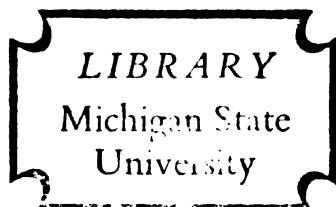




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STUDIES ON THE ISOLATION OF PROTHROMBIN
FROM HUMAN PLASMA FRACTION III
BY PAPER ELECTROPHORESIS

Thesis for the Degree of M. S.
MICHIGAN STATE COLLEGE
Robert Bastian Foy
1955



STUDIES ON THE ISOLATION OF PROTHROMBIN FROM
HUMAN PLASMA FRACTION III BY PAPER
ELECTROPHORESIS

By

Robert Bastian Foy

A THESIS

Submitted to the School of Graduate Studies of Michigan
State College of Agriculture and Applied Science
in partial fulfillment of the requirements
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MASTER OF SCIENCE

Department of Chemistry

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*

VITA

The author was born June 14, 1928. His secondary education was completed in 1946 at Cass City High School, Cass City, Michigan. In 1950 he was graduated from Central Michigan College of Education, Mt. Pleasant, Michigan, with the Bachelor of Science Degree. He was admitted to the School of Graduate Studies at Michigan State College in September 1953 and has been in attendance since.

Employment experience consists of positions as: Medical Technologist from 1950 to 1952 with the Clinical Laboratory at Hurley Hospital, Flint, Michigan; Graduate Teaching Assistant 1953 to 1955 in the Department of Chemistry, Michigan State College; and assistant director of the Clinical Laboratories since January 1955 at the Edward W. Sparrow Hospital, Lansing, Michigan.

He is a Junior member of the American Chemical Society, member of the Lansing Chapter of the Michigan Medical Technologists Society, associate member of Sigma Xi and a member of Alpha Phi Omega.

STUDIES ON THE ISOLATION OF PROTHROMBIN FROM
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ABSTRACT

Blood coagulation studies require the use of several enzyme preparations which are difficult to obtain in pure form. Prothrombin, which catalyzes the conversion of fibrinogen to fibrin, has been shown to be concentrated in Fraction III and III(2-3) by the alcohol methods of Cohn and Oncley. This study was undertaken to prepare purified prothrombin from these human plasma fractions by employing filter paper electrophoresis.

The horizontal strip technique, with apparatus modifications was first tested. Effective resolution of the crude plasma fraction components resulted. Staining with brom-phenol blue aided in the detection of resolved proteins. Quantitative isolation of prothrombin by elution of protein zones from unstained paper was not effected.

Continuous filter paper electrophoresis for isolation and purification of prothrombin was carried out on human plasma Fraction III(2-3). Preliminary treatment of this fraction gave a salt soluble extract which appeared to be about 95 percent one-component by Tiselius electrophoretic analysis. When this solution was treated by continuous paper electrophoresis and the cuts analyzed it was found that two principal components appeared. Prothrombin assays demonstrated activity in all cuts collected. There was no concentration of prothrombin in any sample over that of the material in the sodium chloride extract. The assays showed that the prothrombin activity resided in those cuts

obtained nearest the anode side of the paper. Comparison with Tiselius electrophoresis was made on those samples which afforded sufficient material after dialysis and lyophilization.

Inorganic chemicals have been used as adsorbents for the isolation and purification of prothrombin. Barium sulfate and barium carbonate were tried for further purification of one of the samples obtained by continuous paper electrophoresis. Under the conditions employed further purification of prothrombin was not effected.

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I. INTRODUCTION

Prothrombin is a pro-enzyme which is vitally a part of the blood clotting process. Its complete mode of action in this complex sequence of enzymatic events has not been entirely elaborated. A pure form of human prothrombin has not been readily available for studies on this mechanism. The initial problem was one of isolating a suitably pure preparation that could be utilized in blood coagulation studies.

During recent years two methods of prothrombin isolation have been used. One employs adsorption from plasma or modified plasma and the other utilizes iso-electric precipitation. Either of these involve long tedious procedures and require an ample supply of human blood. The availability of prothrombin-rich plasma Fraction III offered the advantage of using this source as a starting material.

Protein separations by filter paper chromatography and/or ionophoretic means have been applied to plasma. The simple apparatus required suggested the adaptation of filter paper electrophoresis to the purification of prothrombin. It was anticipated that this technique might provide a more easily accessible and reproducible source of this enzyme.

II. HISTORICAL

In connection with this investigation to improve the isolation of prothrombin it is desirable to review blood clotting, bases for prior isolation methods and how filter paper electrophoresis has been developed.

A. Mechanism of Blood Clotting

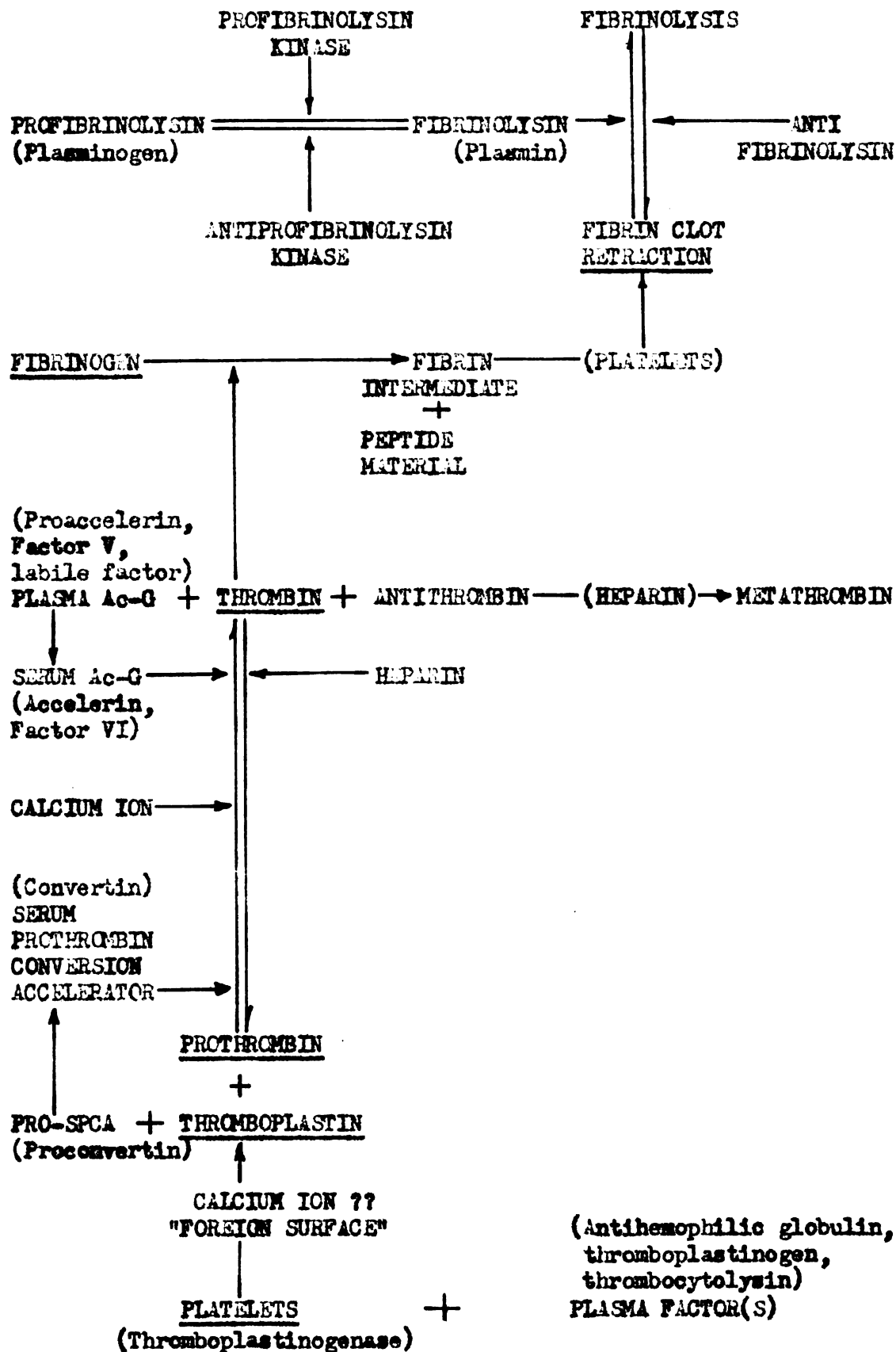
In the early 1900's the accumulated knowledge about human blood coagulation was formulated into what is now known as the "Classical Theory" of blood clotting (33). This mechanism is concerned with three basic phases but at present it is regarded as being much more complicated than originally assumed.

