Subunits of Hydroxylamine-treated Tropocollagen*

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ABSTRACT: The kinetics of reaction of denatured ichthyocol tropocollagen with 1 M hydroxylamine at *p*H 10 and 40° were studied by following the formation of hydroxamic acid, changes in reduced viscosity and weight-average molecular weight, and formation of defined subunits. Two rates of reaction were observed; during the rapid stage hydroxylamine cleaves about six "esterlike" bonds per 100,000 mw. Simultaneously the intrinsic viscosity decreases from 0.4 to 0.16 and the weight-average molecular weight decreases to a value of 27,000. The subunits which are produced are primarily of three molecular weight size classes of *ca.* 34,000, 18,000, and 8000. Their separation by gel filtration on Sephadex G-200 and G-100 and by starch-gel electrophoresis is described. Within each size class there are present several components which probably differ chemically.

The existence of a small but significant number of hydroxylamine- or hydrazine-sensitive bonds, most likely ester or imide in nature, has been demonstrated in tropocollagen (Gallop *et al.*, 1959; Hörmann, 1960; Blumenfeld and Gallop, 1962). These bonds involve both α- and β-carboxyl groups of two aspartyl residues which occur in relatively close proximity along peptide chains. Nucleophilic cleavage of these bonds leads to a decrease in sedimentation constant and molecular weight of the α chains and β chains of the protein (Gallop *et al.*, 1959). In kinetic experiments using 1 M hydrazine at *p*H 10 and 40°, it was found that an end point was reached in approximately 90 minutes with cleavage of about six bonds in each molecule of α component (100,000) (Blumenfeld and Gallop, 1962).

In this paper experiments are reported which define more precisely both the function of these bonds and the size of the protein subunits that are obtained upon their selective rupture. The kinetics of cleavage of ichthyocol by hydroxylamine is examined with respect to hydroxamic acid produced and accompanying changes in molecular weight and intrinsic viscosity. Moreover, description is provided of the isolation on Sephadex columns of subunits of different molecular weight classes which are produced by cleavage of these bonds and their electrophoretic separation on starch gels. On the basis of these experiments, it can be concluded that under defined conditions the action of hydroxylamine on tropocollagen is specific for "esterlike" sites; the action of this reagent results in the production of subunits of definite molecular weight classes.

Materials

Benzyloxycarbonyl-L-aspartic acid α-benzyl ester (mp 84–85°) (Bergmann *et al.*, 1933) and benzyloxycarbonyl-L-aspartic acid β-benzyl ester (mp 108°) (Berger and Katchalski, 1951) were obtained from the Cyclo Chemical Corp., Calif.; α,β-polyaspartic ethyl ester was prepared as described by Blumenfeld and Gallop, and contained about 25% β- and 75% α-ester (Blumenfeld and Gallop, 1962).

Ichthyocol was prepared as described previously (Gallop, 1955). Solutions of denatured ichthyocol (parent gelatin) were prepared by heating 5% (w/v) suspensions of ichthyocol in distilled water at 50–60° for 10 minutes, followed by centrifugation at 100,000 *g* for 20 minutes.

Methods

Buffered 2 M Hydroxylamine Solution, pH 10.0. $NH_2OH \cdot HCl$ (6.95 g) was dissolved in 10 ml of water and the solution was rapidly adjusted with 10 N NaOH to a *p*H value between 9.0 and 9.5; 10 ml of 1 M potassium carbonate solution was added, the *p*H was adjusted to 10.0, and the volume was brought to 50 ml. This reagent was kept on ice until used; it is not stable, and accordingly was prepared daily.

Ferric Chloride Reagent for Determination of Hydroxamic Acid. The reagent was essentially that of Lipmann and Tuttle (1945) to which acetone was added. Acetone suppresses accumulation of bubbles which would be stabilized in solutions of hydroxylamine with a high content of protein; at the same time acetone converts excess hydroxylamine to acetone oxime which offers less interference in determination of ferric hydroxamate. The stock solution contained 18.9 g of

* From the Department of Biochemistry and the Unit for Research in Ageing, Albert Einstein College of Medicine, Yeshiva University, New York. *Received February 2, 1964; revised May 10, 1965.* This work was supported by grants from the National Institutes of Health, U.S. Public Health Service (HE 04762, AM 05821, and HD 00674).

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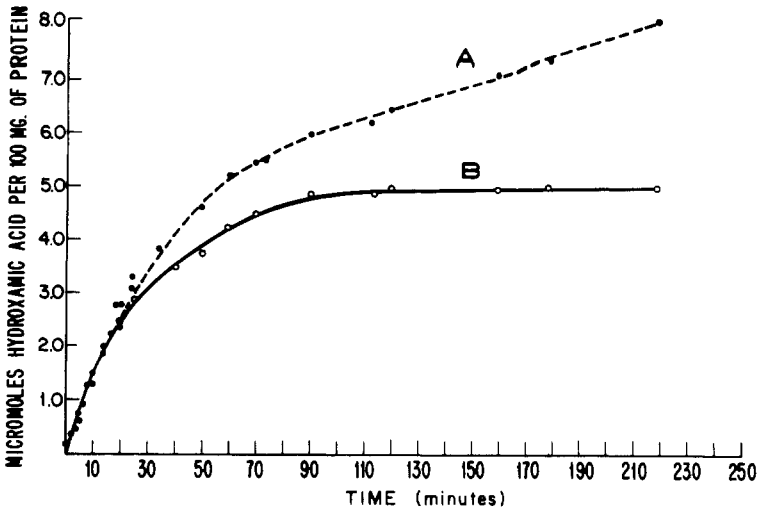

FIGURE 1: Reaction of 1 M hydroxylamine with denatured ichthyocol tropocollagen at 39°, *p*H 10. (A) Experimental values; (B) slow phase of reaction subtracted from curve A.

