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10. I would like to thank C. R. Bentley for furnishing unpublished data. He, G. P. Woollard, E. S. Robinson, J. K. Sparkman, and R. M. Iverson greatly assisted me in obtaining the original data and calibration information for the gravimeters used. The U.S. Geological Survey made the 1966 observation; the earlier data were observed by the Univ. of Wisconsin. Work supported by NSF. The U.S. Navy provided logistic support. Publication authorized by the Director, U.S. Geological Survey.

7 December 1966

Fibrinogen from Human Plasma: Preparation by Precipitation with Heavy-Metal Coordination Complex

Abstract. *Potassium tetrathiocyanato-(S)mercurate II* [$K_2Hg(SCN)_4$] *is used in a mild and rapid procedure for the isolation of human fibrinogen from fresh plasma. The final product, 94 to 99 percent of which is coagulable by thrombin, represents an average yield of 80 percent and is stable in solution. It is free of plasmin, streptokinase-activatable plasminogen, and coagulation factors II, V, VIII, X and XIII. Sedimentation analysis reveals a single peak with a sedimentation coefficient equal to 7.0*S *at infinite dilution. Immunodiffusion on cellulose acetate results in two precipitin lines with rabbit antiserum to whole human serum. The fibrinogen precipitates are unusual in that they are flocculent and readily redissolve.*

The techniques commonly used for the isolation of fibrinogen from blood plasma often result in relatively low yields (*1*, *2*). The final product is unstable in solution and is invariably contaminated with plasminogen and other coagulation factors, especially fibrin-stabilizing factor (FSF). Furthermore, the conditions used are distinctly nonphysiological, and the protein precipitates obtained are difficult to dissolve and have a tough rubber-like consistency. We report a new method for the isolation and purification of fibrinogen with the use of a heavy-metal coordination complex as a reversible protein precipitant. Many of the problems associated with prior techniques are absent from our procedure.

We now report on our use of mercury thiocyanate anions previously reported as an "interesting" specific precipitant of fibrinogen (*3*). (i) In our study fresh plasma (200 ml) was separated from whole blood collected on Dowex 50W-X-8 (sodium cycle) resin with a Cohn fractionator; the plasma was collected in a plastic vessel containing 10 g (wet weight) of barium sulfate (*4*) and enough ϵ-aminocaproic acid so that its concentration in the plasma was 0.1 mole/liter. The plasma was gently agitated during the collection to insure dispersion of the barium sulfate and dissolution of ϵ-aminocaproic acid. Stirring was continued for 1 hour at room temperature or overnight at 2°C. (ii) After the $BaSO_4$ was removed by centrifugation, the plasma supernatant was treated with 5 g of triethyl-aminoethyl-cellulose (wet weight) per 50 ml of plasma. (The cellulose was put on the hydroxyl cycle by exposure to 0.5*M* NaOH, extensively washed with H_2O to neutrality, and then collected by vacuum filtration on a Büchner funnel.) The suspension was stirred intermittently for 10 minutes at room temperature and then centrifuged for 15 minutes at 8000*g*. The cellulose precipitate was washed with one plasma volume of a solution containing 0.15*M* NaCl and 0.1*M* ϵ-aminocaproic acid, *p*H 7.2. The treated plasma and wash solution were pooled (the pool volume was approximately twice that of the starting plasma). (iii) The diluted TEAE-treated plasma was adjusted to *p*H 7.2, with 0.1*M* sodium acetate, *p*H 4.0, cooled to 0°C, and made 4 m*M* in $K_2Hg(SCN)_4$ by the addition of the appropriate volume of a 50 m*M* solution (*5*). The resulting suspension was allowed to stand for 1 hour at 0°C with occasional mixing and then centrifuged for 15 minutes at 8000*g*. (iv) The fibrinogen-containing precipitate was washed twice with 50 ml of cold buffer (0.15*M* sodium acetate, 0.1*M* ϵ-aminocaproic acid, *p*H 6.5) to remove occluded protein. The washed precipitate was then readily dissolved at room temperature in 25 ml (one-eighth of the plasma volume) of a solution containing 0.3*M* NaCl and 0.1*M* ϵ-aminocaproic acid, *p*H 7.2. (v) In order to remove the $K_2Hg(SCN)_4$, the solution (25 ml) was passed through a column of Sephadex G-25 (70 ml) with a solution of 0.3*M* NaCl, *p*H 7.2, as the mobile phase. The column eluate, monitored at 254 mμ, had two peaks. The initial peak contained all the protein, and the second peak contained the coordination complex which absorbed in the ultraviolet. The protein-containing fraction was rendered free of detectable Hg^{++} by the addition of Chelex-100 (4 g/100 ml) (*6*). The resulting solution, after removal of the resin by centrifugation, was the final product and had a protein concentration of 0.7 to 1.2 percent.

