A Hydroxyproline-containing, Collagen-like Protein in Plasma and a Procedure for Its Assay

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The presence of dialyzable hydroxyproline-containing peptides in plasma (1) and urine (2-4) has been known for some time. Although their exact significance is not clear, the levels of these peptides in the absence of collagen or gelatin in the diet are a reflection of endogenous collagen metabolism (1-5). In a previous publication (6) it was shown that, in addition to free hydroxyproline and dialyzable hydroxyproline peptides, there is also present in human plasma a form of hydroxyproline which is nondialyzable, precipitable with protein precipitants, and released by acid or alkaline hydrolysis. The presence in plasma protein hydrolysates of material which interferes with the procedure for hydroxyproline assay made it difficult to quantify or characterize this protein. It has now been possible to modify the existing procedure (7) for hydroxyproline assay to make it sufficiently specific and sensitive for this purpose. With the modified procedure it has been found that plasma from laboratory animals and humans contains 6 to 14 μg per ml of protein-bound hydroxyproline. The behavior of this protein (hypro-protein) to ammonium sulfate precipitation and its migration on Sephadex columns suggest that it is a large molecule. Its resistance to digestion by chymotrypsin and Pronase suggests further that the protein may be a form of collagen.

**EXPERIMENTAL PROCEDURE**

**Materials and Methods**

Hydroxy-L-proline-5-3H·HCl, 750 mc per mmole, was obtained from New England Nuclear Corporation. Unless otherwise specified, determinations were carried out on human plasma prepared either from outdated blood obtained from the blood bank at the National Institutes of Health or from fresh heparinized blood obtained in the fasting state. Plasma was obtained from the blood of animals which had been fasted overnight (except the mouse). It was drawn by intracardiac puncture under light ether anesthesia with heparin as anticoagulant. When necessary, plasma was stored at -20°.

Bio-Rad analytical grade cation exchange resin AG 50-W-X8 (H+, 200 to 400 mesh) was washed successively with 10 volumes of 6 N NaOH, water to pH 7, 10 volumes of 6 N HCl, and water to pH 5. It was stored under water until used. The chromatographic columns were 30 × 1.1 cm with a 50-ml reservoir. Sephadex G-200, bead form, 4- to 20-μ diameter, (Pharmacia, Uppsala, Sweden) was used for the gel filtration experiments.

Δ The abbreviation used is: hypro-protein, the hydroxyproline-containing protein(s).

Pronase was *Streptomyces griseus* protease, B grade. Chymotrypsin was pancreatic α-chymotrypsin, B grade. Both were obtained from California Corporation for Biochemical Research.

**Determination of Hydroxyproline**—The isotope dilution procedure of identification and assay of hydroxyproline was described in a previous communication (6).

Colorimetric determination of hydroxyproline was by Procedure II of Prockop and Udenfriend (7). This involves conversion to pyrrole, extraction into toluene, and reaction of the pyrrole with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) to yield a red chromophore. When samples to be assayed contained less than 3 μg of hydroxyproline, as in the effluent fractions from gel filtration experiments, the following adaptation of the colorimetric assay was used.

Of the final toluene extract containing the pyrrole derived from hydroxyproline, 9 ml were mixed with 2.0 ml of Ehrlich's reagent. After 30 minutes at room temperature, when the color was maximal, 0.6 ml of water was added. After vigorous shaking the mixture separates into two phases, the chromophore appearing in the lower, water-ethanol-sulfuric acid phase. The tube was centrifuged for 2 minutes, the upper phase was removed by aspiration, and 5.0 ml of ethyl acetate were added. After shaking and centrifugation, the ethyl acetate was removed by aspiration. The aqueous phase (about 0.6 ml) containing the chromophore was transferred to a microcuvette and its absorbance was measured at 500 μm. The absorbance of blanks carried through the entire procedure was found to range from 0.100 to 0.190 depending on the purity of the *p*-dimethylaminobenzaldehyde. The absorbance of 1 μg of hydroxyproline carried through the procedure is 0.375 ± 0.025 (above the blank) and color is proportional to concentration up to 3 μg.

