7.

A SIMPLE, SPECIFIC ONE-STAGE PROTHROMBIN ASSAY USING RUSSELL'S VIPER VENOM IN CEPHALIN SUSPENSION

PETER HJORT, M.D.
SAMUEL I. RAPAPORT, M.D.
and
PAUL A. OWREN, M.D.
Oslo, Norway

From the Medical Department A, Oslo University Medical School

Reprinted from
THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE
St. Louis

Vol. 46, No. 1, Pages 89-97, July, 1955

(Printed in the U. S. A.)

A SIMPLE, SPECIFIC ONE-STAGE PROTHROMBIN ASSAY USING RUSSELL'S VIPER VENOM IN CEPHALIN SUSPENSION

PETER HJORT, M.D., SAMUEL I. RAPAPORT, M.D.,* AND PAUL A. OWREN, M.D.
OSLO, NORWAY

INTRODUCTION

A ONE-STAGE prothrombin assay is based upon timing the interval between recalcification of a test system and clotting. This time interval depends upon the initial rate of thrombin accumulation in the clotting mixture. This rate measures the prothrombin content of the test plasma only when the rest of the clotting mixture provides a constant, optimal concentration of all other clotting factors, or their equivalent. Nonspecific factors that influence the interval between recalcification and clotting, such as ionic strength, pH, and temperature, must not vary.

Quick's construction of the first one-stage "prothrombin" test[1] was a big step forward in coagulation methodology. This simple test remains a valuable clinical and research tool. However, its value lies partly in that it is not a specific prothrombin test but is sensitive to changes in the proaccelerin (labile factor, plasma Ac globulin) and proconvertin (stable factor, Factor VII, SPCA) content of the test plasma. Furthermore, it is insensitive to changes in prothrombin concentration in the 50 to 100 per cent of normal range.

These difficulties can be surmounted by diluting the test plasma, which increases the sensitivity of the system, and by introducing reagents that contain a high and constant concentration of the nonprothrombin factors. Adsorbed ox plasma provides a high and constant concentration of proaccelerin and fibrinogen. It has been used for years in this laboratory in a one-stage method, the P. and P. method,[2, 3] for the quantitative determination of the combined effect of prothrombin and proconvertin in patients on Dicumarol therapy. The P. and P. method can be converted into a specific prothrombin assay by the addition of a constant concentration of proconvertin in the form of specially prepared prothrombin "free" stored serum.[3]

Fullerton[4] first substituted Russell's viper venom for brain thromboplastin in the Quick test. However, the clotting time using venom was found to vary with the lipoid and/or platelet content of the plasma. Furthermore, it failed to measure the full effect of Dicumarol therapy. This is because the clotting action of viper venom in the presence of lipoid is equivalent to convertin (the combination of thromboplastin and proconvertin) and therefore independent of the proconvertin content of the test plasma.[5, 6]

From the Medical Department A, Oslo University Medical School, Oslo, Norway.
Received for publication Feb. 28, 1955.
*Fulbright Research Scholar in Norway, 1953-1954. Present address, Hematology Section, Veterans Administration Hospital, Long Beach, Calif.

Preliminary experiments revealed that the use of Russell's viper venom dissolved in crude "cephalin" suspension in place of brain thromboplastin changed the P. and P. test into a specific one-stage assay of prothrombin alone.[7] Further experience has perfected this technique of prothrombin assay into a method which is superior to the older test with stored serum. The present paper describes the reagents, technique, and standardization of the specific one-stage venom-cephalin assay for prothrombin as it is now used in this laboratory.

MATERIALS AND METHODS

Test Plasma.—Four and five-tenths milliliters of blood is drawn into 0.5 ml. of 0.1 M (3.13 Gm. per cent) sodium citrate dihydrate and centrifuged at 2,500 r.p.m. for 15 minutes. *Standard plasma* for preparing the correlation graph is made by drawing 180 ml. of normal blood into 20 ml. of the same anticoagulant. The plasma, obtained by centrifugation at 30 minutes at 2,500 r.p.m., is stored in small quantities in the liquid frozen state at -20° C.

