Chromatographic Fractionation of Acetic Acid-solubilized Rat Tail Tendon Collagen

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It has been known since before 1870 (1) that collagen swells in dilute acetic acid, loses its fibrillar appearance, and eventually dissolves, in part, to yield a viscous solution. If the temperature of this solution is raised steadily, its viscosity decreases, at first slightly, then sharply, a phenomenon which occurs between 13° and 40°, depending on the type of collagen (2). Physical measurements of this relatively nonviscous dissolved collagen and the sudden process producing it, have been undertaken only within the last several years, and include sedimentation studies, viscometry, osmometry, light scattering, polarimetry, and electrophoresis (2-8). Several workers (2, 6-8) have shown that warmed dissolved collagen consists of more than one molecular species.

Attempts at detailed examination of the chemical and physical characteristics of these components have been hindered by the inability to isolate them adequately. The techniques (9-13) used to fractionate these substances known commercially as "gelatin"—collagens more or less drastically treated with acids or alkalies—do not seem satisfactory for this purpose. Ion exchange chromatography (13) requiring sodium hydroxide is drastic for the study of primary structure; for other methods, Orekhovitch and Shipkiter (14) found alcohol precipitation (9, 10) and conservative (11, 12) unsatisfactory for the separation of the components demonstrated by ultracentrifugation of solutions of collagen treated with hydrogen bond-breaking agents. We have now developed a method which we find satisfactory and have succeeded in chromatographing dissolved, gently heated rat tail tendon collagen into at least four components. The method involves gradient elution chromatography on warmed carboxymethyl cellulose columns, under mild conditions of pH and low salt concentrations.

EXPERIMENTAL

Preparation of Collagen Solutions—Pathogen-free male rats of the Walter Reed strain weighing 300 ± 10 g were used as the source of tendons. Each animal was deeply anesthetized with ether, the tail was cut off at its base, stroked several times to remove blood and then skinned. It is easy to pull away several tendons at one time from the rest of the tail by pinching the distal tips of the shiny tendons with a hemostat.

This process was repeated halfway to the base of the tail. The tendons of one tail were then cut into 1-cm pieces, directly immersed in 100 ml of chilled 0.16 or 0.2 M sodium chloride and shaken slowly for 18 hours at 4°. This sodium chloride extract was discarded, and the tendons were washed with 5 portions of chilled distilled water; each rinse was 50 ml and lasted 5 to 10 seconds. After the addition of 35 ml of acetic acid of specified concentration to 240 mg of wet tendon, the mixture was allowed to stand in a tightly stoppered Erlenmeyer flask at room temperature (20°-24°) for 24 hours (Experiment 1), or for other specified times, centrifuged at 18,000 r.p.m. for 15 minutes, and filtered under vacuum through a medium (10 to 15 μ pore size), sintered glass funnel. The result was a clear viscous solution, which was usually chromatographed immediately, although no changes were detected in chromatographic patterns of solutions left 4 months at 4°. Experiments performed on preparations (0.1 M and 1.0 M acid) kept at 4° during the initial 24 hours of solubilization yielded results not noticeably different from preparations solubilized at room temperature.

Chromatographic Procedure—The carboxymethyl cellulose was prepared from Whatman 200 mesh wood pulp cellulose by the method of Peterson and Sober (15). Titration, carried out as described by these authors, showed 0.73 mmole carboxyl groups per gram of material.

Columns were 0.9 cm in diameter and jacketed for water circulation allowing precise control of the 40° temperature required for the chromatography. The absorbent was poured to a final height of 7 cm from a slurry made of the carboxymethyl cellulose, and a solution which was 0.2 M with respect to sodium hydroxide and 0.5 M with respect to sodium chloride. Longer columns gave no better resolution than one of this height.

After the column had packed by gravity, it was washed with the starting buffer until the effluent was no longer base, and then with about 4 ml of distilled water, and allowed to become dry at the top. Collagen solution, 1.5 ml, containing about 0.5 mg of nitrogen was applied to the heated column (40°) at this time. This was the first time that the collagen solution was subjected to a temperature higher than 20°-24°. The sample was allowed to run into the adsorbent, covered with 4 ml of initial buffer, and the elution gradient process was started.