**Protein Precipitation and Dialysis**—Ethanol was used routinely to precipitate plasma proteins. Absolute ethanol, 4 ml, at 0° was added to 1.0 ml of plasma. After 15 minutes at 0°, the mixture was centrifuged at 4° and washed twice with 2.0 ml of 80% ethanol at 0°. Precipitations with tungstic and trichloroacetic acids were carried out in the usual manner and the precipitates were washed twice with the precipitating solution. In the dialysis experiments, 1-ml aliquots of plasma were dialyzed for 24 hours at 4° against deionized water. The retentates were evaporated to dryness, suspended in water, and aliquots assayed for protein and hydroxyproline. Protein was assayed by the procedure of Lowry et al (8). Absorption spectra were determined on a Cary model 11 MS recording spectrophotometer.
RESULTS

In the initial studies, acid hydrolysates of plasma proteins were assayed by the colorimetric method of Prockop and Udenfriend (7). Although this procedure has been found to be specific for the determination of hydroxyproline in hydrolysates of purified proteins, it was found that with whole plasma hydrolysates it gave values which were much greater than those obtained by isotope dilution assay. There were many other indications that the colorimetric assay was measuring material other than hydroxyproline. The most convincing evidence emerged from a comparison of the absorption spectra obtained with authentic hydroxyproline and with those from plasma protein hydrolysates (Fig. 1). As can be seen, plasma hydrolysates exhibit absorption at 450 nm which is not present in the chromophore obtained with hydroxyproline or with pyrrole.

The absorbance at 450 nm was used in subsequent studies designed to characterize the impurity and to separate it from hydroxyproline. After preliminary attempts to separate the material absorbing at 450 nm by simpler procedures, ion exchange chromatography was tried. The system employed was the same as had been used previously for purification of hydroxyproline (3). Tritium-labeled hydroxyproline was added to a plasma protein hydrolysate before chromatography. As shown in Fig. 2, hydroxyproline isolated from plasma gave one peak on elution with 1 N HCl which coincided with the radioactive peak of the added tritium-labeled hydroxyproline. Plasma hydrolysates contained several additional components which reacted with Ehrlich's reagent. Two smaller components were eluted with 1 N HCl subsequent to hydroxyproline, but these reacted to give only chromophores absorbing at 450 nm. A third component required 3 N HCl for elution and gave a chromophore absorbing at both 450 nm and 560 nm. The bulk of the material which interfered with the hydroxyproline assay (absorption at 560 nm) could not be eluted from the column even with large volumes of 3 N HCl. A summary of the chromatographic separation of the 560 nm-absorbing components of plasma protein hydrolysates is shown in Table I. In this representative experiment only about 40% of the apparent hydroxyproline in the hydrolysates had the elution and absorption characteristics

![Fig. 1. Absorption spectra of chromophores from 12.5 μg of authentic hydroxyproline and protein hydrolysate of 1 ml of plasma. Chromophores were concentrated by adding 0.6 ml of water to the final ethanol-sulfuric acid-toluene-Ehrlich's reagent mixture of the colorimetric procedure, shaking the mixture, and separating the water-ethanol-acid phase; the latter was then diluted to 3.0 ml with ethanol.](image1)

![Fig. 2. Chromatographic separation of hydroxyproline from the interfering material of plasma protein hydrolysate. Plasma proteins from 5 ml of plasma were precipitated with ethanol and hydrolyzed with alkali as described in the text (see Section 1 of "Results"). After neutralization and removal of BaSO₄ by centrifugation, the supernatant solution was evaporated to dryness in a vacuum and dissolved in 5 ml of 1 N HCl; a tracer quantity of L-hydroxyproline-5-³H (81,000 c.p.m.) was then added. After removal of 1.0 ml for assay, 3.0 ml were placed on a column (1 X 5 cm) of cation exchange resin and eluted, first with 50 ml of 1 N HCl and then with 150 ml of 3 N HCl, 4-ml fractions were collected. Assays were by the colorimetric procedure (7).](image2)
of hydroxyproline. The spectral characteristics of the chromophore obtained with the hydroxyproline isolated from plasma protein hydrolysates (Peak I; 1 N HCl) are shown in Fig. 3. This spectrum is compared with the spectra obtained with the chromophores of pure hydroxyproline and of Peak II; 3 N HCl. It can be seen that the latter absorb mainly at 450 μm and shows only a shoulder in the region 500 to 560 μm. By contrast the chromophore obtained with Peak I has the characteristic absorption of the chromophore obtained with authentic hydroxyproline.

