0.21 × 0.13	0.06 × 0.43	0.12 × 0.46	0.07 × 0.59
Standard 2	0.30 × 0.19	0.21 × .17	[b]	[b]	.08 × .60
Iodocollagen (1 min.)	.30 × .21	None	None	0.13 × 0.45	.10 × .60
Iodocollagen (15 min.)	.27 × .19	None	None	None	.10 × .60
Iodocollagen (30 min.)	.28 × .19	None	None	None	.10 × .60

[a] Standard 1 was developed by Pauly's reagent. Standard 2 and the iodocollagens were developed by the $CeSO_4$–$HAsO_2$ method for iodine-containing compounds. The time given for the iodocollagen samples is the length of time during which iodination was carried out. [b] The DIH and MIT spots were too close together to be differentiated in this sample. The first solvent was 1-butanol saturated with 2 *N* NH_4OH. The second solvent was 1-butanol-HOAc-H_2O (200:13:44). The R_f values list the R_f in the first solvent first.

sence in collagen after iodination. Chromatograms were run on the untreated and treated collagen after hydrolysis as described under methods. Table I shows the results. After 1 minute of iodination MIT and DIT were found. After 15 and 30 minutes of iodination, DIT was the only detectable organic iodine-containing compound.

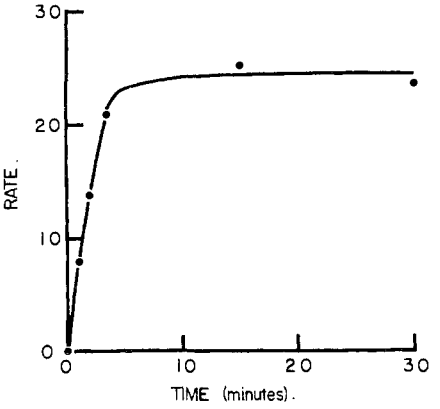

Fig. 3.—The effect of time of iodine treatment on the subsequent rate of fiber formation. The final ionic strength was 0.14, the temperature was 20.0° and the final concentration was 0.035% for the determination of rate. A volume of 0.02 ml. of 0.023 *N* I_2 in 0.3 *M* KI was used for iodination. An equal volume of 0.3 *M* KI was added to the uniodinated control. Thiosulfate (0.1 ml. of a 0.01 *N* solution) was added to all. The uniodinated control failed to show signs of clotting after 24 hours.

The demonstration that iodinated tyrosyl groups are the only iodinated product of the reaction which can be detected by chromatography still leaves at least two possibilities for side reactions. First it is possible that MIH or DIH may be formed but their presence may go undetected due to their partial breakdown during hydrolysis.[17] It was reasoned that if the iodine content of the final product and the iodine uptake were no greater than that which could be accounted for by diiodotyrosyl groups this possibility could be considered unlikely. The other possibility still open is the occurrence of an oxidative reaction. Such a reaction could consume iodine without the formation of an iodinated product. This could be checked by determining the iodine uptake as compared with the amount of DIT formed. The results of a set of these determinations are given in Table II. These results are consistent with the conclusion that the iodination of tyrosyl groups is the only reaction.

TABLE II

THE COMPARISON BETWEEN THE TYROSINE CONTENT OF NATIVE COLLAGEN AND IODINE UPTAKE, DIT FORMATION AND IODINE CONTENT OF IODOCOLLAGEN[a]

Time of iodination (minutes)	Tyrosine content (mmole/100 g.) Column isolation	Tyrosine content (mmole/100 g.) Spectrophotometry	Iodine uptake (0.25 × matom/100 g.)	DIT content (mmole/100 g.)	Iodine content[a] (0.5 × matom/100 g.)
None	3.1	3.3	..	..	..
1	..	..	1.3	2.1	2.6
15	..	..	3.1	3.1	2.1
30	..	..	3.2	3.0	3.3

[a] The number of milliatoms of iodine taken up was multiplied by 0.25 since four atoms of iodine are required for the formation of one molecule of DIT. The iodine content was multiplied by 0.5 to get the millimoles of DIT.

The Effect of Iodination on the Rate of Fiber Formation.—A good demonstration of the relation between the extent of iodination and the rate of fiber formation was obtained when larger amounts of protein were iodinated for varying lengths of time in the presence of 0.1 *M* KI. (In the presence of KI the iodination reaction proceeds at a much slower rate.) The results in Table III show that as the degree of iodination increases, the rate of subsequent fiber formation increases.