Dilution Fluids.—The usual dilution of the test plasma is 1:50. The standard plasma must be diluted from 1:10 to 1:500 to prepare the correlation graph. The diluting technique must keep the citrate concentration and ionic strength constant for any dilution within this range. This is done by making two dilutions. The plasma is diluted first to 10 times the final concentration desired (1:5 for the test plasma and up to 1:50 for the standard plasma) in Diluting Fluid I. This consists of 100 ml. of 0.1 M sodium citrate added to ~~600~~ 500 ml. of 0.9 Gm. per cent sodium chloride solution and has the same citrate concentration and ionic strength as citrated plasma from blood with a normal hematocrit. A second 1:10 dilution of the various primary dilutions is then made in Diluting Fluid II which consists of 200 ml. of Veronal buffer, 200 ml. of 25.66 mM sodium citrate and 600 ml. of 0.9 Gm. per cent sodium chloride solution.

Crude "Cephalin" Suspension.—This is the acetone-insoluble, ether-soluble fraction of human brain prepared according to Milstone's technique.[8] Four hundred grams of human brain are washed free of blood and membranes and ground with 300 ml. of acetone.* After centrifugation, the acetone is discarded and the residue re-extracted with the same volume of acetone a total of six times. The residue is then extracted with 1,800 ml. of ether overnight at room temperature. The ether is siphoned off and evaporated to dryness by suction at 35° C. The resulting residue is washed twice for 15 minutes with 900 ml. of acetone and then redissolved in 200 ml. of ether. The ether is again evaporated to dryness and the suction continued for 75 minutes. About 9.3 Gm. of a waxy cream-colored to brown residue are suspended in 200 ml. of Veronal buffer. This is centrifuged for 20 minutes at 1,700 r.p.m. and again for 10 minutes at 2,500 r.p.m. The sediment is discarded. The supernatant, which contains 2.8 Gm. per cent of crude cephalin in a milky white suspension, is frozen in small quantities at -20° C. Before use, the melted suspension is diluted 1:30 in veronal buffer to a final concentration of 0.09 Gm. per cent.

Russell's Viper Venom-Cephalin Reagent.—The preparation Stypven† is used. The dried venom is dissolved in the 0.09 Gm. per cent cephalin suspension to a concentration of 1:40,000.

Small portions of this reagent were stored at 37° C., 20° C., 4° C., and -20° C. It proved remarkably stable even at 37° C. There was no deterioration after standing for one day at 20° C., or for several days at 4° C. The reagent could be frozen and thawed repeatedly without loss of activity. It proved easiest to store it at -20° C. in aliquots suitable for one day's use. It can probably stay frozen indefinitely without loss of activity.

Prothrombin (and Proconvertin) Free Bovine Plasma.—The bovine plasma should contain no prothrombin, a high and stable concentration of proaccelerin, and a constant and

*Merck & Company, Inc.
†Burroughs Wellcome & Company, Inc.

stable fibrinogen. The technique for preparing the corresponding reagent for the P. and P. method (filtration of oxalated ox plasma once through 20 per cent and once through 50 per cent asbestos filter pads) leaves traces of prothrombin in the adsorbed plasma. This is unimportant in the P. and P. test but reduces greatly the sensitivity of the venom-cephalin test.

A suitable reagent is obtained as follows: Nine hundred milliliters of ox blood is collected at the slaughter house in 1,000 ml. ice-cold bottles packed in ice and containing 100 ml. of 2.5 per cent potassium oxalate monohydrate. The blood is centrifuged as soon as possible for 30 minutes at 2,500 r.p.m. in a refrigerated centrifuge. The plasma is passed through a 20 per cent asbestos filter pad (filter diameter 15 cm., oxygen pressure about 1 kg. per square centimeter, filter changed for every 500 ml.). The filtered plasma is then adsorbed for 5 minutes at 20° C. with barium sulfate (Baker), 75 mg. per milliliter of plasma, and the barium sulfate removed by centrifugation. The adsorbed plasma is stored in small aliquots liquid frozen at -20° C., in which state it remains stable for long periods.

Veronal Buffer.—This buffer of pH 7.35 and ionic strength 0.154 is made by mixing sodium diethyl barbiturate 11.75 Gm., sodium chloride 14.67 Gm., 0.1 N HCl ~~400~~ 430 ml., and distilled water to 2,000 ml.