Further evidence that the peak eluted with 1 N HCl represents the chromophore obtained with authentic hydroxyproline. The spectral characteristics of the chromophore obtained with Peak I has the characteristic absorbance of the chromophore obtained with authentic hydroxyproline. It can be seen that the latter absorbs mainly at 450 μm and shows only a shoulder in the region 500 to 560 μm. By contrast the chromophore obtained with Peak I has the characteristic absorption of the chromophore obtained with authentic hydroxyproline. Further evidence that the peak eluted with 1 N HCl represents the chromophore obtained with authentic hydroxyproline.

1. Procedure for Measurement of Hydroxyproline in Plasma Protein Hydrolysates (Hypro-protein Assay)—A specific procedure for assay of hydroxyproline was developed involving the chromatographic separation of hydroxyproline from the interfering substances in plasma proteins. This procedure will be referred to as hypro-protein assay.

Aliquots of plasma, 1 ml each, were pipetted into 30-ml culture tubes and the proteins were precipitated by the addition of 4.0 ml of ethanol at 0° with mixing. After 15 minutes, the proteins were centrifuged for 15 minutes at 200 × g at 4°, the supernatant solutions were decanted and the proteins were suspended in 2 ml of H2O. A saturated solution of Ba(OH)2, 4 ml, was added and the proteins were hydrolyzed by autoclaving at 124° and 15 pounds pressure for 16 hours. The hydrolysates were neutralized by the addition of 1 drop of phenolphthalein and the dropwise addition of 6 N H2SO4 until colorless. At this point 0.2 ml of 6 N HCl was added, each volume equalized, and BaSO4 was removed by centrifugation at 1000 × g for 10 minutes. The supernatant solutions were placed on 1 × 5-cm cation exchange resin columns previously equilibrated with 1 N HCl. The BaSO4 precipitates were washed with 1 ml of H2O, and after centrifugation the washings were added to the columns. The total volume placed on the columns was 7 to 8 ml. After the solutions had passed through, 5 ml of 1 N HCl was added to each column and the effluents discarded. Then 20 ml of 1 N HCl were added and the eluates were collected in 70-ml culture tubes and the proteins were precipitated by the addition of 4.0 ml of ethanol. They were then assayed by the hypro-protein assay to obtain total nonprecipitable hydroxyproline. The value for free hydroxyproline was subtracted from the total value to obtain peptide hydroxyproline.

Details of the hypro-protein assay are described in the text (see Section 1 of "Results").

### Table I

<table>
<thead>
<tr>
<th>Material</th>
<th>No. of determinations</th>
<th>Hydroxyproline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>µg/ml plasma</td>
</tr>
<tr>
<td>Free hydroxyproline</td>
<td>6</td>
<td>1.5</td>
</tr>
<tr>
<td>Peptide hydroxyproline</td>
<td>5</td>
<td>0.6</td>
</tr>
<tr>
<td>Hypro-protein hydroxyproline</td>
<td>21</td>
<td>8.1</td>
</tr>
</tbody>
</table>

### Table II

Forms of hydroxyproline in human plasma

All determinations were on fresh plasma samples from normal young adult volunteers, aged 21 to 29.

Free hydroxyproline was measured by collecting the supernatant and washings after the ethanol precipitation of protein from 2 ml of plasma, removing the ethanol by evaporation at 50°, and applying the residue in water acidified to pH 1 to a column. Elution and colorimetric measurement were as described for the hypro-protein assay.

Supernatants and washings after the ethanol precipitation of proteins from 2 ml of plasma were hydrolyzed overnight in alkali after the evaporation of ethanol. They were then assayed by the hypro-protein assay to obtain total nonprecipitable hydroxyproline. The value for free hydroxyproline was subtracted from the total value to obtain peptide hydroxyproline.