If the plasma was not immediately treated with $BaSO_4$ and TEAE as described, the solution was cooled to 0°C and allowed to remain at this temperature overnight. The resultant cold-insoluble fibrinogen (CIF) was collected by centrifugation at 8000*g*, a procedure necessary to insure the complete removal of any CIF formed due to action of thrombin. When fresh plasma was treated immediately with $BaSO_4$ and TEAE, the entire procedure required a maximum of 8 hours.

The final preparation was 94 to 99 percent coagulable by thrombin, as determined by the method of Blombäck and Blombäck (*2*), an average recovery of 80 percent of the fibrinogen present in the starting plasma. The fibrinogen solution obtained by our method has

shown no visible signs of instability and no loss of coagulability when stored for several weeks at +4°C. No fibrinolytic activity nor streptokinase-activated hydrolysis of benzoylarginine ethyl ester has been detected. Assays for coagulation factors revealed (*7*) the complete absence of factors II, V, VIII, X, and XIII.

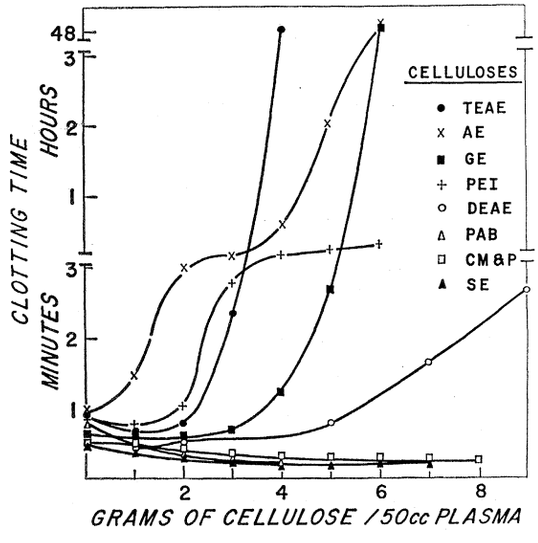
Factors VII and XI were found in small amounts (3 and 5 percent, respectively, of those in the original plasma). No assays were made for factors IX and XII. The lack of factor XIII was determined by the complete solubility of the fibrin clot in 5*M* urea or in 1 percent monochloroacetic acid with calcium and cysteine in the clotting mixture (*8*). Electrophoresis on cellulose acetate, with barbiturate buffer (*p*H 8.6; ionic strength, 0.07), resulted in a single band. Immunodiffusion and immunoelectrophoresis on cellulose acetate, by the serial-dilution technique of Saravis (*9*), revealed a single precipitin line against rabbit antiserum to human fibrinogen, and two precipitin lines against rabbit antiserum to whole human serum. Sedimentation analysis revealed a single peak with a sedimentation coefficient $s_{20,w} = 7.0S$, extrapolated to infinite dilution (*10*).

Each step of the procedure has been checked thoroughly, and the stated conditions are those which gave optimum purity accompanied by optimum yield. The ε-aminocaproic acid is present in the plasma collection vessel, as well as in all solutions, in order to prevent the activation of plasminogen (*11*), to inhibit the fibrinogenolytic action of plasmin (*11*), and to aid in the separation of plasminogen from fibrinogen (*12*). At *p*H 7.2, ε-aminocaproic acid is essentially isoelectric and does not contribute to the ionic strength of the medium (*13*).