Briefly the triphasic mechanism is: 1) An activation of blood platelets with production of thromboplastin, 2) The resulting conversion of the zymogen, prothrombin, to the active enzyme, thrombin, and 3) The transformation of soluble fibrinogen to insoluble fibrin by thrombin. In addition to these basic steps there are several activator and inhibitor systems involved (1). Figure 1 elaborates the above and summarizes the presently regarded enzymatic reactions and biochemical factors concerned with the human blood coagulation process.

B. Isolation of Human Prothrombin

Early prothrombin preparations depended upon either a direct precipitation of the proenzyme or the preliminary removal of fibrinogen from plasma.

Figure 1. Schematic Representation of the Blood Clotting Mechanism



Mellanby (31) in 1909 recorded the first preparation of prothrombin and later offered a modification for its purification (32). The original procedure employed iso-electric precipitation of the globulins from diluted plasma brought to pH 5.3 with one percent acetic acid. The precipitate was resuspended in 0.70 percent sodium chloride and defibrinated with thrombin to yield a prothrombin-rich solution.

Seegers and co-workers (33) modified the above method by adsorbing prothrombin from diluted bovine plasma at pH 5.3 upon magnesium hydroxide and eluting with carbon dioxide. After dialysis and lyophilization the prothrombin preparation was stored with prolonged retention of activity. Seegers, et al. (39), elaborated their earlier directions with more details of procedure in 1945. Seegers and co-workers (40,41) have recently reported the application of these procedures to the isolation of human prothrombin.

Bordet and Delange (3) used twenty percent sodium chloride to suppress thrombin formation and then obtained crude prothrombin by dilution and defibrination of plasma. Howell (21) precipitated plasma proteins with acetone and dried the fractions for storage. When prothrombin was wanted the dry precipitate was extracted with slightly alkaline water and filtered. Cekada (5) removed plasma fibrinogen by heat coagulation and after acetone precipitation dried the prothrombin product with ether. Gratia (19) reported a preparation whereby plasma was inoculated with Staphylococcus aureus and incubated six to twelve hours. Removal of the resulting fibrin left an enriched prothrombin. Eagle (15) proposed the precipitation of a prothrombin-rich fraction

by bubbling carbon dioxide through cold diluted plasma. When centrifuged the active precipitate was peptized in 0.85 percent sodium chloride at pH 7.0.

Parfentjev (36) and Taylor and Adams (49) isolated a plasma globulin fraction with considerable clotting power by repeated precipitations with ammonium sulfate solutions. Orr and Moore (35) also produced a plasma globulin cut with potential thrombic activity by using Butler's (4) phosphate buffer.¹ Precipitation of active material occurred principally between 1.7 and 2.0 molar.

The urgent need for human plasma protein products during World War II accelerated development of the alcohol fractionation method as devised by Colm and co-workers (7,8). Their process involved the careful control of pH, temperature, protein and ethanol concentration and ionic strength. The resulting availability of various plasma protein fractions led Oncley, et al. (34), to investigate their sub-fractionation in order to concentrate prothrombin and any other active proteins of minute occurrence. They reported a preparation of human prothrombin from Fraction III with an activity of 30 units per mg.

A different preparation of human prothrombin produced by Surgenor and co-workers (46,47) involved adsorption upon barium sulfate from plasma of blood that was decalcified by collection over Dowex-50. The prothrombin was subsequently eluted with citrate solutions.

¹ Consists of equimolecular parts of potassium dihydrogen phosphate and potassium monohydrogen phosphate, pH 6.5, with the total ionic strength varying from 0.6 to 3.0 molar.

Certain physico-chemical information concerning bovine prothrombin has been reported (25,26), while the data on human preparations is incomplete. Samples of the latter have been reported to be polydisperse in the ultracentrifuge (42,46) and to have electrophoretic mobility comparable to that of the bovine prothrombin (40,42). Some evidence has been offered for the presence of tyrosine and tryptophane (42), while Lanchantin (27) has suggested the presence of cysteine but the absence of cystine in human prothrombin.

C. The Development of Paper Electrophoresis

Initially the whole field of paper chromatography and subsequent paper electrophoresis received its impetus from Tswett's original (44) experiments on the separation of plant pigments by adsorbents contained in a column. Other workers brought out different elaborations of this technique, such as solvents for elution (37) and frontal analysis (29). Some have applied electrical potentials across the ends of the column (10,13).

Integration of the previous research led to the development of zone electrophoresis. Zone electrophoresis involves the migration of charged components as separate zones in a supporting medium and gives the advantage of securing more complete separations. However, mobilities and iso-electric points are not generally determined by this method. The matter is complicated by adsorption on paper fibers and by electroosmotic phenomena.

One of the first reports about electrophoresis upon paper was that by Konig (23) in 1937. The first application to protein separations appeared in 1939 when von Klobusitzky and Konig (22) described the resolution of a yellow chromoprotein from a snake venom. During the decade of 1938 to 1948 paper chromatography and various electrochromatographic methods appeared to overshadow paper electrophoresis development. The introduction of moving boundary electrophoresis by Tiselius (50) in 1937 also delayed the development and acceptance of the paper work.

In the late 1940's various methods of paper electrophoresis were independently suggested from several laboratories. The simplest apparatus consisted of a circuit which was completed by dipping the ends of an electrolyte-moistened paper into vessels containing electrolyte and electrodes. The problems of supporting the paper to prevent buffer "pooling" and surface evaporation were two disadvantages of this setup.

For paper support there are basically three constructions. One is the glass plate method (11,24) whereby the moistened paper strip is placed between two glass plates which are sealed to minimize surface evaporation. A second construction, known as the gallows type (13,16), employs minimum contact by supporting the paper over a glass rod with the ends pulled outward and dipped into electrolyte. Thirdly, the box-type apparatus (2a,9) keeps the paper horizontal while giving minimum contact to the migration surface. The use of glass rectangles or raised pointed projections on a plastic surface provide support.

A weighted type or stretching frame may also be used to keep the paper in a taut, level position.

For control of temperature and surface evaporation the glass plate seems quite effective, but other setups provide a cover and refrigeration. Non-polar immiscible liquids such as chlorobenzene (9), heptane (2a) or carbon tetrachloride (11) act as sealing agents to aid in heat dissipation. McDonald (30) tried hydrogen and helium as water-saturated gaseous sealants.

Haugaard and Kroner (20) were first to express the idea of combining a flowing buffer with electrophoretic migration on paper. This led to the separation process known as "continuous electrophoresis". Simultaneously yet independently, Grassmann and Hannig (17,18) and Svensson and Brattsten (48) described an apparatus employing the above principles. More recently Durrum (14) and Larson and Feinberg (28) have presented some variations of the continuous electrophoresis apparatus. A notable difference in this continuous technique and other paper electrophoresis is that the slowly migrating components do not necessarily pass over the same path as do the faster moving components.

The principal construction is a filter paper sheet hung over the edge of a buffer vessel such that buffer flows continuously downward by capillarity. The protein mixture to be separated is supplied from a reservoir above to the top of the paper in a continuous manner by the aid of a filter paper wick. The lower edge of the paper is serrated to provide drip points for receiving vessels and the lower corners are

inserted into electrode vessels such that an electrical potential is horizontally applied across the paper.

The problem of surface distillation has been met by coating the edges of the paper with paraffin (51) or by clamping them with glass plates (45). Refrigeration has also aided in temperature control.

III. EXPERIMENTAL

A. Equipment

Horizontal Strip Apparatus -- The power supply unit (0-300 volts) was obtained from Ivan Sorvall, Inc., New York, labeled Type 3290A-LKD. Rods of 1/4 inch stainless steel and 3/16 inch carbon were used as cathode and anode, respectively. Whatman No. 1 filter paper was the supporting medium. Buffer wells were constructed from 3/8 inch Lucite sheets. One end of each vessel was provided with a tap which served to connect them by a 12 inch length of thin plastic tubing. Paper supports were either an 8 x 12 x 1/4 inch glass plate or a plastic frame with tension holders.¹ The housing unit consisted of an 18 x 30 x 4 inch box provided with a Lucite cover. Figure 2 illustrates the apparatus.