### Fig. 3

Absorption spectra of chromophores from hydroxyproline and interfering material separated chromatographically from plasma protein hydrolysates. The chromophores obtained with material in Peak I and Peak II (Fig. 2) and with a standard hydroxyproline solution were concentrated as described in the legend of Fig. 1 and their spectra determined.
of the hydroxyproline in plasma was precipitated. On dialysis, 75% of the hydroxyproline was found in the retentate. Ethanol precipitation was chosen for routine use in the assay because of its simplicity. The distribution of the various forms of hydroxyproline in plasma is shown in Table II.

2. Comparison of Isotope Dilation and Hypro-protein Assay—Values obtained by the unmodified colorometric assay (7) were much higher than those obtained by the isotope dilution procedure (6). However, after chromatographic separation similar values were obtained by colorimetry and by isotope dilution. This is shown in Table III. The same values were also obtained whether acid or alkaline hydrolysis was used. Following acid hydrolysis all the hydroxyproline migrated as 4-hydroxy-l-proline on electrophoresis. When hydrolysis was carried out in Ba(OH)_2, epimerization occurred and comparable amounts of threo-L- and allo-D-hydroxyproline appeared on electrophoretograms. Although Ba(OH)_2 hydrolysis had obvious advantages preceding ion exchange chromatography, the epimerization seemed to create a problem. However, on further study it was found that both epimers gave exactly the same amount of color when carried through the hydroxyproline assay (7). Furthermore, even though the two compounds show partial separation on the cation exchange resin, both are quantitatively eluted with the 20 ml of 1 N HCl used in the hypro-protein assay.

3. Hypro-protein in Plasma and Serum of Various Animal Species—Hypro-protein was found in the plasma and serum of all animals investigated, the rabbit appearing to have the highest levels (Table IV).

Table III
Comparison of hypro-protein and isotope dilation assays of pooled human plasma proteins

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein Hydroxyproline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/ml</td>
</tr>
<tr>
<td>Plasma</td>
<td>68.5</td>
</tr>
<tr>
<td>Serum</td>
<td>65.0</td>
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</table>

Ammonium sulfate fraction

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein Hydroxyproline</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 to 40% saturated</td>
<td>24.0  37  5.7  79</td>
</tr>
<tr>
<td>40 to 50% saturated</td>
<td>1.3   2    0    0</td>
</tr>
<tr>
<td>50 to 60% saturated</td>
<td>22.2  34   0.7  10</td>
</tr>
<tr>
<td>60 to 100% saturated</td>
<td>11.0  17   0    0</td>
</tr>
<tr>
<td>Recovery</td>
<td>58.5  90  6.4  89</td>
</tr>
</tbody>
</table>

Table IV
Comparison of hypro-protein and isotope dilation assays of pooled human proteins

The hypro-protein assay is described in the text. The isotope dilution procedure (6) was briefly as follows. Hydroxyproline-5-3H (0.07 µc, 0.5 µg) was added to plasma protein precipitates and hydrolysis was carried out by autoclaving in alkali. a-Amino acids were removed by reaction with trinitrobenzenesulfonic acid; hydroxyproline was separated from proline by cation exchanger chromatography with elution in 1 N HCl. After desalting and concentration, hydroxyproline was subjected to high voltage electrophoresis at pH 1.9; strips of the radioactive hydroxyproline spots were eluted and the specific activity of hydroxyproline determined on each strip. The amount of hydroxyproline present in the original plasma could be calculated by the equation

\[ E = \frac{B(C/D) - 1}{A} \]

where \( E \) = plasma hydroxyproline, in micrograms per ml, \( B \) = labeled hydroxyproline added, in micrograms, \( C \) = specific activity of added hydroxyproline, \( D \) = specific activity of recovered hydroxyproline, and \( A \) = original plasma aliquot, in milliliters.

Table V
Fractionation of hypro-protein in serum by ammonium sulfate precipitation

Citrate human plasma was treated with thrombin. After standing at room temperature for 30 minutes, with stirring, the clot was removed by centrifugation at 600 X g for 10 minutes. Solid ammonium sulfate was added to the supernatant serum at 25°C to achieve the appropriate concentrations. After each addition the mixture was stirred for 20 minutes and centrifuged for 10 minutes. The precipitated protein was dissolved in distilled water, dialyzed overnight against distilled water at 4°C, and assayed for total protein and hydroxyproline.