$FeCl_3 \cdot 6\ H_2O$ and 0.8 ml of 12 N HCl dissolved in water in a total volume of 100 ml. On the day of intended use, 40 ml of stock solution, 10 ml of 4 N HCl, 10 ml of reagent grade acetone, and 20 ml of water were mixed to provide the working reagent.

Reaction of Ichthyocol Parent Gelatin with Hydroxylamine and Measurement of Hydroxamic Acid Formed. To one volume of solution containing protein (of known concentration as determined by Kjeldahl nitrogen) or solution containing one of the model esters, one volume of buffered hydroxylamine solution (2 M, *p*H 10.0) was added. The solution was mixed and, if necessary, the *p*H was adjusted to 10.0. The mixture was then incubated at 39 or 40°.

In the kinetic experiments, at selected time intervals, samples were removed for determination of hydroxamic acid, for study in the ultracentrifuge, and for starch-gel electrophoresis. A 1.5-ml aliquot of sample and 1.5 ml of diluted ferric chloride–acetone reagent were mixed and, after 10 minutes, examined for absorption at 540 mμ in a Zeiss spectrophotometer against a reagent blank prepared simultaneously. Monosuccinic hydroxamic acid was used as standard and was measured in the presence of buffered 1 M NH_2OH. Under these conditions, its molar absorbance coefficient was 320.

The remainder of the protein sample was adjusted immediately with HCl to a *p*H value between 3 and 4 to terminate the reaction; the mixture was dialyzed first against distilled water to remove the salt and the $NH_2OH \cdot HCl$ and finally against 0.1 M sodium phosphate buffer, *p*H 7.1, prior to examination in the ultracentrifuge.

For the isolation of subunits, the samples were usually incubated with hydroxylamine for 2 hours. The *p*H values were then adjusted to *p*H 3–4 and the $NH_2OH \cdot HCl$ and salt were removed from the protein either by dialysis against distilled water or by gel filtration on Sephadex G-25 or G-50.

Viscosity Measurements. Ostwald-Fenske (series B) viscometers were employed, and flow times for each were determined at 39° with 5 ml of 1 M hydroxylamine buffered at *p*H 10 or 5 ml of 1 M NaCl, *p*H 10. Equal volumes of buffered NH_2OH and protein solution were mixed, the *p*H of the mixture was adjusted to 10.0, and 5 ml was placed in a previously calibrated viscometer at 39°. At convenient intervals, flow times were measured and the reduced viscosities were calculated neglecting protein-density corrections. The flow times of a control protein solution in 1 M NaCl at *p*H 10.0, but free of hydroxylamine, were measured similarly. The intrinsic viscosities of test solutions and controls were measured at the point at which the reduced viscosity showed no significant further decrease (about 2.5 hours). Dilutions of both protein and test samples were made with their corresponding buffers, with or without hydroxylamine.

Reaction of Ichthyocol Parent Gelatin with NaOH (*p*H *11.5*). To 5 ml of ichthyocol gelatin (about 30 mg/ml) was added 0.5 ml of 1 M K_2CO_3 and the *p*H value was adjusted to 11.5 with a small volume of NaOH. This solution (5 ml) was placed in a previously calibrated viscometer at 40° and the reduced viscosity was computed as before. The solution was incubated until the reduced viscosity showed no significant decrease (about 18 hours). At that time the *p*H 11.5–treated protein was treated with 1 M NH_2OH, *p*H 10, at 40° and the bound hydroxamic acid was measured as described.

Molecular Weight Determinations. In the kinetic experiments, molecular weight measurements were carried out by the short-column equilibrium method of Yphantis (1960), which employs interference optics and a synthetic-boundary cell measurement of C_o. Since it was found that the molecular weight of gelatin subjected to treatment with hydroxylamine showed a strong concentration dependence in the early stages, the

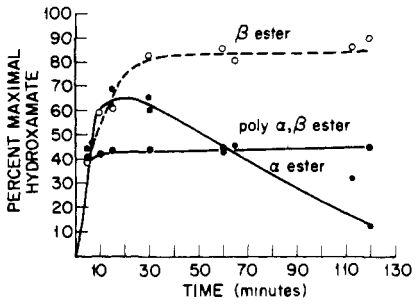

FIGURE 2: Reaction of 1 M hydroxylamine with carbobenzoxyaspartyl α-benzyl ester, carbobenzoxyaspartyl β-benzyl ester, and α,β-polyaspartyl ethyl ester at 39°, *p*H 10.

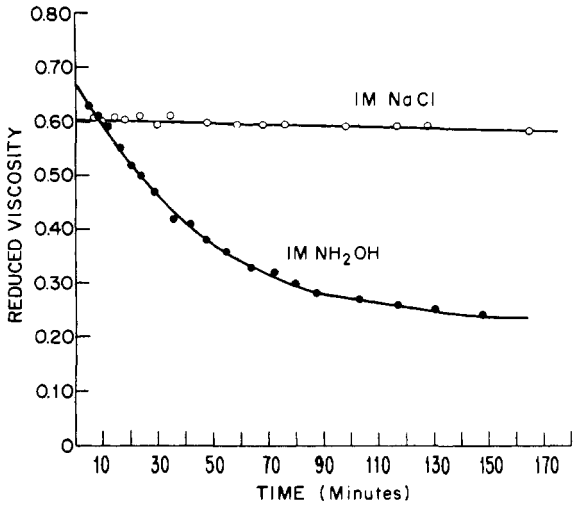

FIGURE 3: Changes in reduced viscosity of ichthyocol parent gelatin during reaction with 1 M hydroxylamine, *p*H 10, 39°.

molecular weights were calculated after extrapolation to infinite dilutions.