Continuous Flow Apparatus -- The power supply unit (0-750 volts) was Model E-800-2 sold by Research Equipment Corporation, Oakland, California. Platinum wire of 25 gauge served as electrodes. One hundred ml. tall-form beakers were the electrode vessels. These were provided with a tap near the bottom for connection by a small bore plastic leveling tube. Paraffin was applied (1/2 inch inward) to the long edges of a 6 x 16 inch sheet of Whatman 3 MM filter paper terminating at five inches from one end. At the untreated end the paper was cut to provide five drip points, each two cm. apart. The apparatus is shown in Figure 3.

¹ The frame was constructed and kindly made available for this work by Dr. J. R. Brunner, Dairy Department, Michigan State College.

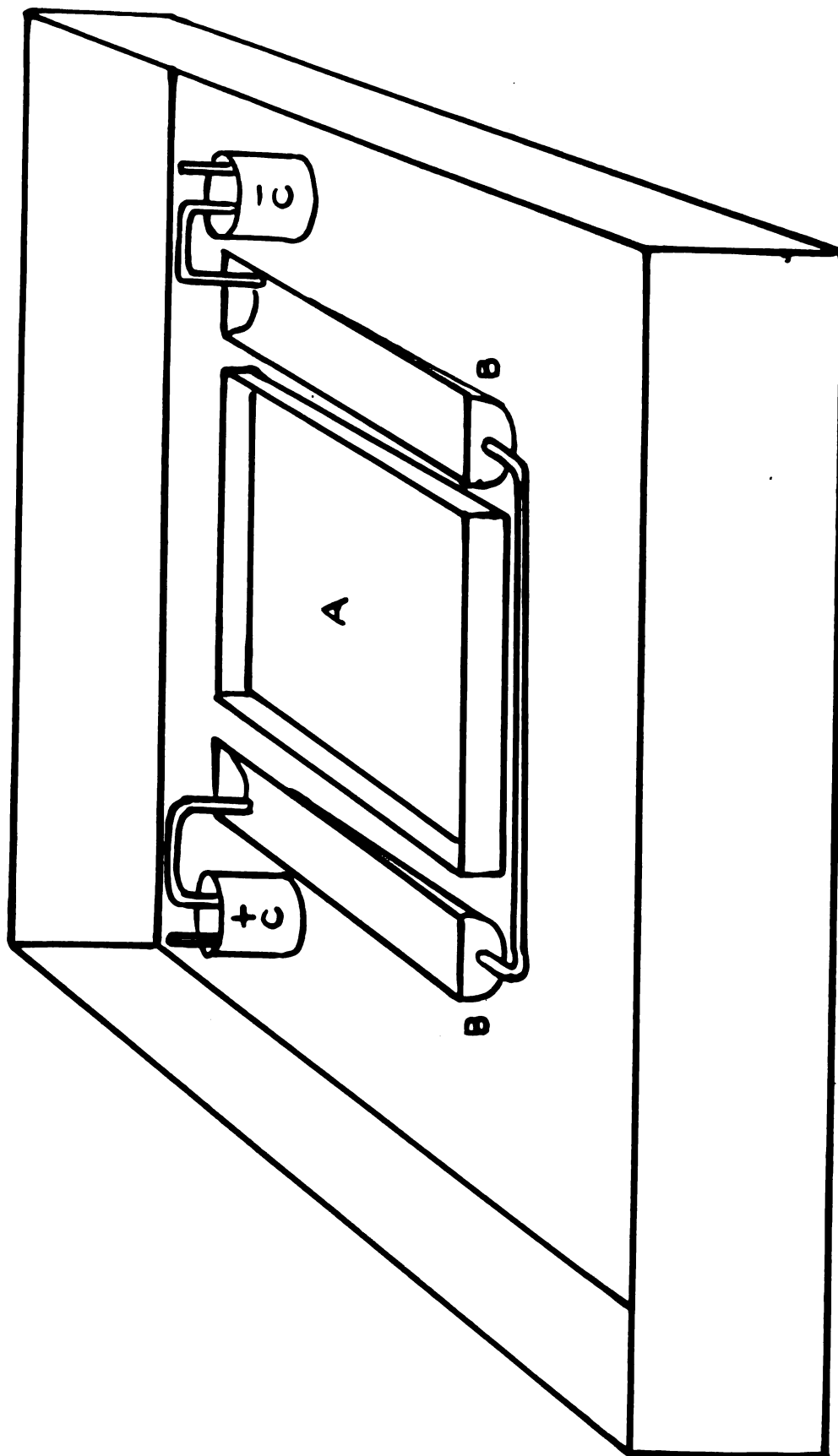


Figure 2. Horizontal Strip Apparatus

A. Water Reservoir

B. Buffer Wells

C. Electrode Vessels

Figure 3. Continuous Flow Apparatus

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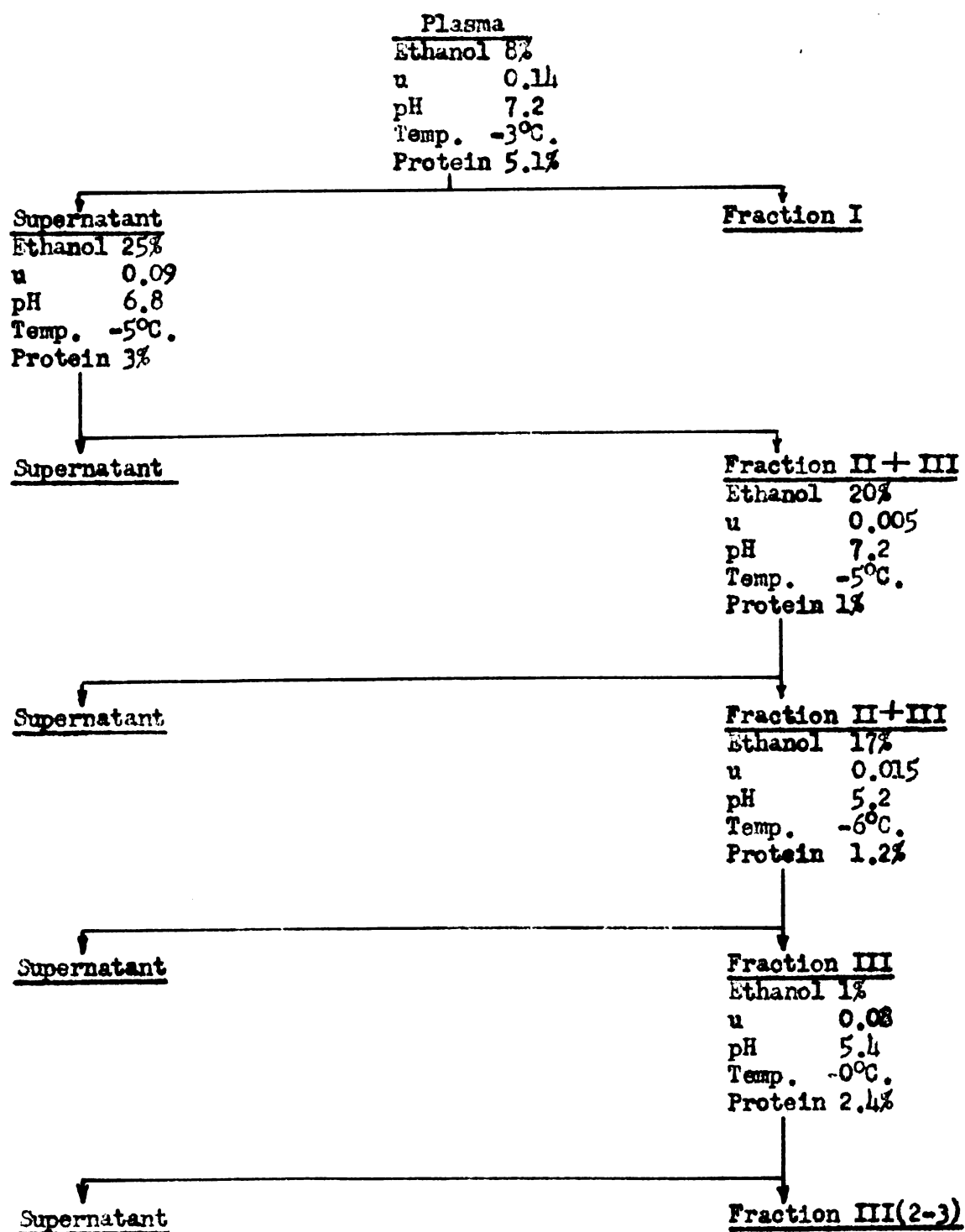
Moving Boundary Apparatus -- Schlieren diagrams and mobility data were obtained by use of a Tiselius electrophoresis instrument, the Perkin-Elmer Model 38. Conductivity measurements were made with a Model RC-1B Conductivity bridge manufactured by Industrial Instruments, Inc., Jersey City, New Jersey. The conductivity cell supplied by Perkin-Elmer had a cell constant of 0.4893. All dialysis was performed with the external rotating liquid dialyzer as constructed by Djang, Lillevik and Ball (12). The equilibration membrane was from Cenco, catalog number 70160-A-1-1/8. Lyophilizations were carried out with an all glass apparatus similar in construction to that advertised by the E. H. Machlett Co., New York. Centrifugations were accomplished with the Sorvall Swedish angle-head, Model 3420, or the Model V International Centrifuge manufactured by the International Equipment Co., Boston, Mass.