The elution gradient arrangement had, as its lower section, an Erlenmeyer flask fitted with a side arm, so that its volume up to the side arm was 160 ml. The top section was a 250-ml separatory funnel with a long stem which could reach almost to the bottom of the Erlenmeyer flask. The two vessels were connected with a one-hole rubber stopper, and the contents of the lower flask were stirred with a magnetic stirrer.

The lower solution was 0.05 M with respect to sodium chloride and 0.05 M with respect to sodium dihydrogen phosphate. The upper (separatory funnel) solution was 0.14 M and 0.05 M with respect to the same salts. Deviations from these specifications resulted in poor resolution.
Amino acid analyses, with the exception of hydroxyproline, were performed by the chromatographic method of Moore et al. (18) on columns of Amberlite IR-120, and determined quantitatively by the method of Rosen (19). Hydrolyses were carried out in sealed tubes with 6 N hydrochloric acid, at 110° for 16 hours. The hydrochloric acid was evaporated with a stream of air.

Hydroxyproline was measured by the Neuman-Logan method (20), itself a modification of the method of Langheli (21). Our results were reproducible to ±2%.

RESULTS

Ultracentrifugation—The acetic acid solutions of collagen were centrifuged in the cold after 24-hour dialysis against acetic acid of the same strength; sedimentation patterns showed only one component (kindly performed by Mr. Edward Kline, Armed Forces Institute of Pathology, Walter Reed Army Medical Center). Fig. 2 shows a representative picture from the 0.1 M acetic acid solution.

Chromatography—Fig. 3 shows the chromatographic elution patterns of tendon collagen solutions ranging from 1.0 M down to 0.005 M with respect to acetic acid. A forepeak (or forepeaks) and three incompletely separated components are consistent features of all the chromatograms; the material in these last three peaks of all chromatograms was not ultrafilterable through cellulose (Visking, with two atmospheres of nitrogen), and upon hydrolysis yielded all the amino acids found in unhydrolyzed collagen. There is a single forepeak in the more dilute acetic acid solutions; at higher concentrations of acetic acid, larger amounts of material are chromatographed in the forepeaks which now assume varying patterns (Fig. 3). The forepeaks of the 0.005, 0.05, 0.10, and 0.25 M acetic acid solutions were composed of low molecular weight material; these isolated fractions were completely filterable through Visking cellulose. Preliminary attempts at fractionation of the 0.1 M acetic acid forepeak on Amberlite IR-120 showed that it is multicomponent.

The forepeaks from the 0.50, 0.60, 0.75, and 1.00 M acetic acid solutions could not, for the most part, pass through the ultrafilter (only 8% for the forepeak of the 1.0 M solution passed through). Thus, the concentration of acetic acid near 0.25 M represents a critical one, at, or above which, major changes occur in the dissolved collagen, which are reflected in the chromatograms by a progressive increase in the “forepeak.” Reference to Table I, column 4, shows this clearly. The “forepeak” comprises only 0.8% of the total in the 0.05 M solution, but 88% of the total in the 1.0 M. Hydroxyproline content of the forepeak(s) is another interesting feature of these chromatograms. Column 5 of Table I shows that the “classical” hydroxyproline content of collagen is approached (although not reached) only in those solutions above 0.25 M. A study of the amino acid composition of the various forepeaks is now underway. Results of analysis of the 0.1 M preparation are shown in Table II.

Since the acid concentrations had so marked an effect on the components produced when dissolved rat tail collagen was chromatographed at 40°, we thought it important to determine whether they might also be influenced by the length of time the tendons were in contact with the acid. An experiment was performed with 0.1 M acetic acid allowed to remain in contact with the tendons for varying times, from 10 minutes up to 148 hours at room temperature. Aside from a slight regular increase in the
total amount of collagen dissolved, the chromatograms were essentially identical.

In all the chromatograms, the cold collagen solutions were first heated to 40° when they were applied to the columns, that temperature being maintained during the 7-hour runs. A third series of experiments was performed to determine the effect, if any, of this temperature alone on the chromatographic pattern. Tendons were solubilized in 0.1 M acetic acid as before, and the solution kept sterile in a test tube at 40° for 78 hours. Chromatography was performed on aliquots at 6-hour intervals in the usual manner at 40°. The first three patterns were identical; during the next 30 hours, there was a slight continuing increase of the height of the forepeak while the last three fractions remained unchanged; after this time the pattern of the last three components became smeared, and at 78 hours no fractionation occurred.