The molecular weight determinations of isolated subunits and some hydroxylamine-treated samples before separation were performed using the equilibrium methods of Yphantis (1964) with a 3-mm cell using a high speed of centrifugation giving a zero concentration of protein at the meniscus. A double-sector centrifuge cell with 12-mm-thick aluminum epoxy centerpiece was used with one sapphire (upper) and one quartz (lower) window, and with Hycar window liners (Beckman-Spinco). Each side of the cell was filled with 0.100 ml of fluorocarbon oil (Fc-43); the sample side was then filled with 0.120 ml of protein sample and the reference side with 0.120 ml of the buffer against which the protein sample was dialyzed. After each run the centrifuge cell was thoroughly cleaned without being disassembled; it was then filled with 0.100 ml of Fc-43 oil and 0.120 ml of distilled water on both sides and centrifuged for 30 minutes under identical conditions to obtain a baseline-interference-fringe pattern. All ultracentrifuge determinations were performed in 0.1 M phosphate buffer, *p*H 7.1, at approximately 34°. With some samples, sedimentation-velocity measurements were obtained using conventional techniques.

Separation of Subunits by Gel Filtration. Ichthyocol gelatin that had been treated with NH_2OH as described was fractionated on columns of Sephadex G-200 and G-100 (Pharmacia, Uppsala, Sweden) using acetic acid as eluent. Columns of various sizes (from 3.5 × 180 cm to 2.5 × 65 cm) were employed. The flow rate was controlled by gravity and ranged from 12 to 30 ml/hour. Ninhydrin determinations (Rosen, 1957) were performed on a suitable aliquot of each fraction.

For rechromatography, suitable fractions were pooled, acetic acid and water were evaporated off *in vacuo*, and the material was redissolved in acetic acid for application to the column. After rechromatography the fractions of the appropriate peaks were pooled, the solvent was evaporated off *in vacuo*, and the residue was dissolved in a small volume of distilled water. An

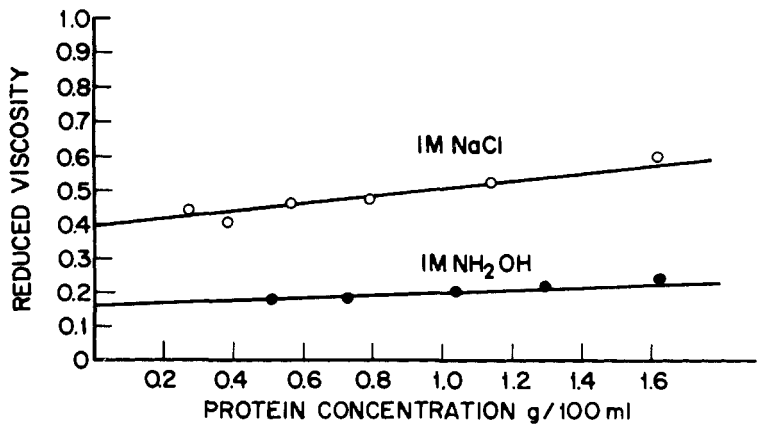

FIGURE 4: Intrinsic viscosity of ichthyocol parent gelatin after treatment for 2 hours with 1 M hydroxylamine at *p*H 10, 39°, and 1 M sodium chloride, *p*H 10, 39°.

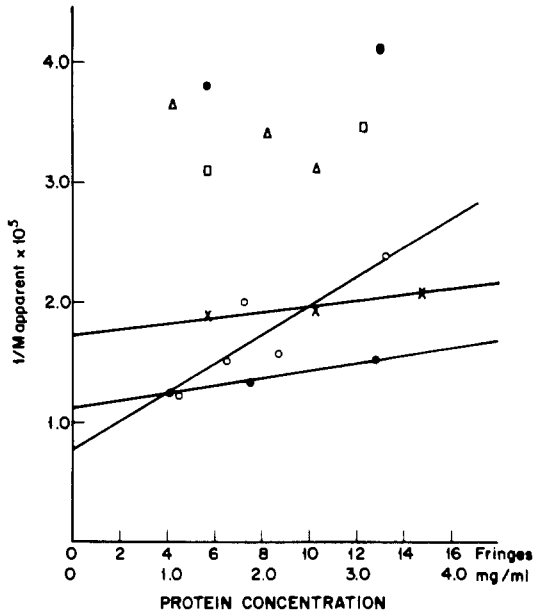

FIGURE 5: Concentration dependence of the apparent molecular weight $1/M_{app}$ of ichthyocol parent gelatin treated with 1 M hydroxylamine at *p*H 10, 39°, for: O, 0 hour; ⊙, 15 min; ×, 60 min; □, 120 min; △, 140 min; ●, 180 min.

aliquot was dialyzed against 0.1 M phosphate buffer, *p*H 7.1, for molecular weight determinations and the rest was saved for starch-gel electrophoresis.