B. Reagents and Materials

Crude Starting Materials -- Fraction III and Fraction III(2-3) were received as frozen pastes after the alcohol fractionation of pooled human plasma. This process is summarized in Figure 4. For this investigation the crude fractions were provided by the Plasma Products Division of the Michigan Department of Health.¹ These fractions were prepared according to Method 9 (34) and Method 6 (7). Fraction III contained approximately 38.4 percent solids, of which about 37.1

¹ Supplied through the kind generosity of Drs. H. D. Anderson and K. B. McCall.

Figure 4. Diagrammatic Representation of the Preparation of Fraction III and Fraction III(2-3) According to Methods 6 and 9.



percent was protein. Fraction III(2-3) consisted of about 26.0 percent solids, of which 27.0 percent was protein.

Buffers -- Barbitol, U.S.P., and sodium hydroxide from Fisher Scientific Co. were used. In the continuous flow experiments glycerol, Baker, U.S.P., was added to the buffer to the extent of twenty percent (v/v). The glycinate buffer was prepared according to Oncley, and co-workers (34). In some instances Baker, C.P. sodium chloride was added to increase the ionic strength.

Electrode Bridges -- Agar agar, U.S.P. from Sargent, and the veronal buffer went into filling the bridges connecting the electrode vessels to the buffer wells.

Sealant -- Chlorobenzene from Eastman Kodak-Distillation Products, catalog number 70, was used in the horizontal strip studies.

Staining -- The dye solution for protein identification was prepared by dissolving one gm. brom-phenol blue (Nutritional Biochemicals Corporation) in one liter 95 percent ethanol saturated with mercuric chloride (Baker, C.P.). Acetic acid and sodium acetate in the rinse solutions were Baker, C.P. chemicals. The drying oven was Model C-400, from Research Equipment Corporation, Oakland, California.

Adsorbent for Prothrombin -- Mallinckrodt analytical reagent barium sulfate and barium carbonate were used.

Semi-micro Kjeldahl Analysis -- Procedures and reagents were followed according to Clark (6). Digestion and distillation equipment for these analyses was that as modified and used in this laboratory.

Prothrombin Assay -- The reagents were all prepared by the analyst at the Michigan Department of Health Laboratories.¹ The thromboplastin was of human placental origin. The prothrombin conversion mixture contained this thromboplastin, gum acacia,² imidazole buffer, sodium chloride and calcium chloride. A one percent (w/v) saline dispersion of human fibrinogen was used as substrate in the assay. The accelerator globulin (Ac-G) diluent was merely fresh bovine serum diluted 1:75 in 0.85 percent (w/v) sodium chloride. The standard thrombin curve (clotting time versus activity units) was prepared beforehand using the above reagents and a sample of thrombin supplied by the National Institute of Health (N.I.H.), Biologics Control Division, Bethesda, Md. One unit of thrombin activity is considered to be derived from one unit of prothrombin. A unit of thrombin has been defined as that amount of thrombin which will clot one ml. of fibrinogen in 15 seconds under standard conditions as given in detail in the Minimum Requirements of Dried Thrombin, Second Revision, September 1946, issued by N.I.H.

1 The analyses were performed through the fine cooperation and courtesy of Dr. L. A. Hyndman and Mr. H. Gallick.

2 This provided 0.68 percent (w/v) calcium.

C. Methods

Horizontal Strip Technique -- The 7 x 12 inch filter paper sheet is placed in the frame with the ends dipping into the buffer wells. One hour is allowed for equilibration with buffer as it moistens the paper by capillary action. The samples for paper electrophoresis are prepared to contain one percent (w/v) protein from Fraction III or Fraction III(2-3) in 0.05 molar veronal buffer of pH 8.6. A 200 μ l sample is applied at a point six cm. from the cathode end and one cm. in from each edge by streaking a filled capillary pipette across the end of the paper. Alternately one may place twenty μ l on each of ten spots 1.5 cm. apart across the end of the paper.

One-half hour is allowed for equilibration with buffer after application of sample. Current is then applied and adjusted to ten milliamperes with the voltage ranging from 250 to 300 volts. Running time varies from 12 to 24 hours. After each run the paper is dried and stained as subsequently described. The elution of protein zones from the unstained paper is accomplished after cutting the damp paper cross-wise into one cm. strips. The cuts start at one cm. on the cathode side of the sample origin and proceed toward the anode until ten one cm. strips are had. Each strip is placed into a beaker containing two ml. of 0.85 percent (w/v) sodium chloride and allowed to soak in the refrigerator one to two hours. The saline extract is then assayed for prothrombin activity (vide infra).

The zone separations achieved with this apparatus may be observed in Figure 8. All samples are in 0.05 M. veronal of pH 8.6 with an

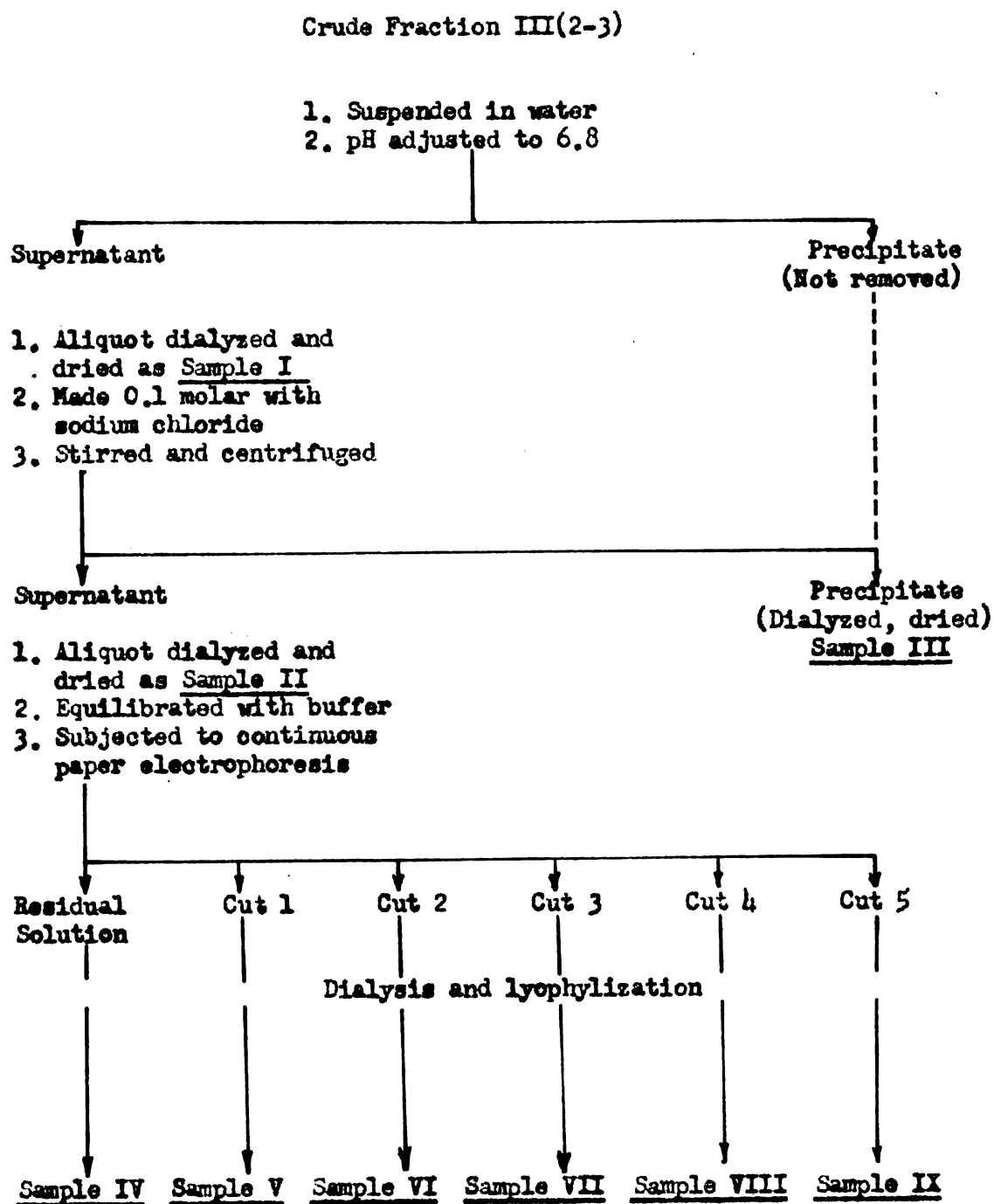
applied potential gradient of 9.8 volts/cm. The only difference in conditions for the samples shown is the length of running time.

