ing with various specific globulins (*6*).

The production of antiserums with acrylamide gel used as the adjuvant and protein carrier is a simple method which results in rapid production of high-titer antibody. The antibody which is produced rapidly after immunization is more specific for macroglobulins than is the antibody obtained after continued immunization. Thus, with continued immunization, the titer may rise but the specificity is decreased (*8*).

MICHAEL WEINTRAUB*
SAMUEL RAYMOND
Pepper Laboratory of Clinical Medicine, University of Pennsylvania, Philadelphia 4

References and Notes

1. F. W. Putnam, *The Plasma Proteins* (Academic Press, New York, 1960).
2. S. Raymond, *Clin. Chem.* **8**, 455 (1962); ——— and Y. Wang, *Anal. Biochem.* **1**, 391 (1960).
3. Hyland Laboratories.
4. J. Lospalluto, J. Chegoriansky, A. Lewis, M. Ziff, *J. Clin. Invest.* **39**, 473 (1960).
5. R. A. Kehwich and P. L. Mollison, *Vox Sanguinis* **6**, 398 (1961).
6. We are indebted to Neva M. Abelson of the Pepper Laboratory for the serological data.
7. N. M. Abelson and A. J. Rawson, *Transfusion* **3**, 469 (1963).
8. We thank H. M. Rawnsley for advice and counsel in preparing this report and for making laboratory facilities available.

* Supported by the Medical Student Research Program of the U.S. Public Health Service.

21 October 1963

Collagen-Like Protein in Human Plasma

Abstract. *Human plasma contains hydroxyproline in a bound form that is nondialyzable, is precipitable with the usual protein precipitants, can be extracted with hot trichloroacetic acid, and is released on hydrolysis in 6N HCl. These properties resemble those of collagen and suggest that small amounts of this connective tissue protein (3 to 5 mg per 100 ml), or a very large peptide derived from it, normally circulate in the blood. The identification and analysis of hydroxyproline in hydrolyzates of plasma protein were based on isotope-dilution procedures.*

Collagen is the most abundant protein in animals, representing as much as 25 to 30 percent of the body protein. The unique abundance of hydroxyproline in collagen has made it possible to study collagen metabolism in the intact animal by measuring the amount of free and peptide-bound hydroxyproline in blood (*1*) and urine (*2*). The procedure developed for hydroxyproline assay (*3*) has been applied to protein-free filtrates and to dialyzates of plasma and yields values of about 1 to 2 μg of peptide-bound hydroxyproline per milliliter of plasma (*1*). The possibility of a circulating form of intact collagen occurred to us in the course of studies on the pathology of the endocardial fibrosis in patients with malignant carcinoid tumors (*4*). Such a possibility was also supported by the finding of Houck (*5*) that hydrolysates of whole plasma yielded much larger values for hydroxyproline than did protein-free filtrates. Further study revealed that normal human plasma contains a nondialyzable substance which is precipitable with several protein precipitants, can be extracted into hot trichloroacetic acid, and yields free hydroxyproline on hydrolysis in 6*N* HCl. These properties resemble those of collagen and suggest that small amounts of a collagen-like protein normally circulate in the blood.

Samples of human serum or plasma prepared from blood of normal volunteers in the fasting state were hydrolyzed directly for 3 hours in 6*N* HCl at 120°C. When assayed by the method of Prockop and Udenfriend (*3*), they contained from 15 to 25 μg of apparent hydroxyproline per milliliter. When plasma proteins were precipitated or purified by dialysis, the bulk of the apparent hydroxyproline was associated with the protein (Table 1). Other indications of the association of the hydroxyproline with protein were (i) precipitation by other protein precipitants including cold 5 percent trichloroacetic acid, tungstic acid, and barium hydroxide, (ii) migration with the plasma proteins on Sephadex-G-25 columns, and (iii) release during acid hydrolysis of plasma proteins at a rate comparable to that observed during hydrolysis of gelatin.

Up to this point identification of the hydroxyproline was based on the color obtained with Ehrlich's reagent in the standard hydroxyproline assay. Comparison of the spectra of the chromophores obtained with hydrolysates of plasma proteins and with authentic hydroxyproline showed that both had peaks at 560 mμ. However, the material from plasma also exhibited an absorption peak at 450 mμ which was not present in the spectrum of the authentic hydroxyproline chromophore. When the plasma proteins obtained by ethanol precipitation were extracted with hot trichloroacetic acid essentially as described for collagen (*6*) and then hydrolyzed, the apparent hydroxyproline content of material extracted was less than 50 percent of the total found by direct assay. Furthermore, the spectral characteristics of the chromophore formed in the assay more closely resembled those obtained with authentic hydroxyproline chromophore. Thus, plasma protein hydrolysates, unlike purified amino acid mixtures and hydrolysates of other proteins (*3*), contain material in addition to hydroxyproline which reacts with Ehrlich's reagent in the standard assay. To measure hydroxyproline in the presence of this interfering material, it was necessary to use isotope-dilution procedure.