Starch-Gel Electrophoresis of Subunits. Starch-gel electrophoresis was performed by the method of Smithies (1959) in 0.54 M sodium formate buffer at *p*H 3.2, 150 v, at room temperature, for about 15 hours. We used 15% gels, which were developed by staining with 1% Amido black.

Results

Formation of Hydroxamic Acid. Figure 1 (curve A) shows the increase in hydroxamate during reaction of 1 M hydroxylamine with ichthyocol gelatin at *p*H 10.0, 39°. As described previously (Gallop *et al.*, 1959), it appears that two reactions occur: a rapid reaction involving a definite number of particularly sensitive bonds, and a slower reaction which is most likely a nonspecific hydroxylaminolysis of amide and peptide bonds. The first reaction is associated with cleavage of linkages we have termed "esterlike" (ester or imide).

The hydroxamic acid values obtained during the fast reaction can be corrected for those due to the slower reaction by subtracting the values obtained by drawing an asymptote to the line in the latter stage of the reaction. Thus curve B (Figure 1) is obtained indicating that about 5 μmoles of hydroxamic acid are formed per 100 mg of protein.

A more direct indication of the extent of nonspecific hydroxylaminolysis was obtained by studying the hydroxamic acid formation of base-treated ichthyocol gelatin. It was found that the hydroxamic acid uptake was much slower than with untreated protein and only approximately 2 μmoles was formed at the end of 150 minutes. This value is in close agreement with the calculated value (curve A-B, Figure 1).

During the fast reaction with hydroxylamine, hydroxamates arising from both α- and β-carboxyl groups of aspartyl residues are formed (Blumenfeld and Gallop, 1962). We have reported previously that dilute solutions of α-aspartyl hydroxamic acids are relatively labile to hydrolysis even in distilled water (Blumenfeld and Gallop, 1962). This is also indicated in Figure 2, in which are plotted the reactions with time at *p*H 10.0 at 39° of 1 M NH_2OH respectively with carbobenzoxy-aspartyl α- and β-carboxybenzyl esters and α,β-polyaspartyl ethyl ester. It can be seen that the initial

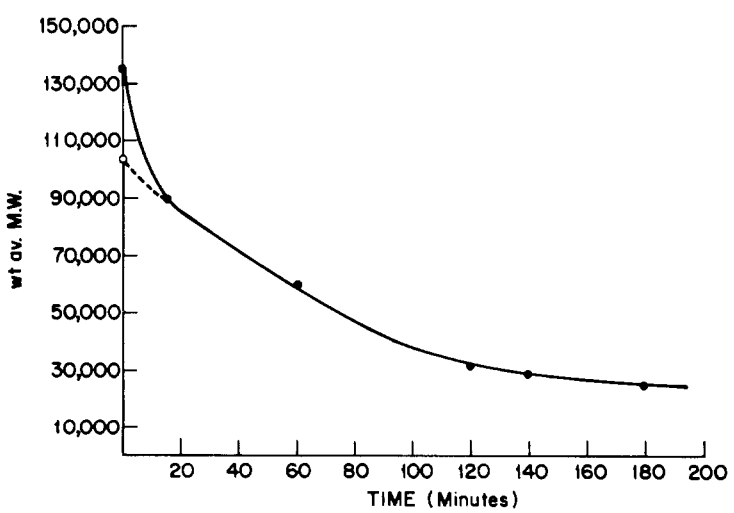

FIGURE 6: Changes in molecular weight of ichthyocol parent gelatin during reaction with 1 M hydroxylamine, *p*H 10, 39°. ●, extrapolated molecular weight; O, molecular weight of α component corrected for 30% β component.

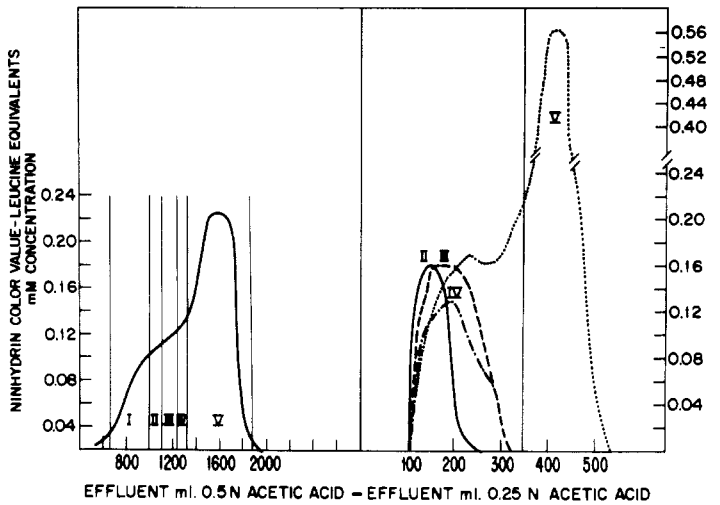

FIGURE 7: Chromatography of hydroxylamine-treated ichthyocol parent gelatin and its fractions. (a) Chromatography of ichthyocol parent gelatin treated with 1 M hydroxylamine for 2 hours at *p*H 10, 40°, on a 3.5- × 184-cm column of Sephadex G-200 at 25° (column 2010). (b) Rechromatography of pooled zones from column 2010 on a 2.2- × 150-cm column of Sephadex G-100 at 25°.