Staining -- The method is a modification of the procedure of Jencks (26). The papers are dried for one-half hour in the chromatography oven at 100°C. The dye is sprayed on in just sufficient quantity to cover the paper without dripping. After air-drying overnight, excess dye is removed by two five-minute rinses in two percent (v/v) acetic acid and one rinse in 0.05 M. sodium acetate contained in two percent acetic acid. The paper is finally allowed to air-dry.

Continuous Flow Technique -- All operations from the beginning through dialysis and preliminary to lyophilization are carried out in the cold room at 0 to 5°C. The separation described as follows is schematically shown in Figure 5.

Four hundred and forty ml. of cold distilled water is added to 15 gm. of crude Fraction III(2-3) and allowed to stand for two hours. The dispersion is slowly agitated with a mechanical stirrer for five more hours. The pH is adjusted to 6.8 with a 0.5 M. glycinate buffer of pH 9.8 and about 0.65 ml. is required. The molarity with respect to sodium glycinate in this protein solution is about 9.4×10^{-7} . At this point a fifty ml. aliquot is dialyzed salt-free against distilled water, lyophilized and the residue stored in the deep-freeze labeled as Sample I. The remaining solution of about 400 ml. is treated with 100 ml. of 2.9 percent (w/v) sodium chloride. Since the glycinate concentration is negligible the addition of salt makes the

Figure 5. Separation Flow Sheet



total ionic strength practically 0.10 molar and the protein concentration approaches 0.9 percent (w/v). Following two hours of stirring the suspension is centrifuged 15 minutes at 1500 RPM to give a precipitate that is resuspended in twenty to thirty ml. distilled water, dialyzed, dried, stored and identified as Sample III.

A 100 ml. aliquot of the above supernatant is dialyzed, dried, stored and referred to as Sample II. The remaining supernatant (about 250 ml.) is dialyzed against four one-half hour changes of 0.05 M. veronal buffer, pH 8.6, which contains twenty percent by volume of glycerol. The equilibrated solution is then placed into the reservoir for continuous paper electrophoresis. To attain equilibrium the paper is kept in contact with buffer for six hours before application of protein solution and two hours prior to separation the current is turned on. The protein is admitted to the paper at the rate of 15 drops per minute but it can vary from 12 to 15 drops per minute. The full amount of 700 volts from the power unit is applied and throughout the run the current ranges from three to seven milliamperes.

After 24 hours the current is shut off and those solutions of this separation referred to as cuts 2 and 3 are dialyzed 12 hours against two changes of three to four liters of distilled water for removal of glycerol and salt. Each is lyophilized, labeled as Samples VI and VII, respectively, and stored in the deep-freeze. The small volumes in cuts 1, 4 and 5 were not dialyzed but were directly dried, labeled as Samples V, VIII and IX, respectively, and stored.

All samples are assayed for prothrombin activity and analyzed for nitrogen content. Electrophoretic patterns are obtained where the amount of sample is sufficient. Information regarding nitrogen content and enzymatic activity is given in Tables I and II. The Schlieren diagrams by Tiselius electrophoresis are shown in Figures 6 and 7.

Adsorption Experiment -- Two hundred mg. of Sample III is suspended in ten ml. distilled water and stirred for twenty minutes at room temperature. One hundred mg. of sodium chloride in two ml. of 0.10 M. veronal buffer of pH 8.6 is added and the stirring continued for one hour.

The mixture is centrifuged in the Swedish angle-head centrifuge and the precipitate discarded. One ml. of the supernatant is transferred to a tared bottle for protein estimation. The rest of the supernatant is placed into a test-tube containing two grams of barium sulfate and gently stirred ten minutes. The barium sulfate is removed by centrifugation. Again one ml. of the supernatant is placed in a tared bottle for protein estimation as is subsequently described.

The final supernatant is equilibrated with 0.10 M. veronal of pH 8.6 with 0.05 M. sodium chloride and electrophoretically analyzed.

A similar experiment was performed with 200 mg. of Sample III and two grams of barium carbonate.

Prothrombin Assay -- Assays are performed according to the directions of Warner, Brinkhous and Smith (53) as modified by Ware and Seegers (52). The procedure is as follows:

To 0.9 ml. of Ac-G diluent is added 0.1 ml. of a saline dispersion of the sample to be tested. The resulting one ml. is added to three ml. of prothrombin conversion mixture (P.C.M.) and allowed to undergo formation of thrombin at room temperature. At varying time intervals (about five minutes) a 0.4 ml. aliquot is added to 0.1 ml. of the fibrinogen substrate which indicates progress of conversion. With a stop watch the time of formation of the first visible fibrin clot is noted as the clotting time. An activity factor is obtained from the standard thrombin curve (clotting time versus thrombin units) using the clotting time of a thirty minute conversion aliquot. Thirty minutes is considered to give maximum conversion of prothrombin. The units of prothrombin in the original undiluted sample are then calculated as shown in Appendix I with Sample I as an example.

Protein Estimation -- An estimation of solids as protein is performed by placing a known volume of test solution in a tared weighing bottle and obtaining its total weight. The sample is dried at 103°C. for eight hours. Extractions are made as follows: 1-2 ml. of distilled water is added to the bottle and then heated for 15 minutes at 103°C. The supernatant is then carefully removed by a capillary tube saving the insoluble denatured protein. This procedure is repeated until an almost constant weight on the dried sample is obtained.

Preparation for the Tiselius analysis -- Samples are dissolved to one percent (w/v) in five ml. buffer. Dialysis is for one to two

hours with 100 ml. buffer at room temperature, then five to seven hours against a 200 ml. change of buffer at 0 to 5°C. and a final overnight equilibration versus 300 ml. fresh buffer at 0 to 5°C. The equilibrated sample if clear is now ready for analysis; otherwise it is centrifuged enough to clarify. All subsequent operational procedures are according to the Manual of Instructions as supplied by the instrument manufacturer.

TABLE I
YIELDS, PROTHROMBIN ACTIVITY AND NITROGEN CONTENT OF
SAMPLES STUDIED

| Sample ¹ | I | II | III | IV | V | VI | VII | VIII | IX |
|------------------------------------------------|------|------|------|-----------------|-----------------|------|------|-----------------|-----------------|
| Volume of Sample in Mls. | 50 | 100 | -- | 30 | 10 | 60 | 100 | 10 | 5 |
| Lyophilized Weight in Mgs. | 220 | 335 | 1820 | 72 ^a | 53 ^a | 75 | 104 | -- ^b | 24 ^a |
| Prothrombin Activity in Units per ml. Saline | 140 | 180 | 114 | 380 | 114 | 85 | 115 | 49 | 10 |
| Mgs. Nitrogen per ml. Saline | 1.40 | 1.78 | 1.00 | 2.39 | 1.72 | 0.99 | 1.13 | 0.88 | 0.41 |
| Prothrombin Activity in Units per mg. Nitrogen | 135 | 101 | 114 | 159 | 65.5 | 86 | 102 | 55.5 | 24 |
| % Nitrogen on lyophilized residues | 10.1 | 8.90 | 10.0 | -- | -- | 9.90 | 11.3 | -- | -- |

¹ Refer to Figure 5.

^a These are not glycerol-salt free weights.

^b Weight was not obtainable.

