Separation and Molecular Weight Estimation of Coagulation and Fibrinolytic Proteins by Sephadex Gel Filtration.* (29177)

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The Sephadex gels act as molecular sieves allowing separation of protein or other mixtures and estimation of molecular size(1,2). These gels are composed of small insoluble granules prepared by crosslinking the polysaccharide dextran. Variations in porosity (pore size) are obtained by varying the degree of crosslinkage. The highly crosslinked gel, G-25, allows entrance into its granules of molecules of molecular weight up to approximately 4000; while the most porous gel currently available, G-200, retains materials of molecular weight up to 200,000. Molecules larger than the pore size pass directly through the gel-packed column, whereas those small enough to enter the grains are retained until replaced by eluent.

These studies were undertaken to see if any useful separation of the various coagulation proteins in plasma could be obtained by gel filtration.

Materials and methods: Human fibrinogen (low in profibrinolysin and fibrin stabilizing factor) was prepared by re-precipitation of Fraction I (Merck Sharp and Dohme) in 0.12 M epsilon amino caproic acid (EACA) at 4% ethanol concentration(3). The final fraction was dialyzed against imidazole buffered saline, pH 7.3, to remove traces of EACA. Final concentration was 0.3% fibrinogen. *Bovine fibrinogen* was Armour Fraction I dissolved to contain 0.3% fibrinogen. *Bovine thrombin* was Parke Davis Thrombin, Topical diluted with buffered saline to contain 50 units per ml. *Special thrombin* was prepared by dissolving 5000 units of Thrombin, Topical in 20 ml distilled water, adjusting the pH to 5.3 with 1% acetic acid, chilling, centrifuging and discarding the precipitate. The pH, salt and thrombin concentrations of the supernatant were adjusted to match those of bovine thrombin.

Coagulation factor assays have been described(4,5). Factor VII assay employed congenitally deficient plasma and factor VII + X assay employed asbestos filtered beef plasma. Fibrin stabilizing factor (FSF) was considered to be present if clots did not dissolve after soaking in 3 ml of 5 M urea for 18 hours. These clots contained 0.1 ml human fibrinogen + 0.1 ml 0.025 M $CaCl_2$ + 0.1 ml fraction + 0.1 ml special thrombin. Assays for *fibrinolytic factors* employed fibrin I^{131}(6,7) as substrate with the exception of those shown in Fig. 2. *Gel filtration* was performed by the method of Porath and Flodin (1,8). Eluent in most experiments consisted of 0.16 M NaCl in 0.004 M trihydroxyaminomethane (Tris) previously adjusted to pH 8.0 with HCl.

Results. G-200 plasma. Fig. 1 presents the distribution of coagulation factors in fractions obtained by Tris-saline elution of normal plasma from G-200. Fractions which emerged first contained fibrinogen, FSF, factor V and factor VIII. Factors XI and XII emerged next; small amounts of both were spread over many fractions. Factor IX emerged just before factors II, VII and X. Antithrombin and antifibrinolysin were the last to leave the column.

Small fraction volumes necessitated the use of clot lysis tests for localization of proactivator-profibrinolysin. Fig. 2 presents the clot lysis times of 2 sets of clots each containing 0.1 ml fraction + 0.1 ml SK. One set contained bovine fibrinogen + bovine thrombin, both known to contain profibrinolysin. The other set contained human fibrinogen, prepared poor in proactivator + profibrinolysin and special thrombin. Lysis of the human fibrin was confined to tubes containing fractions 21-30, whereas bovine fibrin clots containing fractions 16-35 lysed.

Paper strip electrophoresis was carried out on each fraction. Early fractions contained

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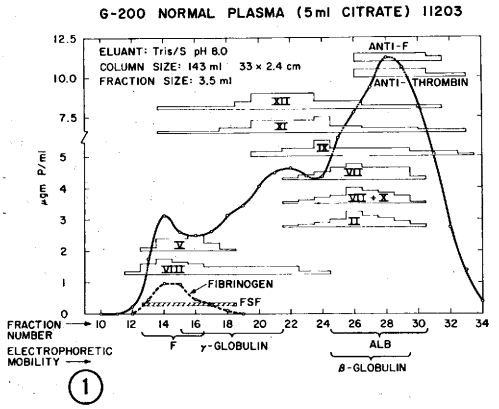
fibrinogen, then γ globulin. β globulin and albumin appeared abruptly in fraction 25. α globulin bands could not be distinguished.

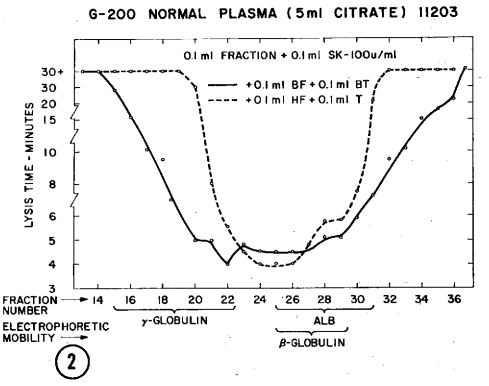
G-200, Fraction I. Fig. 3 illustrates results obtained when Fraction I was passed through G-200. Fibrinogen and factor VIII emerged together and profibrinolysin followed.