There is a direct correlation between the appearance of CIF and the action of thrombin on fibrinogen (*14*). It is, therefore, imperative to remove completely both prothrombin and thrombin as quickly as possible to prevent the formation of CIF. This is accomplished by the immediate treatment of the starting plasma with $BaSO_4$ and TEAE. The most effective prothrombin adsorbent of the available anion-exchange celluloses is TEAE (Fig. 1). However, exposure of whole plasma to $BaSO_4$, or to TEAE alone, was not sufficient for the removal of trace amounts of prothrombin. Furthermore, the TEAE treatment resulted in the complete removal of factors VII, IX, and XIII.

Other isolation methods yield rubbery fibrinogen precipitates, and a blender has usually been used to aid in resolution at elevated temperatures (37°C). Our technique is characterized by flocculent precipitates that readily redissolve with a minimum of handling at room temperature.

Initial studies indicate that the Hg^{++} cation, when added to plasma as $HgCl_2$, is much less selective than the $Hg(SCN)_4^{--}$ anion in its ability to precipitate fibrinogen. Thus, when $HgCl_2$ in water is added to plasma a final concentration of 4 m*M*, the precipitated protein, containing nearly all the plasma fibrinogen, is only 15- to 20-percent coagulable by thrombin. By contrast, under identical conditions $K_2Hg(SCN)_4$ gives a precipitate which contains 80 percent of the plasma fibrinogen and is 70- to 75-percent coagulable (Fig. 2). The proteins precipitated by $HgCl_2$ are relatively insoluble in solutions containing various anions, whereas those precipitated by $K_2Hg(SCN)_4$ are by comparison readily dissolved by solutions. The solubilizing ability of the anions studied is as follows: $SCN^- > I^- > Br^- > Cl^- > NO_3^- > F^- >$ acetate$^-$. The association constants of these anions with mercury follow a series of similar order (*15*). In considering the complex anion as the precipitating species, there is no



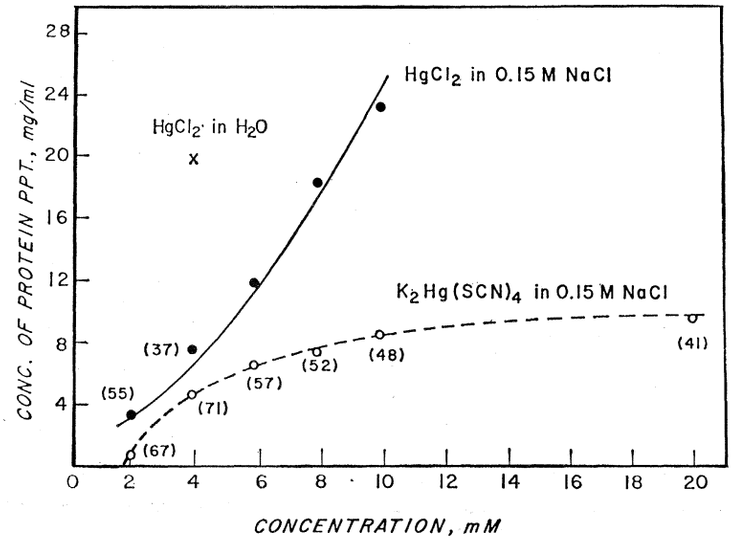


Fig. 1 (left). The relative binding rates and capacities of the various celluloses for human prothrombin. The adsorbents were equilibrated, and various amounts were added to fresh plasma in successive increments of 1 g per 50 ml of plasma. After the plasma was mixed for 10 minutes, centrifuged supernatants were assayed for clotting times by the addition of thromboplastin and calcium. There is a change in the time axis marked by the horizontal lines. Abbreviations: TEAE, triethylaminoethyl; AE, aminoethyl; GE, guanidoethyl; PEI, polyethyleneimine; DEAE, diethylaminoethyl; PAB, paraaminobenzyl; CM, carboxymethyl; P, phosphoric acid; and SE, sulfoethyl. Fig. 2 (right). Effects of increasing the millimolar concentration of $HgCl_2$ and $K_2Hg(SCN)_4$ on the precipitation of proteins from plasma. The values are expressed in milligrams of protein per milliliter of whole plasma, and the values in parentheses indicate the percentage of thrombin-coagulable protein.

evidence to eliminate the possibility that the $Hg(SCN)_4^{--}$ anion is acting as an Hg^{++} buffer and that the precipitating species is the Hg^{++} cation.