TABLE III

THE CORRELATION BETWEEN IODINE UPTAKE, DIT CONTENT AND RATE OF FIBER FORMATION

Time of iodination (minutes)	Iodine uptake[a]	DIT content	Approximate[b] time of clotting (hours)
5	23	20	3
10	33	23	2
15	35	30	1
30	50	50	0.5
45	63	65	0.3

[a] The values are given as the % of the total tyrosine iodinated using a tyrosine content of 0.60%. [b] The time was taken when the first visible sign of clotting was noted.

Figure 3 gives the rates of fiber formation after different times of iodination. Under the conditions given in the legend, iodination appears to be essentially complete by 3 minutes. Although it is not apparent from Fig. 3, the relative difference in the rate of fiber formation between the uniodinated and the 1 minute sample is far greater than that between the 1 minute and the 3 minute sample.

Effect of Iodination on Energy of Activation.—In order to compare rates of fiber formation for the native and the iodinated collagen, it was necessary to make determinations under different conditions. In determinations of energies of activation it is,

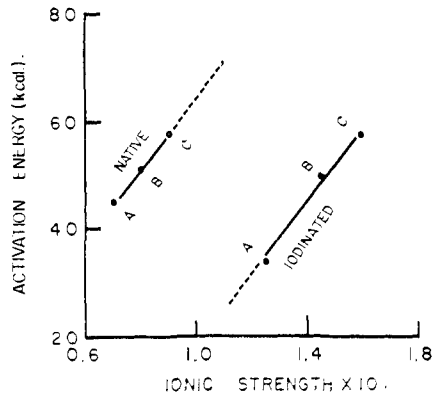

Fig. 4.—The effect of ionic strength on the energy of activation of native and iodinated collagen. The points marked A, B and C were determined over a temperature range of 10–13°, 15–19° and 20–23°, respectively, in steps of 1°.

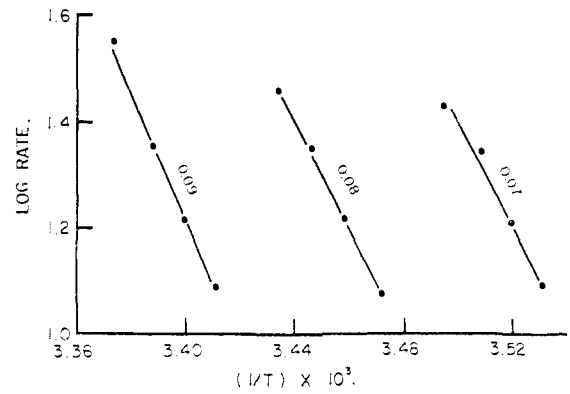

Fig. 5.—The Arrhenius plots used in the determination of the energy of activation for the native collagen shown in Fig. 3. The values of the ionic strength are given to the right of each line.

therefore, necessary to determine the effect of the environmental conditions as well. Tris buffer changes *p*H with changes in temperature. Therefore, in determinations of rates, even over a narrow range of temperatures, it was deemed advisable to ascertain the effects of small *p*H differences on the energies of activation. For this reason, we determined the energies of activation at three different *p*H values. The results of these determinations are presented in Table IV. It can be seen that *p*H differences had little effect. On the other hand, large differences in the energy of activation between the native and the iodinated collagens were found. It should be noted that the ionic strength of the media for the iodinated collagen was higher than that for the untreated protein.

TABLE IV

THE EFFECT OF IODINATION ON THE ENERGY OF ACTIVATION FOR FIBER FORMATION

*p*H		Energy of activation (kcal.)[a]		
Native	Iodinated	Native (E_n)	Iodinated (E_i)	Difference ($E_n - E_i$)
7.79	7.98	74	42	32
8.11	8.06	83	48	35
8.15	8.13	74	41	33

[a] The activation energies were based on the rates at temperatures of 12–15° for the iodinated sample and 25–28° for the native sample. The ranges were covered in 1° steps. The ionic strength for the native sample was 0.125 while that for the iodinated protein was 0.150. The final protein concentration was 0.024%. Iodination was carried out for 1 minute.