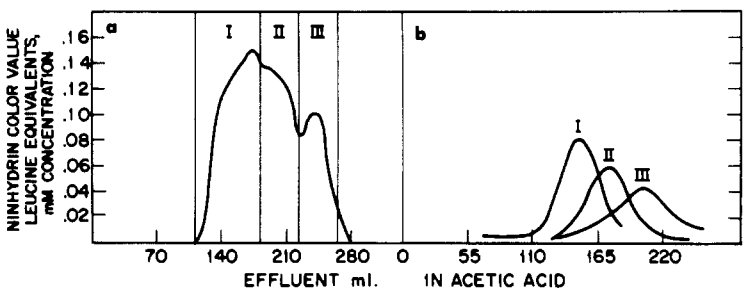

FIGURE 8: Chromatography and rechromatography of hydroxylamine-treated ichthyocol parent gelatin. (a) Chromatography of hydroxylamine-treated ichthyocol parent gelatin (1 M NH_2OH, 40°, 2 hours) on a 2.5- × 72-cm column of Sephadex G-100 at 25° (column 107-1). (b) Rechromatography of pooled zones from column 107-1 on a 2.5- × 67-cm column of Sephadex G-100 at 25° (columns 107-2,3,4).

rates of reaction are similar for all three model esters, but that the measurable α-carboxyl hydroxamic acid decreases after 15 minutes, showing that this group in particular is unstable under these conditions. The reaction with α,β-polyaspartyl ethyl ester also shows the lability of α-hydroxamic acid (Blumenfeld and Gallop, 1962), but indicates that close proximity in the same molecule of the two types of hydroxamic acid groups modifies the lability of the α-hydroxamic acid groups. The 86% of theoretical reaction obtained with the carbobenzoxyaspartyl β-benzyl ester suggests that the molar color yield of the aspartyl β-hydroxamic acid is lower than that of the standard used, i.e., succinic monohydroxamic acid.

In view of the lability of α-aspartyl hydroxamates, the effects of neighboring groups on this lability, and the uncertain color yields, the values of hydroxamates obtained cannot establish an exact number of bonds cleaved in ichthyocol at the end of the fast reaction with hydroxylamine. Nevertheless, the value of 5 μmoles/100 mg obtained in these experiments, although lower than the value of 6–7 obtained in kinetic experiments with 1 M hydrazine at *p*H 10.0 at 40° (Blumenfeld and Gallop, 1962), indicates that approximately the same number of bonds are cleaved by hydrazine or hydroxylamine during the rapid reaction.

Changes in Viscosity during Reaction with Hydroxylamine. The kinetics of changes in reduced viscosity of ichthyocol parent gelatin during reaction with 1 M hydroxylamine, *p*H 10, 39°, are shown in Figure 3. It can be seen that the reaction levels off at about 2 hours; this is the approximate time required for the conclusion of the first stage of the hydroxylamine reaction (see Figure 1). If the intrinsic viscosity is measured at that time, a value of 0.16 is obtained (see Figure 4). The reduced and intrinsic viscosities of a control

ichthyocol parent gelatin solution at *p*H 10.0 at 39° in the presence of 1 M NaCl but in the absence of hydroxylamine do not change as rapidly. As seen in Figure 4, the protein under these conditions retains an intrinsic viscosity of 0.40, a figure which is in good agreement with the previously reported values for ichthyocol parent gelatin (Gallop, 1955; Boedtker and Doty, 1956).

Changes in Molecular Weight during Reaction with Hydroxylamine. Molecular weight determinations were carried out on samples of ichthyocol parent gelatin at various times of incubation with hydroxylamine. The short-column equilibrium method of Yphantis was used for these measurements (Yphantis, 1960). In Figure 5 is shown the apparent dependence of determined molecular weights on concentration. It can be seen that at the initial time, and subsequently at early stages in the reaction, a strong concentration dependence is found for the apparent $1/M$ values. The concentration dependence decreases as the reaction proceeds and is not seen at later times when a plateau of molecular weight values is reached. In Figure 6, the extrapolated molecular weight values are plotted as a function of time of reaction with hydroxylamine. A leveling off value of about 27,000 is apparent near the conclusion of the first stage of the hydroxylamine reaction (about 2 hours), as measured by hydroxamic acid formation and decrease in reduced viscosity (see Figures 1 and 3). The zero-time molecular weight value is given as 135,000 (uncorrected) and 104,000 (corrected for 30% β component, reported by Piez *et al.* (1963) to be present in ichthyocol). The corrected value for the molecular weight of α chains is in agreement with those of Lewis and Piez (1964).

Sedimentation-velocity measurements were carried out on samples of gelatin incubated for 2 hours with hydroxylamine. No concentration dependence of sedimentation constant was found, and the value of $s_{20,w}$ 1.81 S obtained agrees with the value previously reported (Gallop *et al.*, 1959). It should be noted that the β component of gelatin was no longer distinguishable in sedimentation-velocity experiments after 15 minutes of incubation with hydroxylamine, although the rapid reaction as measured by hydroxamic acid formation was not more than one-third completed. At this time, the extrapolated apparent molecular weight was 89,000 and $s_{20,w}$ 2.87 S. Two samples of ichthyocol gelatin incubated with hydroxylamine for 90 and 120 minutes were examined in the ultracentrifuge by the equilibrium-sedimentation method of Yphantis (1964), using the 3-mm cell, at a speed of 42,040 rpm. This method is particularly sensitive for detection of heterogeneity and can give an approximate range of values of molecular weights of species present. It was found that the samples examined contained subunits of molecular weight sizes ranging from about 50,000 to 10,000.