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20 December 1966

Sarcoplasmic Reticulum of Striated Muscle: Localization of Potential Calcium Binding Sites

Abstract. *Fish branchial muscle stained at a low* p*H with thorium dioxide shows localization of the stain over the sacroplasmic reticulum. Binding of the positively charged thorium micelles with dissociated acid groups of polyanions in this region suggests a possible mechanism for the storage and release of divalent cations such as calcium.*

The importance of calcium ions in the contraction mechanism of striated muscle has been established (*1*). Membrane depolarization spreads to the interior of the fiber via the transverse tubular system (T-system) and causes the subsequent release of calcium ions from the sarcoplasmic reticulum (SR) into the sarcoplasm (*2, 3*). The calcium ions then activate the adenosine triphosphatase of the actomyosin system, and contraction occurs. Relaxation takes place when calcium reaccumulates in the SR (*4*). The exact mechanisms of these steps are not known. Depolarization may extend to the membranes of the SR via bridges between the transverse tubules and the terminal sacs of the SR located at the triads (*5, 6*). The subsequent release of calcium ions by the SR may or may not be immediately dependent on such a depolarization (see *7*). Also the steps involved in the uptake of calcium by the SR have not been clearly defined (*8*).

The data of Hasselbach (*9*) show that calcium ions are accumulated inside the vesicles of the SR, and recent studies by Weber *et al.* (*10*) suggest that part of this calcium exists in a bound form. The staining technique with thorium dioxide discussed in this paper demonstrates potential binding sites for calcium localized in the regions occupied by the SR.

Branchial muscle from *Fundulus grandis* was fixed for 90 minutes in cold, phosphate-buffered (*11*) 1 percent osmium tetroxide at a *p*H of 7.4. After being rapidly dehydrated through increasing concentrations of ethanol, tissue was embedded in a prepolymerized methacrylate mixture composed of three parts butyl- and one part methylmethacrylate. Thin sections were cut on a Model MT-2 Porter Blum microtome and stained with colloidal thorium dioxide (Thorotrast) (*12*). This technique is essentially similar to the classical Hale reaction (*13*) in that positively charged colloidal particles bind with acidic groups in the tissue. Since the staining is done at a *p*H of about 2, only strong acidic groups remain dissociated and, hence, free to interact with the positively charged thorium dioxide micelles.

Unstained control sections were transferred directly from the distilled water of the sectioning trough to copper grids. All sections were stabilized by a thin film of evaporated carbon before being examined at 60 kv with a Philips 200 electron microscope.

The overall morphology of the branchial muscle is not of the same high quality as that obtained with conventional preparations of striated muscle embedded in epoxy. In general, the epoxies are not as permeable to surface stains as the methacrylates, making them unsuitable for use here. Nevertheless, familiar features of striated-muscle fine structure are easily recognized in the methacrylate sections (Figs. 1 and 2), and the preservation is certainly adequate for relating sites of thorium localization to the muscle fine structure.

The longitudinal section of *Fundulus* branchial muscle (Fig. 1) shows that this moderately fast acting muscle has a well-developed SR with the triad located at the level of the Z-line. Although some of the mitochondria are quite long, they are not particularly numerous. These observations generally support Porter and Franzini-Armstrong's (*5*) correlation between ultrastructure and metabolic needs for skeletal muscles that contract rapidly but in short bursts.

Figure 2 shows localized thorium micelles over regions occupied by the sarcoplasmic reticulum. At this magnification it is difficult to ascertain whether these particles are directly associated with the membranes or whether they are dispersed over the spaces of the cisternae. A survey of images at higher magnifications, however, indicates a greater tendency for the particles to be directly associated with the membranes. That the micelles are seldom concentrated over the dilated terminal sacs of the SR further supports this interpretation. Also attesting to the high specificity of the stain for binding sites of the SR is the finding that neither the membranes of the T-system nor the mitochondrial membranes react with the colloidal thorium. Particles are, however, found over smooth-surfaced membranes that occu-