22. H. Henney is a U. S. Public Health predoctoral fellow; National Institutes of Health fellowship No. 1F1 GM-20, 586-01. Work supported by U. S. Public Health grants AI-04431-02 and AI-04431-02S2 and by a grant from the University of Texas Research Institute.

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Antiserums Prepared with Acrylamide Gel Used as Adjuvant

Abstract. *Antiserums to macroglobulins were prepared by a convenient and time-saving procedure in which acrylamide gel was used as adjuvant. After electrophoresis in acrylamide gel, the macroglobulin zone from the gel pattern was homogenized and injected directly into rabbits without intermediate isolation of the purified antigen. The resulting antiserums exhibited high titers of antiserum to macroglobulin and showed only weak cross-reactions with other gamma globulins in serologic tests.*

The preliminary isolation and purification of protein antigens, which is usually required before they can be used for the preparation of specific antiserums, is laborious, time-consuming, and often wasteful of starting material (*1*). The purified antigen is usually mixed with an adjuvant to stimulate an increased antibody response. We describe here the application of acrylamide gel as the isolation medium and as the adjuvant in a technique which is simple, time-saving, and economical of material.

Acrylamide gel is a hydrophilic polymer of acrylamide, cross-linked with methylene-*bis*-acrylamide. The monomers are reported to be neurotoxic, but the gel itself has not shown evidence of toxicity. The use of acrylamide gel as an electrophoresis medium of high resolution was described previously (*2*).

A standard acrylamidegel electrophoresis pattern, prepared from 0.2 ml of serum from a patient with Waldenstrom's macroglobulinemia, demonstrated a macroglobulin zone adjacent to the sample slot. This zone was excised from the unstained pattern, homogenized in an iced Potter-Elvejem tissue grinder, and injected into the posterior thigh muscles of a female albino rabbit. The 7 ml of homogenate injected contained a calculated dose of 0.03 g of macroglobulin and 0.35 g of acrylamide. Seven days after injection, the antibody response was 1750 μg of antibody nitrogen per milliliter against macroglobulin eluted from another sample of the gel. Similar results were obtained in three other rabbits. Controls injected with gel alone produced no detectable macroglobulin antibodies.

Repetition of the injections of the gel-antigen combination for a 3-week period not only increased the antibody titers, but also stimulated the formation of antibodies to γ-globulin, presumably from the small amount of this component which was present in the macroglobulin zone. Six injections were given, totaling 0.09 g of macroglobulin and 1 g of acrylamide gel. Antibody titers of 3140 μg and 960 μg of antibody nitrogen per milliliter were obtained in two rabbits 10 days after the last injection. Controls receiving 1 g of acrylamide gel alone, according to the same time schedule, produced no demonstrable antibody.

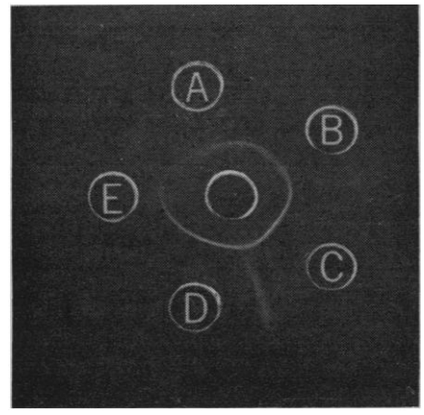
The antibodies reacted strongly in the Ouchterlony plate when the macroglobulin was used as antigen (Figs. 1 and 2). A commercial antiserum to macroglobulin (*3*) was placed in one antiserum well, and serum from each rabbit separately in other antiserum wells. The center well contained either macroglobulin prepared by diethylaminoethyl column chromatography (*4*) or material eluted from a gel electrophoresis pattern. The band formed by the commercial antiserum to macroglobulin (horse antiserum to human macroglobulin) was identical to the band formed by the rabbit antiserums. This identity was seen with both macroglobulin preparations tested, but the bands were heavier and sharper with the macroglobulin eluted from the gel. No components of the antiserums to β-globulin, α-globulin, or albumin were found on the Ouchterlony plates in which whole serum was used in the antigen well.

Table 1. Endpoints of titers obtained when rabbit antiserums were tested against coated human red blood cells.

Coating on red blood cells	Antiserum rabbit No.		Control antiserums*	
	24	E3	19S	7S
Anti-Rh (7S)	32	2		>512
Anti-Fy^a (7S)	4	0		>128
Anti-Le^a (19S)	16	16	16	Trace
Incomplete anti-HC′	4	0	2	0
Uncoated cells	0	0	0	0

* These antiserums are described by Abelson and Rawson (*7*).

Information about the nature of the antibody to γ-globulin produced by prolonged immunization was obtained from serologic data. Serums from rabbits 24 and E3 were tested with human red blood cells coated with blood-group antibodies of the 7S class (anti-Rh and anti-Fy^a) and of the 19S class (anti-Le^a) (*5*). They were also tested with human red blood cells sensitized to incomplete anti-H in the presence of complement, C′. The results (Table 1) indicated that both serums contained, in addition to antibodies to macroglobulin, small amounts of antibodies react-



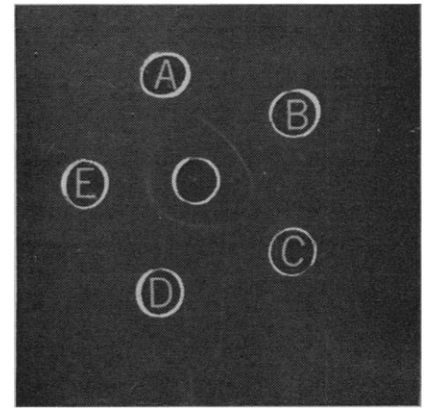


Fig. 1 (left). Photograph of Ouchterlony plate. *A*, Rabbit 21 (after 3-week course of immunization); *B*, rabbit 24 (after 3-week course of immunization); *C*, horse antiserum to human macroglobulin; *D*, rabbit 21 (after 3-week course of immunization); *E*, rabbit B20 (control—received gel alone); *center*, macroglobulin prepared by diethylaminoethyl column chromatography. Fig. 2 (right). Photograph of Ouchterlony plate. *A*, Rabbit B20 (control—received gel alone); *B*, horse antiserum to human macroglobulin; *C*, rabbit E3 (one immunization injection given 1 week previously); *D*, horse antiserum to human macroglobulin; *E*, rabbit 24 (after 3-week course of immunization); *center*, macroglobulin eluted from a gel pattern.

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*).

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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,

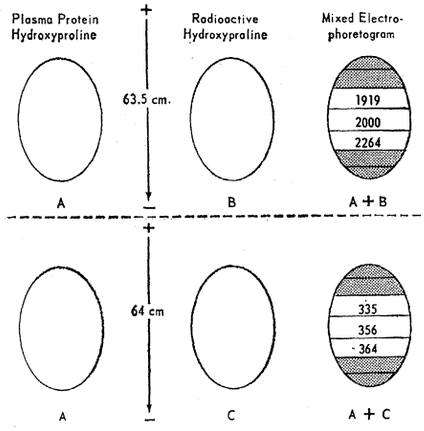

Fig. 1. Diagram of electrophoretograms of authentic and plasma-protein hydroxyproline. Samples containing plasma-protein hydroxyproline (*A*) (purified by column chromatography), tritium- (*B*) or C^{14}- (*C*) labeled hydroxyproline standards, or mixtures of *A* + *B* or *A* + *C* were placed in spots 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.