Isolation of Subunits and Determination of Their Molecular Weight. Chromatography of ichthyocol gelatin treated with 1 M hydroxylamine at *p*H 10, 40°, for 2 hours, on Sephadex G-200 and G-100 confirmed the presence of components of different molecular weight sizes. This is seen in Figures 7a and 8a. Separation of subunits of homogeneous molecular weight sizes was achieved by rechromatography of various zones of these chromatograms. Fractions corresponding to the different zones as indicated in Figure 7a were pooled and rechromatographed on a column of Sephadex G-100. This is shown in Figure 7b. Of all the peaks obtained, only peak V was homogeneous, as indicated by long-column equilibrium ultracentrifugation. In a similar experiment (column 101-1) the region corresponding to peak II was also shown to be homogeneous. The plot of fringe displacement versus (radius)^2 for that peak is shown in Figure 9. The molecular weights of subunits present in these peaks are indicated in Table I.

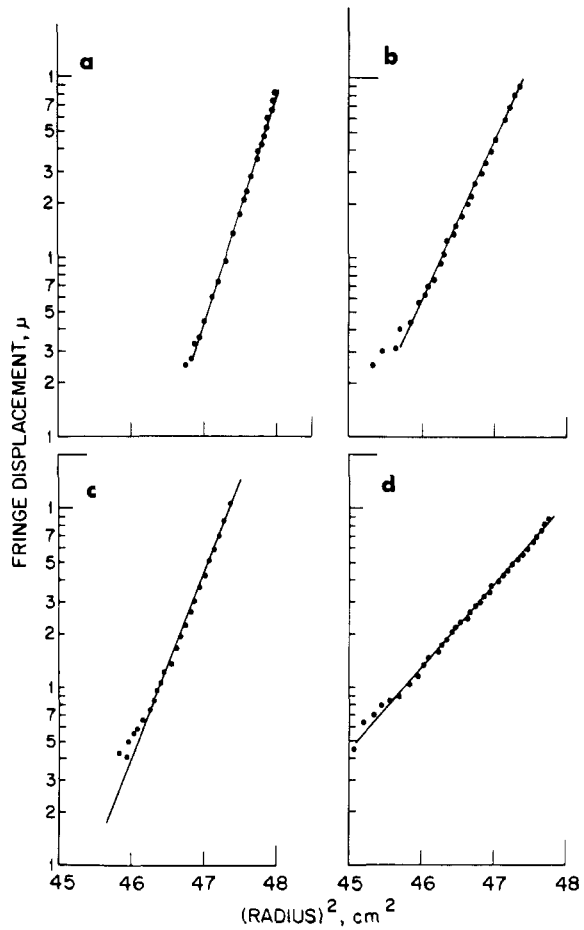

FIGURE 9: Plots of fringe displacements as a function of (radius)^2. (a) Peak I, column 101-1, mw 47,650; (b) peak I, column 107-3, mw 33,243; (c) peak II, column 107–2, mw 18,525; (d) peak III, column 107-4, mw 8030.

In subsequent experiments chromatography was carried out directly on a column of Sephadex G-100 of a smaller size. A typical chromatogram obtained with a sample of hydroxylamine-treated gelatin is shown in Figure 8a. Rechromatography of fractions of zones I, II, and III on the same column under similar conditions gave three peaks, as shown in Figure 8b. The homo-

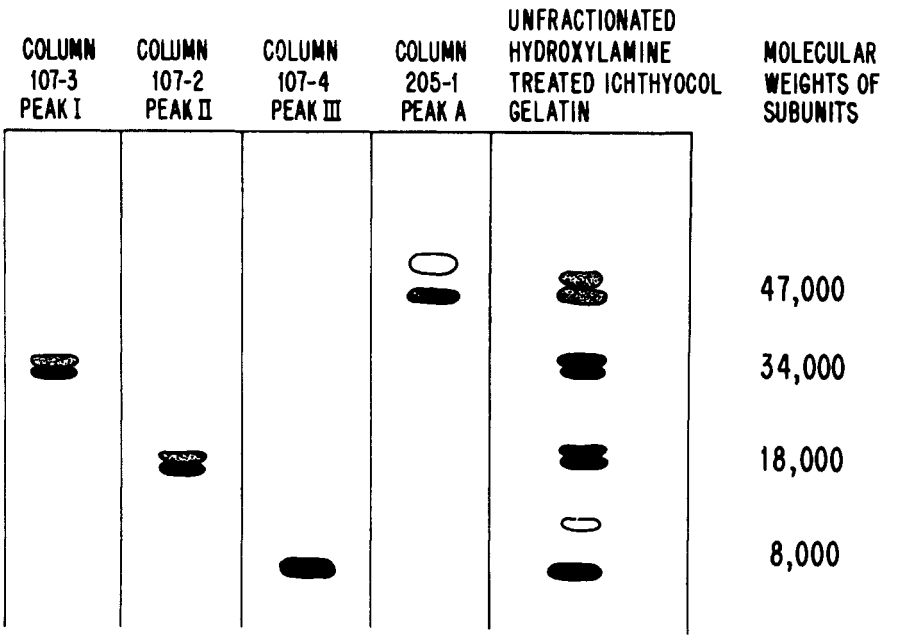

FIGURE 10: Starch-gel electrophoresis of peaks obtained from columns 107-2, -3, -4, 205-1, and unfractionated hydroxylamine-treated ichthyocol gelatin (1 M NH_2OH, *p*H 10, 40°). Gel (15%) in 0.054 M sodium formate buffer, *p*H 3.2; tray buffer, 0.54 M sodium formate buffer, *p*H 3.2, 150 v, 34 ma, 15 hours, 25° (in vertical direction).