Calcium Chloride.—A 35 mM solution is made by dilution of a 1 M aqueous stock solution with distilled water.

The test is done by mixing 0.2 ml. of adsorbed bovine plasma, 0.2 ml. of the Russell's viper venom-cephalin reagent, and 0.2 ml. of a dilution of the test plasma in a serologic test tube which is then incubated for 5 minutes in a water bath at 37° C. Then 0.2 ml. of calcium chloride is blown into the tube and the clotting time measured. All tests are done in duplicate.

RESULTS

Determination of the Optimal Concentration of Russell's Viper Venom.—A 1:40,000 dilution of Russell's viper venom in cephalin suspension was chosen as standard on the basis of the results summarized in Table I. They show the clotting times obtained with different dilutions of venom in 0.09 Gm. per cent cephalin. The shortest times were found in the 1:40,000 to 1:80,000 concentration range.

TABLE I. DETERMINATION OF OPTIMUM CONCENTRATION OF VENOM WHEN DISSOLVED IN 0.09 GM. PER CENT CEPHALIN SUSPENSION

CONCENTRATION OF VENOM DISSOLVED IN CEPHALIN SUSPENSION	CLOTTING TIME (SECONDS)
1:1,000	>600
1:5,000	45.3
1:10,000	32.0
1:20,000	28.6
1:40,000	28.0
1:80,000	28.0
1:160,000	33.4
1:320,000	41.1
1:640,000	51.5
1:1,280,000	65.5

Clotting mixture: 0.20 ml. ox plasma reagent, 0.20 ml. of various concentrations of venom dissolved in 0.09 Gm. per cent cephalin suspension, 0.20 ml. 1:50 dilution of standard plasma, 0.20 ml. 35 mM $CaCl_2$.

Determination of the Standard Concentration of Cephalin.—Table II gives the clotting times obtained when a 1:40,000 dilution of venom was made in varying concentrations of the cephalin suspension. A wide optimal concentra-

TABLE II. DETERMINATION OF THE OPTIMAL CEPHALIN SUSPENSION CONCENTRATION FOR VENOM CONCENTRATION OF 1:40,000

CONCENTRATION OF CEPHALIN SUSPENDED IN VERONAL BUFFER (GM. PER CENT)	CLOTTING TIME (SECONDS)
2.44	63.7
1.22	41.7
0.24	26.5
0.12	26.6
0.06	26.4
0.02	33.2
0.01	43.1
0.00	210 (?)

Clotting mixture: 0.20 ml. 1:40,000 venom dissolved in cephalin suspension of various concentrations. Otherwise as in Table I.

tion range for our particular cephalin preparation was found. A 1:30 dilution of the original 2.8 Gm. per cent suspension with buffer was selected as the standard procedure. This gives a cephalin suspension of 0.09 Gm. per cent.

Dissolving the venom in cephalin suspension eliminates variation in the clotting time due to the platelet or lipoid content of the test plasma. Table IIIA illustrates that platelet-rich (centrifuged 30 minutes at 900 r.p.m.) and platelet-poor plasmas (centrifuged 30 minutes at 2,500 r.p.m.) give the same

TABLE IIIA. COMPARISON OF PLATELET-RICH AND PLATELET-POOR SAMPLES OF THE SAME PLASMA

PLASMA	CLOTTING TIME (SECONDS)	
	PLATELET-RICH	PLATELET-POOR
1	26.4	26.4
2	26.3	25.9
3	24.0	24.3

TABLE IIIB. COMPARISON OF FASTING AND LIPEMIC PLASMA SAMPLES FROM THE SAME SUBJECT

PLASMA	PROTHROMBIN (PER CENT)	
	FASTING	LIPEMIC (90 MIN. AFTER DRINKING 330 ML. CREAM)
1	98	98
2	109	108
3	97	99

Clotting mixture: As in Table I, with Russell's viper venom 1:40,000 in 0.09 Gm. per cent cephalin suspension.