TABLE II
PROTHROMBIN ANALYSES

| RT | CT | Diln. | F | U/ML | RT | CT | Diln. | F | U/ML | RT | CT | Diln. | F | U/ML |
|------------|------|-------|------|------|-------------|-------|-------|------|------|------------|-------|-------|------|------|
| Sample I | | | | | Sample II | | | | | Sample III | | | | |
| 2 | 68.1 | 50 | -- | -- | 3 | 76.9 | 100 | -- | -- | 9 | 36.8 | 100 | 0.30 | 30 |
| 5 | 26.9 | 50 | 1.42 | 21 | 7 | 26.0 | 100 | 0.46 | 46 | 15 | 20.4 | 100 | 0.62 | 62 |
| 10 | 11.9 | 50 | 1.44 | 72 | 10 | 17.7 | 100 | 0.77 | 77 | 20 | 17.0 | 100 | 0.82 | 82 |
| 15 | 17.3 | 150 | 0.80 | 120 | 15 | 19.2 | 200 | 0.66 | 136 | 30 | 13.8 | 100 | 1.14 | 114 |
| 20 | 20.0 | 200 | 0.64 | 128 | 20 | 16.8 | 200 | 0.84 | 108 | | | | | |
| 30 | 18.9 | 200 | 0.70 | 140 | 30 | 16.0 | 200 | 0.90 | 180 | | | | | |
| Sample IV | | | | | Sample V | | | | | Sample VI | | | | |
| 3 | 28.7 | 100 | -- | -- | 5 | 35.4 | 100 | -- | -- | 5 | 60.1 | 100 | -- | -- |
| 5 | 14.5 | 100 | -- | -- | 10 | 19.0 | 100 | -- | -- | 10 | 28.5 | 100 | 0.29 | 39 |
| 15 | 19.0 | 400 | -- | -- | 15 | 15.5 | 100 | 0.95 | 95 | 15 | 20.7 | 100 | 0.61 | 61 |
| 26 | 16.5 | 400 | -- | -- | 20 | 23.9 | 200 | 0.49 | 98 | 23 | 16.6 | 100 | 0.64 | 84 |
| 28.5 | 15.5 | 400 | 0.95 | 380 | 30 | 21.4 | 200 | 1.14 | 114 | 25 | 16.7 | 100 | 0.65 | 85 |
| Sample VII | | | | | Sample VIII | | | | | Sample IX | | | | |
| 5 | 50.2 | 100 | -- | -- | 5 | 104.2 | 100 | -- | -- | 5 | 72 | 100 | -- | -- |
| 10 | 25.5 | 100 | 0.45 | 45 | 10 | 47.8 | 100 | -- | -- | 10 | 72 | 100 | -- | -- |
| 15 | 18.8 | 100 | -- | -- | 20 | 25.3 | 100 | -- | -- | 20 | 115.4 | 100 | -- | -- |
| 20 | 15.4 | 100 | -- | -- | 30 | 24.0 | 100 | 0.49 | 49 | 30 | 91.9 | 100 | 0.1 | 10 |
| 30 | 13.8 | 100 | 1.15 | 115 | | | | | | | | | | |

RT = Reaction Time, Minutes; CT = Clotting Time, Seconds; Diln = Sample Dilution, volume basis; F = Activity Factor; U/ML = Units per ML.

TABLE III
DATA ON ELECTROPHORESIS PATTERNS OBTAINED IN
0.1M VERONAL BUFFER OF pH 8.6

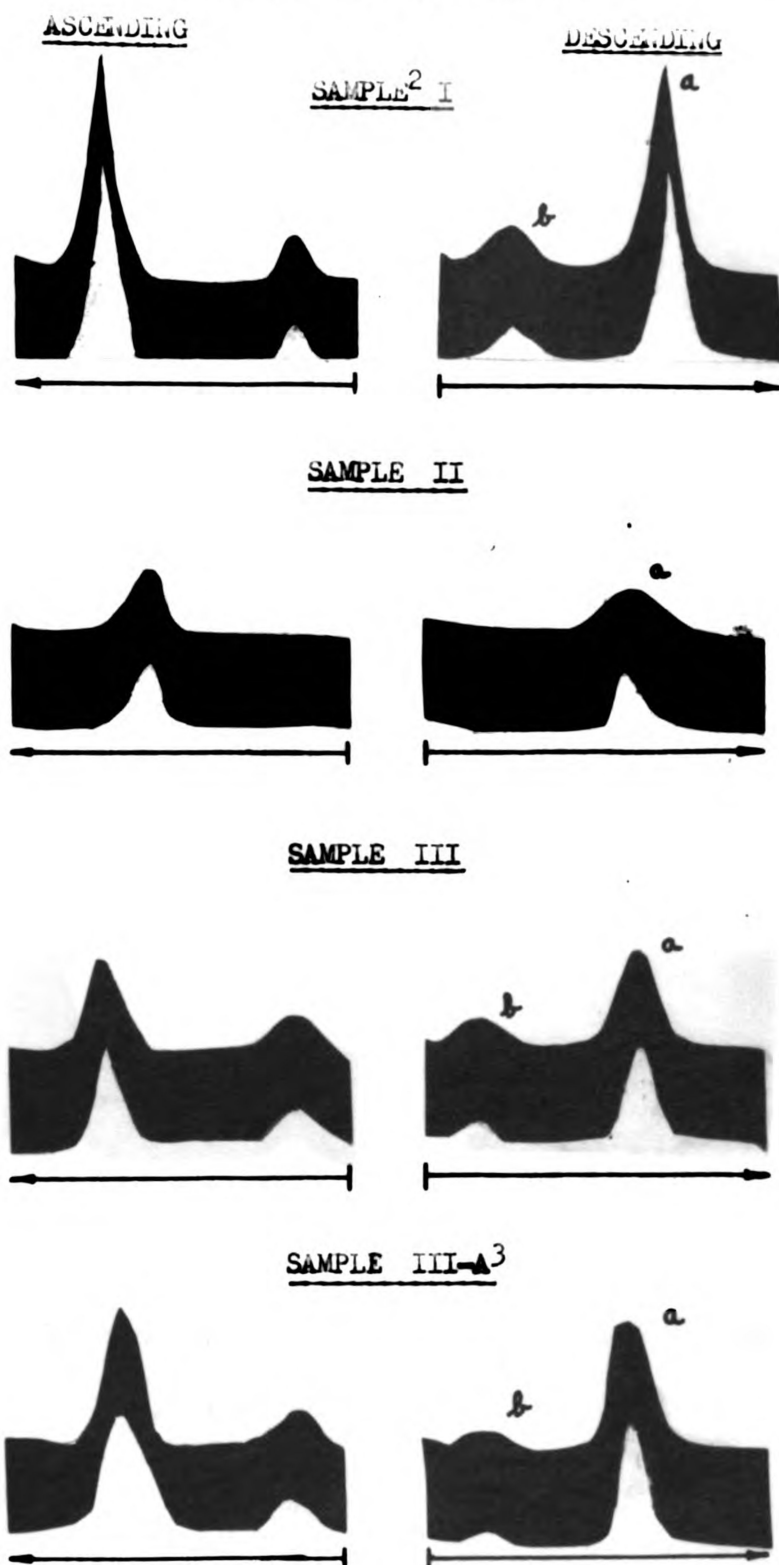
| Sample ¹ | Ionic ² Strength (Molar) | Time (Secs) | Potential Gradient (Volts/cm) | Mobility ³ Component | | Concentration Component | |
|-------------------------------|-------------------------------------------|----------------|-------------------------------------|------------------------------------|------|----------------------------|----|
| | | | | a | b | a (%) | b |
| I | 0.15 | 5400 | 7.51 | 7.43 | 2.29 | 81 | 19 |
| II | 0.10 | 5400 | 8.94 | 5.84 | -- | 93 | 7 |
| III | 0.15 | 5400 | 7.45 | 7.69 | 2.00 | 76 | 24 |
| III-A (BaSO ₄) | 0.15 | 5400 | 6.89 | 7.11 | 1.82 | 86 | 14 |
| VI | 0.10 | 5060 | 7.97 | 7.35 | 2.96 | 83 | 17 |
| VII | 0.10 | 4500 | 8.94 | 7.69 | 1.84 | 83 | 17 |

¹ Protein concentrations are one percent.

² Values over 0.10 M. include 0.05M NaCl.

³ Mobilities are calculated from descending patterns and are expressed in Tiselius units, cm./sec. per volt/cm. x 10⁸.