In a number of runs, we carried out the entire procedure steriley up to the chromatography itself. All solutions used in these procedures gave negative results when cultured for aerobes and anaerobes on thioglycolate broth at 37° in room air, and for fungi on Sabouraud's broth at 37° in room air. These chromatograms were identical to the ones in which no sterile precautions were taken.

**DISCUSSION**

An accumulating body of evidence from physical studies indicates that dissolved collagen gives rise to a polydisperse system...
Fig. 3. Chromatographic elution patterns of acetic acid solutions of rat tail tendon collagen. Conditions were as described in the text; the abscissa represents optical densities of color obtained for 2-ml fractions by the modified Lowry method.

**TABLE I**

<table>
<thead>
<tr>
<th>Acetic acid molarity</th>
<th>TN</th>
<th>HP N (as % of TN)</th>
<th>&quot;Forepeak&quot; N (as % of total chromatogram N)</th>
<th>&quot;Forepeak&quot; HP N (as % of TN)</th>
<th>HP N of 3 last fractions (as % of their TN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>0.475</td>
<td>8.3</td>
<td>58.0</td>
<td>7.4</td>
<td>8.5</td>
</tr>
<tr>
<td>0.75</td>
<td>0.475</td>
<td>8.0</td>
<td>74.0</td>
<td>7.1</td>
<td>8.9</td>
</tr>
<tr>
<td>0.60</td>
<td>0.480</td>
<td>8.1</td>
<td>56.5</td>
<td>7.8</td>
<td>9.0</td>
</tr>
<tr>
<td>0.50</td>
<td>0.540</td>
<td>8.1</td>
<td>59.0</td>
<td>7.5</td>
<td>8.4</td>
</tr>
<tr>
<td>0.25</td>
<td>0.475</td>
<td>8.2</td>
<td>21.3</td>
<td>4.7</td>
<td>7.0</td>
</tr>
<tr>
<td>0.10</td>
<td>0.415</td>
<td>8.5</td>
<td>3.6</td>
<td>3.8</td>
<td>7.0</td>
</tr>
<tr>
<td>0.05</td>
<td>0.515</td>
<td>7.3</td>
<td>3.8</td>
<td>0.7</td>
<td>7.2</td>
</tr>
<tr>
<td>0.005</td>
<td>0.360</td>
<td>6.9</td>
<td>14.2</td>
<td>3.7</td>
<td>7.5</td>
</tr>
</tbody>
</table>

"heavy" components prepared by Tomlin and Turner (8) did not differ appreciably in their electrophoretic velocities from one another or from the unfractionated material. Our own results with rat tail tendon, we think, amply confirm the observations that more than one type of molecule arises from mildly heated collagen solutions, and provides a tool for the ready separation of these components.

Our experiments suggest that the determining factor involved in the production of the components from rat tail tendon collagen heated and chromatographed on carboxymethyl cellulose at 40\(^\circ\)C, is the concentration of acetic acid of the various solutions of collagen, since all the solutions were otherwise treated identically. Seventy-two-hour dialyses at 3\(^\circ\)C of the 0.1 M and 1.0 M acetic acid collagen solutions in a Visking cellulose bag against acetic acid of corresponding concentration had no effect on the subsequent chromatograms. This suggests that the small molecular weight forepeak obtained by chromatography at 40\(^\circ\)C is integral with, or bound to, the dissolved protein. The ultracentrifugal sedimentation patterns of the same cold starting solutions also showed only one component and were not changed if the samples had previously been dialyzed. Chromatography of the solutions at any temperature below 40\(^\circ\)C was not possible.