TABLE IV. DETERMINATION OF THE OPTIMUM CALCIUM CHLORIDE CONCENTRATION

CALCIUM CHLORIDE CONCENTRATION (mM)	CLOTTING TIME (SECONDS)
5	75.4
10	33.4
15	30.3
20	29.0
25	27.5
30	27.5
35	26.9
40	27.0
45	27.4
50	27.3
75	33.3
100	43.7

Clotting mixture: As in Table I, with Russell's viper venom 1:40,000 in 0.09 Gm. per cent cephalin suspension.

clotting times in this system. Table IIIB shows that lipemia produced by drinking cream does not alter the prothrombin values obtained by this technique.

The Optimal Calcium Chloride Concentration.—This was found, by the experiments summarized in Table IV, to lie between 25 and 50 mM. As cited above, a concentration of 35 mM has been chosen as standard.

Preparation of the Correlation Graph and Selection of the Dilution of the Test Plasma.—The prothrombin concentration of the test plasma is read from a correlation graph, such as Fig. 1, which relates the clotting time to the prothrombin content of dilutions of standard plasma. This graph is readily reproducible but requires meticulous care in making the dilutions. The times, when plotted on double logarithmic paper, are a straight line except for a bend at about 20 seconds. The reason for the bend is unknown. Above 20 seconds the line has the same slope as a thrombin-fibrinogen curve. The end point loses its sharpness above 80 seconds.

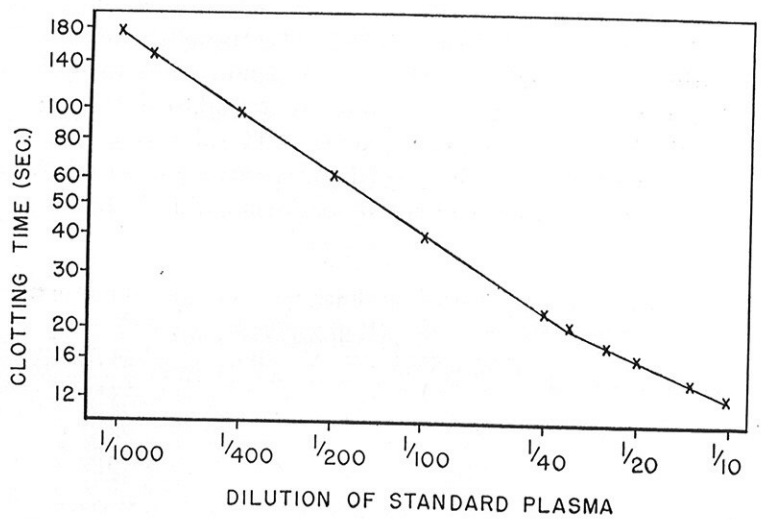

Fig. 1.—Correlation graph obtained by plotting the clotting times of dilutions of standard plasma on double logarithmic scale.

The optimum clotting times, therefore, are between 25 and 80 seconds. The clotting time will fall within this range for a 1:50 dilution of a test plasma with a prothrombin concentration between 20 and 120 per cent. (The clotting time of a 1:50 dilution of the standard plasma is arbitrarily called 100 per cent.) If the clotting time of a 1:50 dilution of the test plasma falls above or below this range, a 1:25 or a 1:100 dilution should be used.

Sensitivity of the Method to Heparin.—The P. and P. time is unaffected by small amounts of heparin in the test plasma or anticoagulant solution. However, heparin can lengthen the specific prothrombin time with venom-cephalin. Table V illustrates the increase in clotting time produced by a final concentration of about 1.4 μg of heparin ("A-L", Oslo) in the dilutions of the test plasma. In these experiments, 100 μg of heparin were added to each

milliliter of the citrate anticoagulant. The first dilution to ten times the final concentration was made with Diluting Fluid I, which contained enough added heparin to keep the heparin concentration the same as in the undiluted citrated plasma sample. The final 1:10 dilution was made in the usual non-heparin-containing Diluting Fluid II.

TABLE V. THE EFFECT OF HEPARIN (1.4 μG PER MILLILITER OF DILUTED TEST PLASMA) UPON THE CLOTTING TIME

PLASMA DILUTION	CLOTTING TIME (SECONDS)	
	HEPARIN	NO HEPARIN
1:10	11.3	11.3
1:20	15.7	14.4
1:40	22.6	21.0
1:100	37.4	34.3
1:200	61.0	53.8
1:400	108.0	81.3

Clotting mixture: As in Table I, with Russell's viper venom 1:40,000 in 0.09 Gm. per cent cephalin suspension.