Figure 6. Electrophoresis Patterns from Fraction III(2-3)
Preliminary Treatment.¹

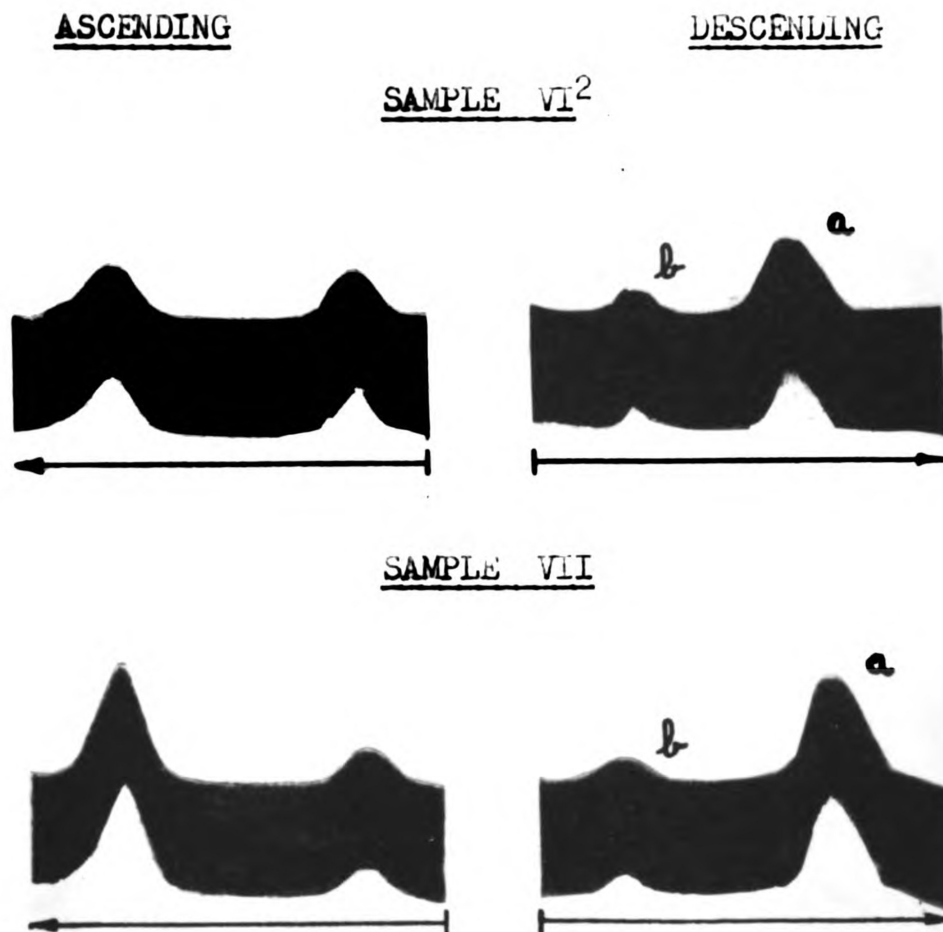


¹All in 0.10 M veronal or pH 8.6, see Table III for conditions of each sample.

²For this and all other samples refer to Figure 4.

³After barium sulfate adsorption.

Figure 7. Electrophoresis Patterns of Gups After Continuous Flow Technique.¹



¹All in 0.10 M veronal of pH 8.6, see Table III for conditions of each sample.

²For this and all other samples refer to Figure 4.

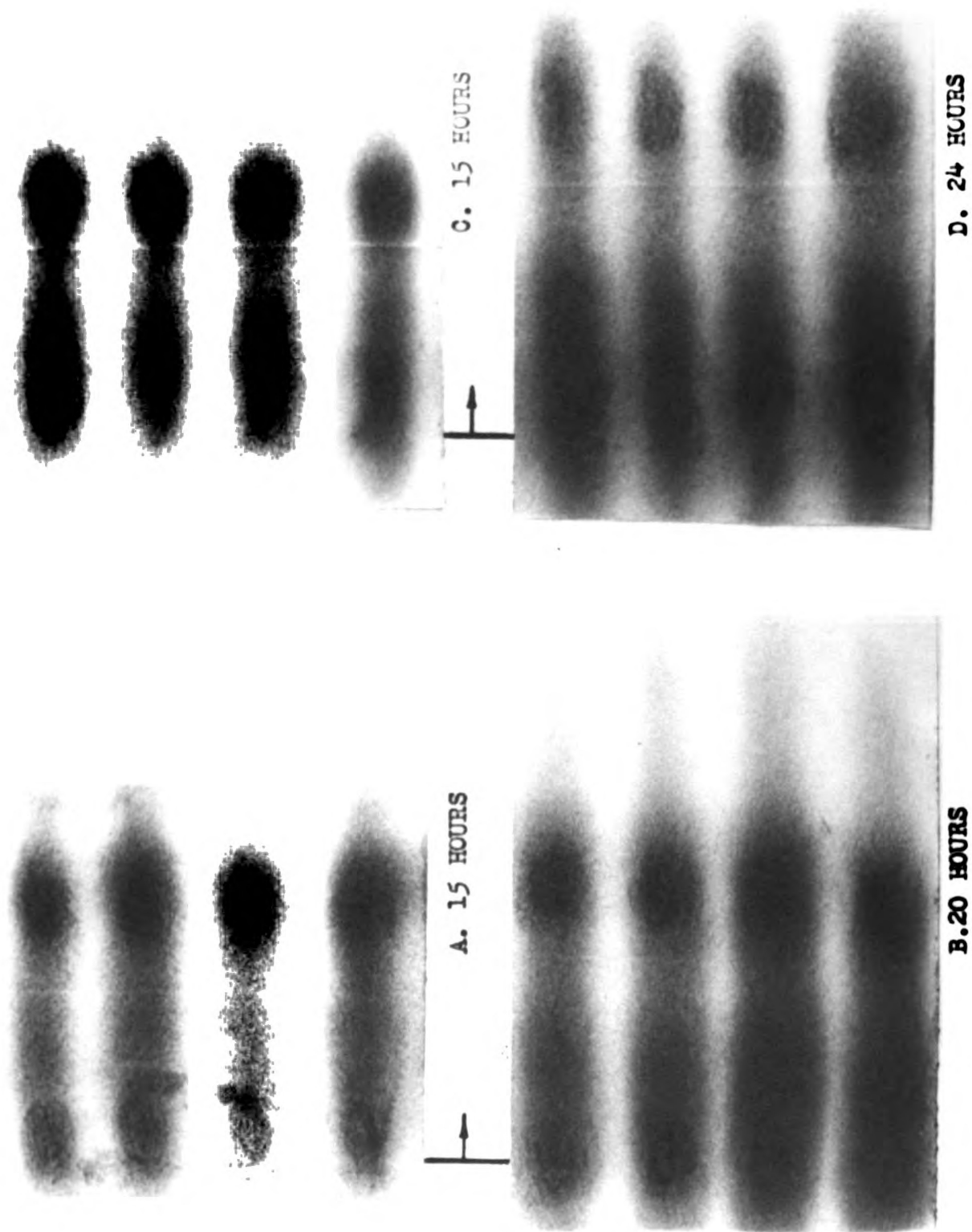


Figure 8. Paper Electrophoresis of Fraction III(2-3).

IV. DISCUSSION

This discussion with reference to prothrombin isolation will attempt to evaluate the results and significance of experiments concerning: (1) Apparatus testing and modification, (2) The separation of proteins in Fraction III by horizontal-strip techniques, (3) The use of continuous paper electrophoresis to produce separation, and (4) the utilization of adsorption methods.

Horizontal-Strip Technique -- Use was made of the box-type apparatus to establish some of the procedures inherent in the isolation or separation of proteins by means of paper electrophoresis. In developing surface evaporation control and maintenance of paper tension Fraction III served as test material.

The initial runs were made with a glass plate as paper support and chlorobenzene as sealant. A "smear-type" migration was the result. The components did not separate sharply but appeared to migrate as a mass. Therefore the glass-plate-chlorobenzene combination was discarded. A plastic frame for holding the paper was substituted into the setup. This proved to be more satisfactory when using either strips or a single paper sheet.

The center compartment which had held the glass plate was filled with water to provide a saturated atmosphere within the closed box. Migration was of much better quality. The application of the protein

solution to the paper by the spot method was found to give much sharper resolution than by the streaking technique.

Considerable electrolysis occurred in the buffer wells when they also served as the electrode vessels. This led to loss of buffer ability. This problem was improved by replacing the steel anode with a carbon rod. Furthermore, the electrodes were moved to containers separate from the buffer wells but connected with an agar-bridge. The final construction is shown in Figure 2.

Several runs were made with no attempt to remove the resolved proteins from the paper. The sheets were merely stained in order to observe the effects of various apparatus modifications. Reproducible results, similar to such as shown by Figure 8, were obtainable when the apparatus and techniques as described in the experimental section were followed. These developments were then directed toward definite isolation of prothrombin from Fraction III(2-3). The four runs of Figure 8 indicate that the longer time gives a better separation. Two components are distinguishable after bromphenol blue staining. This observation was confirmed by Tiselius electrophoretic analysis and the Schlieren diagram can be seen in Figure 6, Sample I.