It was necessary to determine whether or not a comparison of energies of activation could legitimately be made at different temperature ranges and at different ionic strengths. We, therefore, determined the energy of activation for the native and the iodinated samples over the same ranges of temperature but at different ionic strengths. The results in Fig. 4 show that an increase in ionic strength increases the energy of activation.[18] If the lines are extrapolated to a value of identical ionic strength, the difference in energies of activation amounts to approximately 57 kcal. The slopes of these lines are similar and amount to about 65 kcal. per 0.1 increment in μ in the range considered. Correcting the difference in energies of activation shown in Table IV for the differences in ionic strength using this value gives an average difference of 49 kcal. between the untreated and the iodinated collagen. It should be noted that the collagen was iodinated for 2 minutes in the experiment illustrated in Fig. 5 and for one minute in the experiment given in Table IV. Therefore, the differences in energy of activation would be somewhat greater with the longer time of iodination.

The differences in energies of activation could conceivably be accounted for by the different temperature ranges. This would mean that the Arrhenius plot of log rate *vs.* $1/T$ would have to be severely concave. We have uniformly obtained straight lines. Any tendency to deviate from linearity has most usually been a slight trend toward convexity. As an example, the actual curves for the native protein are shown in Fig. 5.

The *pK* of the Phenolic OH Group in Iodinated Collagen.—In determining the *pK* for the phenolic group of the diiodotyrosyl groups of iodinated collagen, the increase in optical density at 310 mμ was taken as the criterion of ionization of this group. The difference in optical density between iodinated collagen in 0.1 *N* HCl and 0.1 *N* NaOH was taken as the total optical-density increase on ionizing the phenolic group. The fraction ionized at different *p*H values is given in Fig. 6. The *pK* value as determined by this method is 7.0. The *pK* of the phenolic group of the untreated tyrosyl residues is taken to be greater than 10.[19]

Discussion

The increased rate of fiber formation upon iodination of soluble collagen appears to result from the decrease in *pK* of the tyrosyl OH group when the

(18) The increase in energy of activation which we observed with an increase in ionic strength contradicts our previous finding.[8] The lack of sensitivity of our former method of determining the rate is the obvious explanation for our not observing this difference. As a result, our previous interpretation of the effect of salts must be slightly modified. Thus, the "inactive" form postulated does not have to include some sites which are saturated at low concentrations of salts. For our more recent interpretation of the effects of salts see the succeeding paper.

(19) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. I, Academic Press, Inc., New York, N. Y., 1958, p. 464.

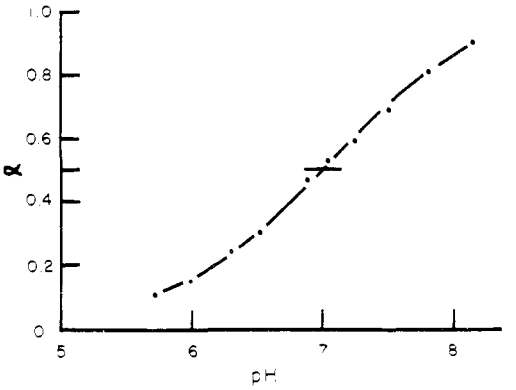

Fig. 6.—The determination of the pK for the phenolic group in iodinated collagen.

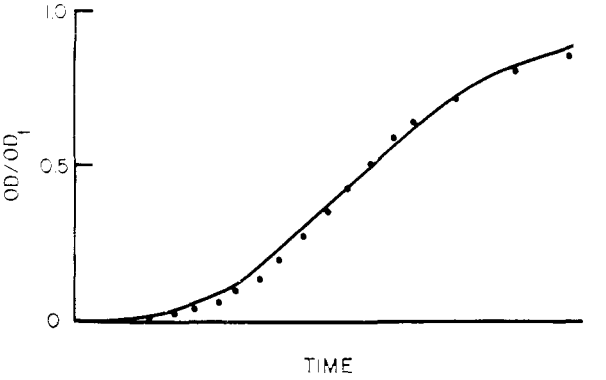

Fig. 7.—A plot of the integral of equation 2 (solid line) as compared with experimental data (solid circles). The time coordinate was normalized by equating the time when the experimental o.d. was 0.4 of the final o.d. with the arbitrary time unit determined for the same point in the theoretical expression.