Absence of Variation Due to Glass Contact.—Tocantins[9, 10] first noted that the clot accelerating effect of glass contact is not found when Russell's viper venom is the thromboplastic agent. The reason is that glass contact increases the activity of both the antihemophilic B factor (PTC, Christmas factor) and proconvertin.[11] Venom plus cephalin acts like convertin and would therefore mask changes in the test plasma content of antihemophilic B factor or proconvertin.

TABLE VI. ABSENCE OF ACCELERATING EFFECT OF GLASS CONTACT UPON THE CLOTTING TIME

PLASMA	CLOTTING TIME (SECONDS)	
	SILICONE SAMPLE	GLASS-ACTIVATED SAMPLE
1	22.1	22.6
2	22.7	23.6
3	23.8	23.3
4	24.0	24.5
5	24.0	24.0

Clotting mixture: As in Table I, with Russell's viper venom 1:40,000 in 0.09 Gm. per cent cephalin suspension.

The data in Table VI verify that the specific prothrombin time with venom-cephalin does not vary with exposure of the test plasma to glass. Blood for these experiments was drawn with silicone technique (General Electric Dri Film 9987 for glassware and Arquard 2 C "Armour" for needles) and centrifuged for 15 minutes at 2,500 r.p.m. One portion of the plasma was shaken with quartz glass powder (Quartz gewaschen u. geglüht, E. Merck, Darmstad, ground in a glass mill to particles smaller than 0.068 mm.) for 10 minutes. A 1:50 dilution of this glass-activated plasma was then compared with a sample of the plasma exposed only to silicone surfaces. As can be seen, glass contact does not shorten the clotting time with venom-cephalin.

The insensitivity of the method to the effects of glass contact and the stability of prothrombin permits storage of the plasma for several days with-

TABLE VII. THE EFFECT OF STORAGE UPON THE PROTHROMBIN CONCENTRATION OF NORMAL PLASMA

STORAGE TIME (DAYS)	PROTHROMBIN (PER CENT)			
	PLASMA 1	PLASMA 2	PLASMA 3	PLASMA 4
0	100	97	91	107
1	98	96	87	107
2	97	96	90	98
4	99	92	86	98
6	101	97	86	96
8	95	96	85	100
10	83	79	73	86
14	81	75	74	84

Clotting mixture: As in Table I, with Russell's viper venom 1:40,000 in 0.09 Gm. per cent cephalin suspension.

out influencing the result of the test. This is illustrated by the data in Table VII which show that the plasma may stand under sterile conditions for as long as eight days at room temperature without introducing an error.

Concentration of Prothrombin in Normal and Pathologic Plasmas.—When the arbitrarily selected standard plasma was called 100 per cent, the average value for the plasma prothrombin content of 35 normal subjects was 105 per cent. The range was 92 to 120 per cent. As expected, normal values for prothrombin concentration were found in patients with hemophilia A and B, parahemophilia, and congenital hypoproconvertinemia (Table VIII). The normal values in the patients with congenital hypoproconvertinemia again confirms the independence of the clotting action of venom-cephalin and proconvertin.

TABLE VIII. THE PROTHROMBIN CONCENTRATION IN VARIOUS PATHOLOGIC PLASMAS

DIAGNOSIS	PROTHROMBIN (PER CENT)
1. Hemophilia A	93
2. Hemophilia B	105
3. Hemophilia B	108
4. Hemophilia B	126
5. Hemophilia B	103
6. Congenital hypoproconvertinemia	122
7. Congenital hypoproconvertinemia	113
8. Congenital hypoproconvertinemia	108
9. Parahemophilia	120

DISCUSSION

The venom-cephalin test is a simple one-stage clotting test sensitive only to variation in the prothrombin content of the test plasma. This is so because the venom-cephalin reagent supplies the clotting equivalent of an optimum amount of convertin, while the high proaccelerin and fibrinogen content of the ox plasma protects against changes in the test plasma content of these factors. The ox plasma also helps to stabilize the antithrombin titer of the system. A 1:50 dilution of the test plasma will permit a sensitive and accurate measurement of a prothrombin concentration between 20 and 120 per cent of normal.