TABLE I: Molecular Weight Determinations on Subunits Isolated from Ichthyocol Gelatin after Action of Hydroxylamine.

Column	Peak	Mol Wt
101–1	I	47,650
102–5	V	17,913
107–3	I	33,501[a]
107–2	II	19,259[a]
107–4	III	8,094[a]
205–1	a	47,762
103–3	I	49,561[b]
205–1	b	34,788
109–5,6		33,483
105–4	IVc	8,677
106–4	III	7,795

[a] Average of two determinations at two different speeds. [b] Isolated from $\alpha_1(\alpha_1 + \alpha_3)$ fractions.

geneity and molecular weight size of the protein in each peak were determined by long-cell equilibrium centrifugation. A linear relationship of plot of fringe displacement (*h*) versus (radius)^2 (Figure 9) suggests that protein of a homogeneous weight size was present in each peak; the molecular weights found are shown in Table I. A tabulation of molecular weights of pure fractions isolated in other experiments is also shown in Table I. It should be noted that the first slopes of inhomogeneous fractions which were observed in the ultracentrifuge were indicative of the molecular weight species present. Thus seven samples showed 16,000–20,000 molecular weight range as first slopes and two showed 28,086 and 33,500 mw as first slopes.

Homogeneity as to molecular weight size of these peaks was confirmed by electrophoresis on starch gels. In Figure 10 is shown the pattern obtained after electrophoresis on starch gel at *p*H 3.2 of aliquots of peaks I, II, and III (column 107–2,3,4), of a pure 47,762 mw subunit separated on a different occasion on a column of Sephadex G-200 (column 205–1), and an unfractionated sample of hydroxylamine-treated gelatin. Electrophoresis on starch gels, under conditions used, depends not only on the net charge of various components but also on their molecular weight size (Smithies, 1959). As can be seen, each peak migrates to only one region on the gel even though within each region more than one band is present. Electrophoresis of these subunits on polyacrylamide gels (Nagai *et al.*, 1964) showed from three to six bands within each molecular weight region. Since each peak is of a homogeneous molecular weight size it must be concluded that the multiplicity of bands in each region arises from the presence of components of same molecular weight but different net charge. The unfractionated ichthyocol gelatin after hydroxylamine treatment shows the same bands as the isolated subunits plus an additional light band between the 18,000- and 8000-mw subunits.

Identification of Subunits Produced at Various Stages of Reaction with Hydroxylamine. Samples of ichthyocol gelatin incubated with hydroxylamine at *p*H 10, 40°, for various times up to 4.5 hours were subjected to gel electrophoresis. As seen in Figure 11 the 34,000-,

18,000-, and 8000-mw subunits are present in all samples in which the extent of deesterification was greater than 66%; the 47,000-mw subunit is seen only in the early samples (up to 1 hour) and disappears in the sample in which a total of 8 μmoles of hydroxamic acid are bound.

Discussion

It has been shown in our laboratory (Gallop *et al.*, 1959; Blumenfeld and Gallop, 1962) and in others (Bello, 1960; Hörmann, 1960) that nucleophilic reagents under specified mild conditions react with a small number of bonds in various collagens; the sensitive bonds have been designated "esterlike," and this terminology should be retained until their chemistry is more thoroughly defined. A similar conclusion is apparent from the present kinetic investigation of the reaction of hydroxylamine with ichthyocol gelatin under the conditions defined in the experimental section. The three parameters measured, hydroxamate formation, viscosity, and molecular weight decreases, show two rates of reaction with 1 M hydroxylamine, at *p*H 10, 39°; first a rapid reaction is found which levels off in rate at about 2 hours, followed by a slower reaction. It is during the first stage of the reaction that hydroxylamine reacts with specific "esterlike" bonds in collagen. From the molecular weight changes, it can be concluded that the cleavage of these bonds gives rise to defined subunits whose weight-average molecular weight is about 27,000.

Isolation of defined subunits from ichthyocol parent gelatin after reaction with hydroxylamine further supports the specific effect of hydroxylamine. These subunits are about 47,000, 34,000, 18,000, and 8000 mw in size. It is likely that the 34,000- and 18,000-mw subunits are the most significant since they are present in largest amount at the end of the rapid reaction; the 8000-mw size class is probably also significant, but since the weight-average molecular weight of the unfractionated hydroxylamine-treated gelatin is 27,000, the 8000-mw size is probably not predominant. The 47,000-mw class is transient and present only in the early stages of reaction. Indeed this size probably reflects still attached 34,000- and 18,000-mw double subunits. Early in the reaction with hydroxylamine, other higher-size intermediates are also seen in starch-gel electrophoresis.