* In these experiments hydrolysis was in 6 N HCl.
hypro-protein was precipitated between 0 and 40% saturation. Little if any was associated with fibrin; however, about 10% appeared at higher ammonium sulfate concentrations.

Fractionation of Serum by Sephadex Gel Filtration: When human serum was fractionated on a Sephadex G-200 column, two peaks of hypro-protein appeared (Fig. 4). A major peak, accounting for 55% of the hypro-protein, was eluted with the earliest serum protein fractions which Flodin (10) has identified as α2-macroglobulins and α- and β-lipoproteins. A second, smaller peak appeared in the later serum fractions which represent mainly albumin. This accounted for about 15% of the hypro-protein. There was also a small amount of hydroxyproline-containing material which trailed between the two peaks. About 89% of the total hypro-protein was recovered from the column.

Digestion with Proteolytic Enzymes: All the findings up to this point were consistent with the supposition that the hypro-protein is a form of collagen. Since native collagen is not attacked by the usual proteolytic enzymes, it was thought that studies with proteolytic enzymes would provide a more rigorous test of the similarity between plasma hypro-protein and collagen. On treating plasma for varying periods of time with chymotrypsin or Pronase, and then dialyzing the incubation mixtures, it was possible to hydrolyze 50 and 90% of the protein, respectively. With both enzymes the bulk of the hypro-protein remained resistant to dialysis. The data for one such experiment with Pronase are shown in Fig. 5. It can be seen that a small amount of hypro-protein was hydrolyzed initially but that 80% was resistant to proteolysis. This procedure provided a 20- to 30-fold purification of hypro-protein and is being used as a basis for further purification.

Attempts were made to use collagenase and trypsin but these enzymes do not function well in whole plasma and such studies must await purification of the hypro-protein.

DISCUSSION

It is apparent from these studies that the bulk of plasma hydroxyproline is in the form of protein (hypro-protein). Two questions that remain are (a) what is the nature of the interfering substances in plasma proteins which make it necessary to modify the hydroxyproline assay, and (b) what is the nature of the hypro-protein.

All assays were carried out in this laboratory except those marked with an asterisk, for which the values represent data taken from the reference cited.

![Graph](image-url)
With respect to the method of assay, the most widely used procedures for hydroxyproline involve oxidation and decarboxylation to pyrrole, followed by condensation with p-dimethylaminoazobenzaldehyde to yield a chromophore absorbing at 560 mp. The original Neuman and Logan procedure (11), although simple and reproducible when applied to pure solutions, presents difficulty when applied to complex mixtures owing to the uncontrolled concentration of reductants and the presence in the final reaction mixture of substances other than hydroxyproline which are capable of reacting with p-dimethylaminoazobenzaldehyde (e.g. indoles, tyrosine, imidazoles). The modification of Prockop and Udenfriend (7) stabilized the oxidation-reduction environment during the formation of pyrrole and separated the pyrrole from interfering substances before addition of the color reagent. This procedure continues to be reliable for urinary hydroxyproline peptides, for purified proteins (7), and for the assay of nonprotein hydroxyproline in plasma (1). In plasma protein hydrolysates, however, an interfering material was present which was not completely separated from pyrrole by the extraction of the Prockop and Udenfriend procedure. The chromatographic separation of this interfering material from hydroxyproline prior to the conversion to pyrrole and formation of chromophore is the basis of the modified procedure described. A comparison of plasma hydroxyproline values obtained by several modifications of the Neuman and Logan procedure is shown in Table VI. The enormous values obtained by the unmodified procedure represent large amounts of interfering material present in 5 to 15 times the concentration of authentic hydroxyproline in plasma proteins. The assay of hydroxyproline without removal of this interference would obviously be meaningless. In tissue, for example, where collagen is often estimated by hydroxyproline measurement in crude tissue hydrolysates, the presence of these interfering substances would invalidate measurements based on the Neuman and Logan colorimetric assay.