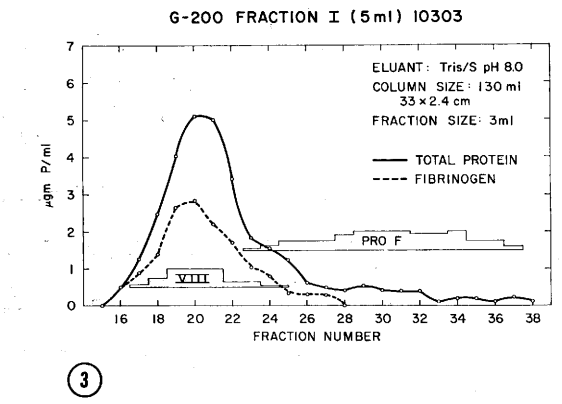
G-100 Plasma. Fig. 4 illustrates results obtained after passing whole plasma through G-100. All of the coagulation factors except II and VII + X emerged together.

G-75 Plasma. Fig. 5 illustrates the passage of plasma through G-75. All coagulation factors appeared at the same time.

Molecular Weights. Estimates of molecular weights obtained by gel filtration are compared to those obtained by other, more precise, methods in Table I. As all of the coagulation factors passed directly through G-75 their molecular weights are probably all







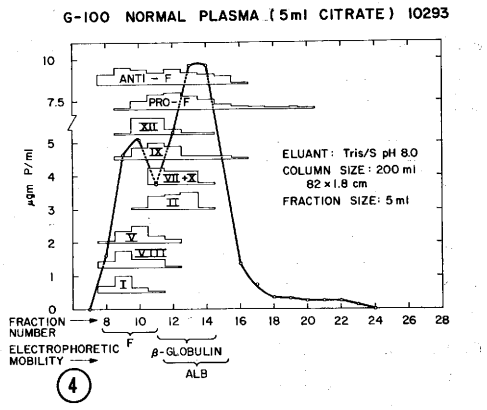


FIG. 1. Distribution of coagulation factors and protein concentration in fractions obtained after passage of normal plasma through Sephadex G-200.

FIG. 2. Lysis times of clots containing fractions described in Fig. 1.

FIG. 3. Distribution of fibrinogen, factor VIII and profibrinolysin after passage of Fraction I through Sephadex G-200.

FIG. 4. Distribution of coagulation factors after passage of normal plasma through Sephadex G-100.

FIG. 5. Distribution of coagulation factors after passage of normal plasma through Sephadex G-75.

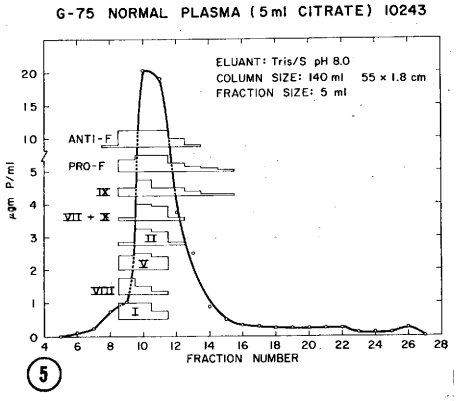


TABLE I. Estimation of Molecular Weight by Sephadex Gel Filtration.

Factor No.	Gel filtration estimate	Other estimate
I	>200,000	341,000 (9)
VIII	"	196,000 (10)
		180,000 (11)
V	"	
XIII (FSF)	"	
Plasminogen	100–200,000	143,000 (12)
		83,000 (13)
XI	"	
XII	"	
IX	"	110,000 (11)
II	50–100,000	68,900 (14)
VII	"	
X	"	

greater than 50,000. Factors I, V, VIII and FSF are excluded from G-200, suggesting molecular weights greater than 200,000. Only factors II, VII and X are retained by G-100 suggesting that their molecular weights lie between 50 and 100,000.

G-25. This gel excludes all materials of molecular weights greater than 4,000. We have used it to remove citrate, sulfate or oxalate from plasma and plasma fractions and unbound I^{131} from iodinated fibrinogen. The first fractions obtained after passage of urine through G-25 contain UP (urinary procoagulant) and UK (urokinase) free from pigments, salts, urea, etc.

Discussion. Considerable separation of the protein coagulation factors was achieved by G-200 filtration. Probably the most useful application will be in preparation of fibrinogen free from profibrinolysin. In this our results confirm the findings of Berg and Korsan-Bengtsen(15). The difference between the lysis times of human and bovine fibrinogen clots containing fractions 16-20 and 31-36 (Fig. 2) may be due to the presence of proactivator without profibrinolysin in these fractions.

Although molecular weight is not synonymous with molecular size, these two must correspond closely. Molecular weight estimates agree fairly well with those obtained by other methods. A series of gels of more closely graded porosity would be useful in further fractionations and molecular weight determinations.

G-25 proved particularly helpful in the rapid desalting of plasma fractions. These observations confirm the results of other investigators(1,16).

Summary. Gel filtration of plasma separated coagulation factors into 3 groups: 1) those with molecular weights greater than 200,000: I (fibrinogen), V (proaccelerin), VIII (AHF) and FSF, 2) those with molecular weights between 100,000 and 200,000: profibrinolysin and XI (PTA), XII (Hageman) and IX (PTC), and 3) those with molecular weights between 50,000 and 100,000: II (prothrombin), VII and X (Stuart).

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