It is also clear from the starch- and polyacrylamide-gel electrophoresis patterns that within each size group of subunits several closely spaced bands are present. This suggests that several subunits of very similar molecular weight with different net charge are produced in the hydroxylamine reaction. These might arise either from the same and/or different chains of the tropocollagen molecule; the slightly different mobilities which they show must reflect differences in the amino acid composition of polypeptide chains from which they arise. Recently, Piez (1964) has shown that codfish skin tropocollagen is composed of three unique chains of similar molecular weight but different amino acid

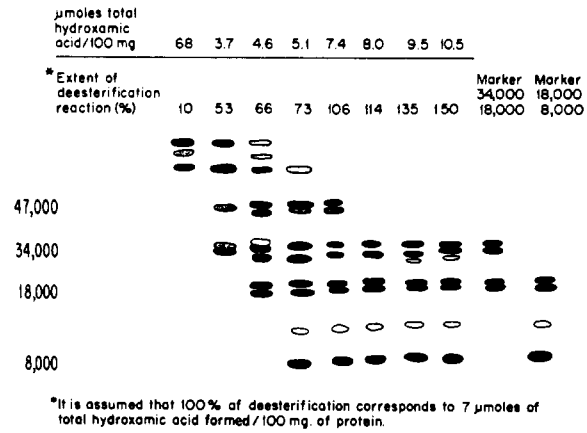

FIGURE 11: Starch-gel electrophoresis of ichthyocol parent gelatin at various steps of reaction with 1 M hydroxylamine, *p*H 10, 40°. Gel (15%) in 0.054 M sodium formate buffer, *p*H 3.2; tray buffer, 0.54 M sodium formate, *p*H 3.2; 150 v, 34 ma, 17 hours, 25° (in vertical direction).

composition. Results obtained by Bornstein and Piez (1965) using cyanogen bromide cleavage of chains further show differences in the amino acid composition and sequence of all three chains of rat skin collagen so that it appears that each α chain in all species is unique.

Separation of subunits by size classes from individual α chains will clarify the origin of various classes of subunits and establish whether or not all classes are present in all chains. A chemical separation of subunits from each molecular weight size class both from hydroxylamine-treated unfractionated parent gelatin and from individual α chains is clearly essential to answer many questions regarding their origins and relationships to each other as well as relating to the architecture of the complete tropocollagen molecule. That such separations are feasible is already apparent from the results obtained with gel electrophoresis, reported in this paper.

Finally, as described in this paper, a partial separation of subunits by virtue of their different sizes was accomplished. Their existence as defined entities and their specific release by hydroxylamine action indicate conclusively that "esterlike" bonds are present and function at subunit attachment sites in tropocollagen.

Acknowledgments

The authors are thankful to Professor Sam Seifter for his advice, help, and encouragement, and to Miss Sybil Herschkopf and Mr. Norman Kinard for expert technical assistance.

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Alkali Metal Binding by Ethylenediaminetetraacetate, Adenosine 5′-Triphosphate, and Pyrophosphate*

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ABSTRACT: From titration curves for ethylenediaminetetraacetate (EDTA), adenosine 5′-triphosphate (ATP), and pyrophosphate (PP) in the presence of the chloride salts of Li, Na, K, Rb, or Cs, the affinity constants are calculated for the binding of the alkali metal ion to the last two dissociation products of the acid H_4Y. Analytical expressions give correction factors applicable when measurements for the binding of one monovalent cation are made in the presence of another such cation. Comparison of the affinity constants shows that EDTA is better able to distinguish among the cations in the series than is either ATP or PP. PP binds each of the cations more strongly than does ATP or EDTA.

In order to understand the metal ion dependence of enzyme activity for an enzyme such as myosin ATPase,[1] it is important to know the affinities of the metal ions for the various reactants in the system. Under appropriate conditions, the ATPase activity of myosin in the presence of KCl or RbCl can be considerably enhanced by the addition of a modifier such as EDTA or PP. The enhancement with EDTA has been attributed to its ability to combine with the inhibiting ion, Mg^{2+} (Friess, 1954; Friess *et al.*, 1954; Offer, 1964). However, sequestering of Mg^{2+} would seem not to be the sole basis for activation, at least with PP, since PP can actually intensify the inhibition brought about by addition of a small amount of Mg^{2+}. Since activation with EDTA or PP occurs only in the presence of a specific metal ion, M^+, interaction of the modifier with M^+ may play a role in the activation process. In any event, it is desirable to know the state of the modifier and of the substrate in free solution for a given reaction system. From analysis of titration curves for EDTA, PP, or ATP in MCl the affinities of these substances for M^+ can be calculated. For a given *p*H and [M], it then is possible to find the extent to which the substrate and the modifier are combined with the metal ion.

Reactions between the tetravalent compound (EDTA, PP, or ATP) and a monovalent cation (H^+ and Li^+, Na^+, K^+, Rb^+, or Cs^+) were analyzed in accordance with the following equilibrium scheme:

$$\begin{array}{ccccc} & & \overset{K_3}{\rightleftarrows} & & \overset{K_4}{\rightleftarrows} \\ H_2Y^{2-} & & HY^{3-} & & Y^{4-} \\ & \overset{K_5}{\updownarrow} & & \overset{K_6}{} & \overset{K_7}{\updownarrow} \\ & MHY^{2-} & \rightleftarrows & MY^{3-} & \\ & & & \overset{K_9}{\updownarrow} & \\ & & & M_2Y^{2} & \end{array} \qquad (1)$$

where Y represents the tetravalent compound, and H and M are the hydrogen and monovalent metal atoms. Here K_3, K_4, and K_6 are dissociation constants whereas K_5, K_7, and K_9 are stability constants. (Titration curves did

* From the Cardiovascular Research Institute, University of California Medical Center, San Francisco. *Received March 24, 1965; revised June 8, 1965.* Supported by a U.S. Public Health Service grant (HE-06285).

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[1] Abbreviations used in this work: ATP, adenosine 5′-triphosphate; ATPase, adenosine triphosphatase; PP, pyrophosphate.