Bacterial processes seem to play no role in our procedure. It seems reasonably certain that hydrolysis also played no significant part in the production of the components, since our chromatographic procedure required only 7 hours and the forepeak at lower acetic acid concentration was completely eluted in as little as 10 minutes. The solutions retained their chromatographic properties after being kept at 25\(^\circ\)C for 148 hours and changed only slightly up to 48 hours at 40\(^\circ\)C. After 48 hours at 40\(^\circ\)C the chromatograms were smeared; this may be due to hydrolysis. Boedtker and Doty (7) found what seems to have been a similar process of hydrolysis for ichthyocol dissolved in pH 3.7 citrate buffer, the viscosity of which fell slowly with time when the material, heated to 36\(^\circ\)C, was kept at that temperature for over 40 hours.

The process by which the fractions are created seems to be an "all or none" one, since our time experiment showed that the characteristic four fraction pattern appeared at the earliest time we could manage to complete the steps preliminary to chromatography (10 minutes). The only observable change for the next 48 hours was a slight consistent increased solubilization and a concomitant increase in all four fractions. Such an "all or none" process was also suggested by Boedtker and Doty (7) for the breakdown of dissolved ichthyocol in warm citrate buffer.

The forepeak obtained from the relatively dilute acetic acid solutions of collagen, followed by acid hydrolysis.
solutions (below 0.25 m) is interesting in that it contains few amino acids, and that its hydroxyproline content is so low. It is difficult to imagine this component as a "contaminant," since it has such large amounts of glycine and proline. Elastin, a likely candidate, has these amino acids in high concentration, but has several other amino acids which are not found in the forepeak, e.g. alanine, leucine, and phenylalanine. Further, elastin has very little aspartic acid, aspartic, or glutamic acid, which are prominent in the forepeak. This fraction may represent an easily detachable portion of the collagen structure, and probably is composed of a number of small peptides and amino acids.

Three basic questions emerge from these results: (a) by what internal process is the collagen fractionated? (b) What is the source of the forepeak obtained from warm acetic acid solutions below 0.25 m, and (c) what is the nature of the process that occurs at an acetic acid concentration between 0.25 m and 0.50 m, that produces the different and great quantities of forepeaks and that leads to the disappearance of most of the three last peaks?

With respect to the chromatographic separation of the last three components, we have no evidence for the nature of the bonds cleaved, nor for the structural features upon which the fractionation depends. These features may be physical, e.g. molecular weights, or chemical, e.g. differences in frequency of occurrence of amide groups. Preliminary analysis indicates that the three last fractions almost certainly differ slightly in amino acid composition. Gallop et al. (23) have recently suggested the existence of ester-like bonds within ichthyocoll which give rise to nondialyzable components when split with hydroxylamine or hydrolyzed in such a way that peptide bonds are not disrupted. It is possible that such bonds are also involved in the phenomena which we are discussing.

With respect to the second question, our results point to a multicomponent protein which gives rise to a hydroxyproline-poor fraction, weakly bound to our adsorbent, and three tightly bound hydroxyproline-rich fractions. It is possible that those portions of the structure which have the least chance to form hydroxyproline, possibly the "youngest," are the least tightly bound. They may be mainly, or only, those involved in interchain hydrogen bonding, postulated by Weir and Carter (24) to be the sole bonds involved in thermal shrinkage. These workers calculated that imbibed water and thermal energy of heated tail tendons increase the interchain distances from 4.4 A to irregular distances of 11 to 17 A. The cross links break, and the unsup-producted chains then revert to a folded configuration of higher entropy.

The fractionation pattern was markedly dependent upon the acetic acid concentrations of the different rat tail tendon collagen solutions; below 0.25 m, the solution chromatographed at 40° gave rise to a small forepeak which is probably composed of lower molecular weight peptides, low in hydroxyproline. At the higher acid concentrations, the major part of the total chromatographable material appeared in the forepeaks which assumed varying patterns, and contained high molecular weight material.

Three of the other major components were high molecular weight fractions; the relative proportions of these components varied little with increasing acid concentrations, whereas their total amounts decreased steadily.

It has been demonstrated that the process producing these fractions is rapid, is not dependent on bacterial or fungal action, and apparently is not due to hydrolysis.

5. The significance of these components is discussed from the point of view of the structure of native collagen, particular attention being paid to the low molecular weight, low hydroxyproline component.

REFERENCES

Chromatographic Fractionation of Acetic Acid-solubilized Rat Tail Tendon Collagen
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