Pooled human plasma (6 to 8 ml) was treated with four volumes of absolute ethanol and the protein precipitate was centrifuged. The supernatant was decanted and the precipitate was resuspended in four volumes of 6*N* HCl and hydrolyzed. A measured amount of radioactive hydroxyproline,

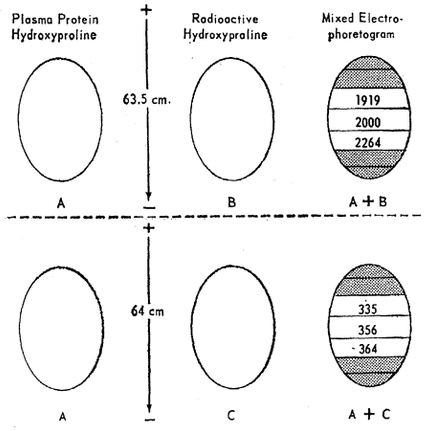

Fig. 1. Diagram of electrophoretograms of authentic and plasma-protein hydroxyproline. Samples containing plasma-protein hydroxyproline (*A*) (purified by column chromatography), tritium- (*B*) or C^{14}- (*C*) labeled hydroxyproline standards, or mixtures of *A* + *B* or *A* + *C* were placed in spots on Whatman No. 3 MM paper and subjected to high-voltage electrophoresis in 4 percent formic acid at 4700 v, 250 ma, for 4 hours at 20°C. The migration of all samples was identical with that of authentic hydroxyproline. Radioactive peaks coincided with hydroxyproline spots developed with an isatin-Ehrlich's spray. Specific activity (in counts per minute per microgram) is given for the actual strip from which it was determined. Strips represented by shaded areas contained radioactivity but there was insufficient material for colorimetric assay.

Table 1. Distribution of apparent hydroxyproline in human plasma.

Subject	Apparent hydroxyproline (μg/ml plasma)			
	Ethanol precipitate *	Supernatant after ethanol precipitation	Retained after dialysis †	Dialyzable
D.D.	16.7	0.8		
M.M.	16.9	1.0		
P.R.	16.4	1.5		
R.T.	18.0	1.0		
L.C.	22.5	0.9		
Pooled plasma ‡	17.4	1.5	14.4	3.0

* Absolute ethanol (2 ml) was added to 0.5 ml of heparinized plasma at 0°C. After 30 minutes the precipitate was separated by centrifugation at 4°C and the supernatant was decanted. The supernatants were evaporated to dryness and taken up in a measured volume of water. † Heparinized plasma (1 ml) was dialyzed twice against 250 ml of distilled water at 4°C for 24 hours. Dialyzates were evaporated to dryness and taken up in a measured volume of water. ‡ The plasma was obtained from "outdated" blood. Values shown represent the averages of from three to nine determinations.

either C^{14}- or tritium-labeled (*7*), was added to a sample of the hydrolysate. The subsequent procedures were then applied to samples, both with and without added labeled hydroxyproline. Primary amino acids were reacted with 2,4,6-trinitrobenzene-1-sulfonic acid by the method of Satake *et al.* (*8*); excess reagent and α-amino acid derivatives were removed on a Dowex-1 column (acetate form). Imino acids do not react with the reagent and pass through the Dowex-1 column. This effluent was desalted by adsorbing on a Dowex-50 (H^+) column, and then by eluting from the column with 1*N* ammonium hydroxide (*9*). Ammonia was removed by repeated evaporation to dryness and the residue was taken up in 1*N* HCl and chromatographed on a Dowex-50 column equilibrated and developed with 1*N* HCl (*9*). Hydroxyproline is eluted from this column very early and is completely separated from the much larger amounts of proline.

The fractions containing hydroxyproline were pooled, concentrated, and subjected to high-voltage electrophoresis on paper along with hydroxyproline standards. Radioactivity on the paper was detected in a windowless gas flow counter (Vanguard 4 pi). As shown in Fig. 1 all hydroxyproline samples migrated approximately 64 cm. To ascertain homogeneity, the hydroxyproline area in the mixed electrophoretogram (Fig. 1, $A + B$ and $A + C$) was cut into 0.6-cm strips; each strip was eluted with distilled water and the specific activity was determined (*10*). The constancy of specific activity within the hydroxyproline areas (Fig. 1) is indicative of homogeneity, and it established the presence of hydroxyproline in the protein hydrolysates. The amount of hydroxyproline in the plasma-protein hydrolysates could be calculated from the extent of dilution of the added radioactive hydroxyproline (Table 2).