tyrosyl group is converted to diiodotyrosyl residues. At the pH at which we have made our determinations, the diiodotyrosyl residues would be essentially ionized. At the same pH the tyrosyl residues of native collagen would be essentially un-ionized. We have found that the energy of activation for the iodinated collagen is 50–57 kcal. less than that of the untreated protein under the conditions used. Taking the heat of ionization of the phenolic OH group to be 6 kcal.,[19] we concluded that the ionization of approximately 8–10 tyrosyl residues is necessary during the activation step in the formation of fibers from solutions of untreated collagen in the pH range used. Taking the molecular weight of soluble collagen to be 360,000,[20] we calculate from our value of the tyrosine content that our collagen samples contain 12 residues per molecule. Therefore, the number of our theoretically important tyrosyl residues is consistent with the number actually found to be present in our samples.[21]

It appears logical to assume that the tyrosyl residues are a part of the collagen molecule and that they are strategically positioned in the chain. The formation of ionic bonds in these positions would then be a requisite to fiber formation. Grassmann, *et al.*,[22] reported on the isolation of a peptide from collagen containing both tyrosine and hydroxyproline. This together with our findings strengthens the hypothesis that tyrosine is indeed a true constituent of collagen and not part of an impurity.

Acknowledgments.—The authors wish to express their gratitude to Dr. Leonard Frank for his valuable suggestions, to Dr. Melvin D. Schoenberg for the electronmicroscopy involved in this work and to Dr. Elliot Q. Adams for the kinetic derivation reported in the Appendix.

Appendix

It is obvious that the slope of the apparently linear portion of our curves of optical density (o.d.) *vs.* time would depend on the final o.d. of the gel. We have surveyed the effect on the final o.d. of changes in the various experimental conditions used in this and the following paper. Within the range of conditions used only the initial protein concentration altered the final o.d. significantly. For this reason we have maintained a constant protein concentration throughout any experiment in which rates were compared.

The soluble collagen molecule has a reported length of 3300 Å. and a diameter of 13.5 Å.[20] The fibers formed have a diameter ranging widely around 2000 Å. and an unknown length which is perhaps measured in millimeters. We picture the first step to be a nucleation step involving end-to-end associations admixed with side-by-side association. Although the kinetics of this process would be different from the subsequent growth of the fiber, they may be ignored for the sake of simplicity since the nucleation step would involve a very small proportion of the total protein and would have been completed before the measurement of the optical density began. Once the nuclei were established, subsequent growth would depend on the area of the fiber and the concentration of the protein in solution at any time. This may be expressed in the general form

$$dV/dt = KAC$$

where V is the volume of the fibers, A is the surface area and C is the concentration of soluble protein at any time, t. We have found experimentally that the final o.d. of the clot is directly proportional to the initial protein concentration, indicating that the final o.d. is directly related to the final fiber volume. It is known from the theory of light scattering that the turbidity of particles larger than the wave length of light is a complex function of particle size, shape and concentration. A general expression relating o.d. to the volume of fibers is presented. Assume

$$C = a(D_f - D),\ V = f(D) \text{ and } A = g(D)$$

where D_f and D are the final and instantaneous o.d., respectively, and $f(D)$ and $g(D)$ are different functions of o.d. Then

$$dV/dt = \frac{df(D)}{dD}\frac{dD}{dt} = f'(D)dD/dt$$

and

$$dD/dt = \frac{aK\, g(D)(D_f - D)}{f'(D)} \qquad (1)$$

If dD/dt is measured in the vicinity of some definite value of D (call it D_1) then

$$(dD/dt)_{D_1} = \frac{aK\, g(D_1)(D_f - D_1)}{f'(D_1)} = bK$$

where the constant b depends on D_f and on D_1 as

$$b = \frac{a\, g(D_1)(D_f - D_1)}{f'(D_1)}$$

Under these conditions $(dD/dt)_{D_1}$ is proportional to the

(20) P. Doty and T. Nishihara, "Recent Advances in Gelatin and Glue Research," ed. by G. Stainsby, Pergamon Press, New York, N. Y., 1958, p. 92.

(21) It must be emphasized that the tyrosyl residues are not the only ionizable groups of importance. Obviously, if the tyrosyl groups participate in the ionic interactions, the ionization of basic amino-acid residues is also required. We have evidence, to be published later, indicating that the ionization of the ε-amino groups of lysyl residues increases the rate of fiber formation.

(22) W. Grassmann, K. Hannig, H. Endres and A. Riedel, *Z. physiol. Chem.*, **306**, 123 (1956).

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

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

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 $0.4\ D_t$. 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 $(0.37 \pm 0.02)D_t$. 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

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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.