After locating the proteins by staining, an attempt was made to elute them from unstained paper. Assays upon saline extracts showed presence of prothrombin activity. However, clotting times were not of sufficient magnitude to warrant designation of maximum activity for any particular region along the strip. Although this development produced clear zone formation with sharp resolution, it may be concluded

that sufficient material can not be applied to the paper for a preparative concentration of active prothrombin by the horizontal-strip technique.

Continuous-Flow Technique -- As a consequence of the above discussed experiments, the continuous-flow technique was next explored as a possible way of preparing prothrombin from Fraction III(2-3). The presence of the two components in the starting material suggested the desirability of preliminary separation. A scheme as shown in Figure 5 was devised before attempting continuous paper electrophoresis. Principally the procedure of Oncley and co-workers (34) was followed. The supernatant (which should have been essentially prothrombin) was found by the Tiselius method to contain about ninety percent of the total material migrating as one component. Figure 6, Sample II demonstrates this result. The precipitate, presumably plasminogen in part, was found by similar analysis to contain two principal components as can be seen in Figure 6, Sample III. Since this pattern is very similar to that of Fraction III(2-3) it is quite probable that the precipitate also consisted of undissolved starting material.

Glycerol in the buffer served to increase the viscosity of the protein solution, thereby decreasing its flow rate on the paper. Concentration changes across the paper due to surface distillation were kept at a minimum by paraffin treatment of the edges. As Valmet and Svensson (51) suggested, electrical equilibrium was established by turning the current on two hours before application of sample.

Heating of the paper occurs when the current is first applied and as the current rises there is produced a swelling of the paper fibers. It evidently causes flow of liquid from the electrode vessels producing an even greater rise in current. Hence, pre-equilibration lessens subsequent complications after application of the protein solution.

After the continuous-flow apparatus was put into operation, five cuts of Sample II were collected. Data concerning volume and lyophilized residue weight are given in Table I. In three of the five cuts such small volumes were collected that glycerol and salt-free weights were not obtained upon the lyophilized residues. However, all samples were subjected to nitrogen analysis and prothrombin assay. Only Samples VI and VII provided adequate residue for Tiselius analysis. Tables I, II and III report the results of these analyses.

Samples which gave sufficient glycerol and salt-free weighable residue were found to contain approximately ten percent nitrogen. This may suggest uniform drying but if 14 to 16 percent nitrogen in prothrombin is assumed there is evidence of incomplete dehydration.

The prothrombin assays¹ demonstrated no enrichment of activity in any particular cut. There is no significant activity difference between Sample II (uncut) and Sample VII (the principal fraction of the cuts). The latter is obtained from the drip point directly beneath the point of sample application. Perhaps the gravitational force on the flowing protein solution was greater than the horizontal

¹ The samples were also checked for the possible presence of thrombin and found to be essentially free.

electrical vector. Although the experiment was of relatively short duration it is possible that channeling of the protein solution occurred.

Prothrombin activity is present throughout all the cuts indicating the difficulty of completely segregating it from its co-proteins in Fraction III(2-3).

Although no cut showed any activity greater than that of Sample II (see Table I) there is quantitative evidence that the prothrombin moiety was most attracted towards the anodic side of the paper and that very little activity resided in the cuts nearest the cathode. This agrees with results of previous experiments where the components of Fraction III(2-3) migrated anodically in the horizontal-strip apparatus.

Most remarkable was the finding of greatest prothrombin activity in the residual solution, Sample IV. One would expect activity loss if any change at all. The activity units in this solution approximate the values of prothrombin concentrates reported by Oncley and co-workers (34) as about 187 units per mg. nitrogen.

Schliaren diagrams were obtained on the two largest cuts, Samples VI and VII. Information concerning the composition by electrophoresis as well as conditions of analysis may be had in Figure 7 and Table III. According to the patterns both cuts possess two components, a and b. Component a in both Sample VI and VII has essentially the same mobility and comprises the same percent of the total material. This observation is also true for component b. The mobilities of components

a and b in Samples VI and VII (allowing for difference in effect of ionic strength) are comparable to those of crude Fraction III(2-3). No outstanding difference in percentage composition can be noted in the three cases.

Electrophoretic analysis of Sample II, as seen in Figure 6, (material prepared for continuous electrophoresis) reveals a principle component that amounts to about 93 percent of the total. Its mobility is less, by 2 Tiselius units, than the fastest component of any other sample so analyzed. This material was the soluble portion of an aqueous extract of Fraction III(2-3) after treatment with sodium chloride. Yet when it was subjected to continuous electrophoresis the samples obtained showed the presence of two components again. It may be surmised that the sodium chloride precipitation left in solution a complex of materials that under conditions of moving boundary electrophoresis migrated singly whereas upon paper there was resolution into two fractions. The similarity of mobilities of the components in Samples I, VI and VII leads one to suspect that Sample II could represent such a complex.

Adsorption Experiments -- Prothrombin has been reported to be adsorbed from whole plasma upon barium sulfate (47). Sample III, available in substantial amount, was tested in this regard. The results with barium sulfate indicated partial revision of the proportions of components a and b by electrophoretic analysis, as seen in Figure 6, Sample III-1. Apparently a ten percent loss of component b with a corresponding increase in a occurred. Protein estimation

revealed about a four percent weight loss after barium sulfate treatment.

Barium carbonate has been used to purify preparations of bovine prothrombin (42). An attempt was made to purify Sample III with this compound. The result was an opaque solution which upon electrophoretic analysis gave only partial patterns. These, however, indicated no particular change in composition as did the protein estimation.

Hence the proteins of Sample III and presumably Fraction III(2-3) under the conditions employed and with the adsorbents used do not seem to be conducive to separation by this technique.

V. SUMMARY

- 1) The best resolution of Fraction III(2-3) by horizontal strip paper electrophoresis was obtained when: a) surface evaporation was controlled by a saturated aqueous atmosphere at room temperature, b) the paper was supported by a tension holder, and c) the protein was applied by the spot method.
- 2) Fraction III(2-3) separated into two principal components, but gave no distinct resolution of prothrombin activity.
- 3) Assays of protein eluates from unstained papers showed no preparative concentration of prothrombin by the horizontal-strip technique.
- 4) A salt-treated extract of Fraction III(2-3) with prothrombin activity showed essentially a singly migrating component by Tiselius electrophoretic analysis.
- 5) Continuous paper electrophoresis of the above material gave five cuts, of which two comprised about eighty percent of the total volume.
- 6) Prothrombin was present in all cuts with no enrichment of activity in any one sample. Thrombin was found to be absent.
- 7) The highest prothrombin activity was located in the residual solution from continuous electrophoresis.
- 8) The use of barium sulfate and barium carbonate as adsorbents was not found to be adaptable to the purification of prothrombin in the manner tried.

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APPENDIX I

Sample Calculation of Prothrombin Activity

- 1) Ten mg. of Sample I is dissolved in one ml. 0.9 percent sodium chloride.
- 2) To 0.1 ml. of solution (1) is added 0.9 ml. of Ac-O diluent. This gives a 1:10 dilution of solution (1).
- 3) The one ml. of solution (2) is combined with three ml. of prothrombin conversion mixture to yield four ml. of reaction mixture. The dilution of solution (1) is now 1:40.
- 4) To 0.1 ml. of fibrinogen is added 0.4 ml. of reaction mixture and the time of clotting obtained. (If clotting times are less than 20 seconds before 15 minutes of reaction time has passed then a dilution of solution (3) is made with Ac-O diluent such that at 30 minutes of reaction time the clotting time will be 12 to 20 seconds.) Total dilution of solution (1) is now 1:50. In this case an additional dilution was made to give a final value for solution (1) of 1:200.
- 5) The clotting time is referred to the standard thrombin curve. This gives thrombin units per ml. of the diluted sample. For Sample I the activity factor for an 18.9 second clotting time after 30 minutes of reaction is 0.70. The prothrombin activity is (0.70 times 200) 140 units/ml. of solution (1).

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A thick, dark, curved line is drawn on a grid background. The line starts at the top left corner of the grid and curves downwards and to the right, ending near the bottom right corner. The line is very thick and has a slightly irregular, hand-drawn appearance. The grid consists of horizontal and vertical lines forming a series of squares.

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