Although the precise nature of the interfering material is not known, the following characteristics have been established: it is associated with or is part of plasma proteins; it is precipitated with protein precipitants; and it is nondialyzable, is releasable by hydrolysis in strong acid or alkali, and is stable to nitrous acid treatment as performed by Hamilton and Ortiz (15). Furthermore, the material requires both oxidation and heating before it can be extracted into toluene and condensed with the color reagent. It so closely resembles hydroxyproline in these respects that it would have remained undetected but for the differences in absorption spectra and the discrepancy between colorimetric and isotope dilution assays. The distinctly different elution pattern of Peak II and the material remaining on the chromatographic column (Fig. 2) suggests the presence of more than one interfering substance. The strong affinity for the cation exchange resin suggests both a basic and an aromatic nature. It is possible that the carbohydrate moieties of the plasma proteins could be converted by hydrolysis to substituted pyrrole, pyrrolone, or pyrrolidine compounds which on oxidation and heating would yield materials capable of reacting with Ehrlich's reagent. The nature of these substances is being investigated.

At the present stage of its characterization, the hydroxy-protein material appears to be more than one substance. The major component, representing 60 to 80% of the total, appears to be of large molecular weight since it precipitates in the 0 to 40% ammonium sulfate fraction and is eluted with the 10 S macroglobulin peak from Sephadex columns. It is also resistant to hydrolysis by Pronase and chymotrypsin, suggesting sufficient tertiary structure to protect its peptide bonds from enzymatic hydrolysis. These properties are certainly consistent with those of collagen, and it will be interesting to attempt to relate this component to the "soluble" precursors of collagen synthesis already characterized (16). The minor component, representing 10 to 15% of the total, is of distinctly lower molecular weight since it is eluted from Sephadex columns with the 3 S albumin peak; also, the material appearing in the 50 to 60% ammonium sulfate fraction may represent this same component. The small amount of hydroxy-protein hydrolyzed by Pronase (Fig. 5) and chymotrypsin may represent proteolysis of this second component. If so, this component may represent a collagen fragment, perhaps a denatured collagen in the form of one of the subunits (16). It is conceivable but unlikely that both hydroxy-proteins represent smaller hydroxyproline peptides tightly bound to globulins or albumin; studies under way in this laboratory may answer these questions.

The significance of these hydroxy-proteins remains to be determined. They may represent collagen released from cells into the blood in the same way that intracellular enzymes are released. If so, it will be of interest to determine from which tissues they arise and whether they represent new collagen in the process of synthesis or a solubilization and release of older collagen. There is the suggestion that the former is more likely since the levels of hydroxy-protein in very young children are about twice the levels in adults. The excretion of dialyzable hydroxyproline peptides is also increased during growth (17). Thus a whole spectrum of collagen metabolites appears to be continually released from the tissues into the blood. These include (a) intact collagen or material of similar size and properties, (b) partly degraded collagen, (c) dialyzable peptides, and (d) free hydroxyproline. Both the relative and absolute amounts appearing in the blood may be a reflection of collagen biosynthesis and metabolism. Studies are in progress to determine the effects on the levels of plasma hydroxy-protein of several factors (e.g. hormones, tumor growth, and wound repair) which influence collagen metabolism, in order to ascertain whether measurement of hydroxy-protein may be of value in clinical diagnosis.

SUMMARY

A hydroxyproline-containing protein has been delineated in the plasma of man and several laboratory animals. A specific and sensitive procedure for the measurement of hydroxyproline in solutions as complex as hydrolysates of plasma proteins or tissue proteins is also described. The procedure is capable of removing a nonhydroxyproline chromophore which interferes with the existing assays for hydroxyproline. The possible nature of the interfering substance or substances is discussed. With the use of this procedure, the hydroxyproline-containing protein has been partially characterized. Gel filtration and enzymatic digestion indicate that it is a large molecule which is resistant to hydrolysis by chymotrypsin and Pronase. These characteristics are consistent with those of collagen and raise the possibility of a collagen-like protein in the blood. The implications of this possibility with regard to collagen metabolism are briefly discussed.

* Unpublished observations.
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A Hydroxyproline-containing, Collagen-like Protein in Plasma and a Procedure for Its Assay
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