The results of three separate isotope-dilution determinations on the pooled plasma indicated 6.4 to 7.3 μg of protein hydroxyproline per milliliter of plasma. In contrast, by colorimetric assay of the precipitated plasma proteins there was about 15 to 20 μg of apparent hydroxyproline per milliliter of plasma which confirmed the presence of some other Ehrlich-reacting substance or substances, and indicated the extent of the interference. Although colorimetric assay of hot trichloroacetic acid extracts (*6*) of plasma-protein precipitates yielded values close to those obtained by the isotope-dilution procedure, this agreement was partly coincidental since significant amounts of the impurity were also extractable by hot trichloroacetic acid and the extraction of hydroxyproline-containing protein was not complete. A tentative procedure based on hot trichloroacetic acid extraction of ethanol-precipitated proteins has been applied to plasmas from ten patients. The values obtained range from 3.5 to 6 μg of protein-bound hydroxyproline per milliliter of plasma and are generally comparable to those obtained by isotope-dilution assay of the pooled plasma. If the hydroxyproline represents collagen or a protein of similar amino acid composition, this value would indicate approximately 3 to 5 mg of the protein per 100 ml of plasma.

Tissue proteins other than collagen do occur in human plasma. Tissue enzymes such as the transaminases, amylases, and peptidases have been observed repeatedly in both normal and pathological plasma. The source of this circulating "collagen" is unclear and warrants further investigation. The source may be "leakage" from cells which are rapidly synthesizing collagen or may be the result of collagens slowly becoming soluble. Houck (*11*) observed that dermal injury led to the mobilization of collagen from distant uninjured sites where it was released presumably into the circulation and to the site of injury, thus implicating a circulating collagen in the processes of

Table 2. Isotope dilution assay of plasma-protein hydroxyproline in three separate samples of pooled plasma. Hydroxy-L-proline-5-H^3 hydrochloride was added to acid hydrolyzates of pooled plasma-protein precipitates. The final specific activity in each case represents the average of at least three determinations on elutions from paper strips; each strip contained 1.1 to 8.3 μg of hydroxyproline by colorimetric assay and 2000 to 12,000 count/min. The following equation was used to calculate the amount of authentic hydroxyproline (E) in plasma protein: $E = B[(C/D) - 1]/A$.

Plasma (ml)	Hydroxyproline			
	Amount (H^3-labeled) added (μg)	Specific activity (per μg)		Authentic, by isotope dilution (μg/ml)
		Initial (10^5 count/min)	Final (10^3 count/min)	
A	*B*	*C*	*D*	*E*
5.5	0.45	1.615	1.96	6.6
7.5	.48	1.695	1.469	7.3
7.2	.48	1.695	1.771	6.4

inflammation and repair. Zucker and Borrelli (*12*) have shown that connective-tissue suspensions and purified collagen produce platelet clumping which implies a relation between collagen and hemostasis. The cardiac lesions of malignant carcinoid (*4*) could result from the deposition of circulating collagen on the endocardial surfaces of the valves rather than from exudation of intracellular collagen. Finally, circulating soluble collagen may be a factor in the excessive fibrosis of many disorders of connective tissue.

H. KEISER, E. C. LEROY
S. UDENFRIEND, A. SJOERDSMA
Experimental Therapeutics Branch and
Laboratory of Clinical Biochemistry,
National Heart Institute,
Bethesda, Maryland

References and Notes

1. D. J. Prockop, H. R. Keiser, A. Sjoerdsma, *Lancet* **II**, 527 (1962).
2. S. Lindstedt and D. J. Prockop, *J. Biol. Chem.* **236**, 1399 (1961); M. Ziff, A. Kibrick, E. Dresner, H. J. Gribetz, *J. Clin. Invest.* **35**, 579 (1956); D. J. Prockop and A. Sjoerdsma, *ibid.* **40**, 843 (1961).
3. D. J. Prockop and S. Udenfriend, *Anal. Biochem.* **1**, 228 (1960).
4. W. C. Roberts and A. Sjoerdsma, *Am. J. Med.*, in press.
5. J. C. Houck, personal communication.
6. S. M. Fitch, M. L. R. Harkness, R. D. Harkness, *Nature* **176**, 163 (1955).
7. *L*-Hydroxyproline-5-H^3-HCl, 750 mc/mmole, New England Nuclear Corp., Boston, Mass. Uniformly labeled C^{14}-DL-hydroxyproline 0.2 mc/mmole, supplied by Dr. C. Mitoma.
8. K. Satake, T. Okuyama, M. Ohashi, T. Shinoda, *J. Biochem. Tokyo* **47**, 654 (1960).
9. B. Peterkofsky and S. Udenfriend, *Biochem. Biophys. Res. Commun.* **6**, 184 (1961).
10. B. Peterkofsky and D. J. Prockop, *Anal. Biochem.* **4**, 400 (1962).
11. J. C. Houck, *Clin. Proc. Children's Hospital, Dist. Columbia* **17**, 243 (1961).
12. M. B. Zucker and J. Borrelli, *Proc. Soc. Exptl. Biol. Med.* **109**, 779 (1962).

8 October 1963