The reagents are simple to prepare. The viper venom is readily available commercially.* A substitute for cephalin is also available.† However, the cephalin and ox plasma reagents are easily prepared in the ordinary laboratory. The anticoagulant solution should not contain heparin as recommended for some brain thromboplastin methods.[12] Small amounts of heparin can increase the clotting time with venom-cephalin. Recent administration of heparin to the patient could also alter the results.

Neither this nor any other test which measures prothrombin alone should be used to control anticoagulant therapy with Dicumarol or similar drugs. These drugs produce a proconvertin deficit that can lead to bleeding without a striking depression of prothrombin. Obviously, this proconvertin depression will be missed by a test which is specific for prothrombin. A test which utilizes brain thromboplastin, such as the P. and P. test, must be used to control Dicumarol therapy.

The venom-cephalin test should prove valuable to those who use prothrombin consumption tests. One of the difficulties with the prothrombin consumption test is that the prolongation of the serum prothrombin time determined with brain thromboplastin is partially masked by the formation of convertin and active proconvertin during clotting.[11] This source of error is eliminated with the venom-cephalin method. Venom-cephalin should also prove useful as a substitute for tissue thromboplastin in the usual two-stage prothrombin methods. The reduced converting time with venom-cephalin will diminish the proportion of thrombin inactivated by antithrombin in the incubation mixture.

TABLE IX. DIFFERENTIATION OF COAGULATION DEFECTS NOT CONCERNED WITH THROMBOPLASTIN FORMATION

COAGULATION FACTOR INSUFFICIENT	PROLONGED CLOTTING TIME		
	QUICK TEST	P. AND P. TEST	VENOM-CEPHALIN
Prothrombin	Yes	Yes	Yes
Proconvertin	Yes	Yes	No
Proaccelerin	Yes	No	No
Fibrinogen	Yes	No	No

The use of the Quick test, the P. and P. test, and the venom-cephalin prothrombin test provides a rapid and simple way to differentiate clotting defects caused by deficiencies of prothrombin, proconvertin, proaccelerin, and fibrinogen. The findings in each of these deficiencies are given in Table IX.

SUMMARY

A simple one-stage test for prothrombin is described. The principal reagents are (1) Russell's viper venom dissolved in cephalin suspension, and (2) adsorbed ox plasma. The method is sensitive and specific. It is uninfluenced by variation in the test plasma content of lipoid, platelets, plasma thromboplastin precursors, proconvertin, proaccelerin, or fibrinogen. Neither this nor

*Stypven, Burroughs Wellcome & Co., Inc.
†Asolectin, Associated Concentrates, Inc., New York.

any method which measures prothrombin alone should be used to control Dicumarol therapy, for the proconvertin deficiency which this drug also produces will not be measured.

We wish to thank Nurse Solveig Mikkelsen for her help in these experiments.

REFERENCES

1. Quick, A. J., Stanley-Brown, M., and Bancroft, F. W.: A Study of the Coagulation Defect in Hemophilia and in Jaundice, Am. J. M. Sc. **190**: 501-511, 1935.
2. Owren, P. A.: The Coagulation of Blood. Investigations on a New Clotting Factor, Acta med. Scandinav., supp. 194, pp. 1-327, 1947.
3. Owren, P. A., and Aas, K.: The Control of Dicumarol Therapy and the Quantitative Determination of Prothrombin and Proconvertin, Scandinav. J. Clin. & Lab. Invest. **3**: 201-208, 1951.
4. Fullerton, H. W.: Estimation of Prothrombin, Lancet **2**: 195-196, 1940.
5. Rapaport, S. I., Aas, K., and Owren, P. A.: The Coagulant Activity of Russell Viper Venom, Scandinav. J. Clin. & Lab. Invest. **6**: 81, 1954.
6. Jenkins, J. S.: The Thromboplastic Activity of Russell's Viper Venom and Its Relationship to Factor VII, J. Clin. Path. **7**: 287-289, 1954.
7. Rapaport, S. I., Aas, K., and Owren, P. A.: The Clotting Action of Russell Viper Venom, Blood **9**: 1185-1194, 1954.
8. Milstone, J. H.: The Problem of the Lipid Thromboplastins, Yale J. Biol. & Med. **22**: 675-687, 1950.
9. Tocantins, L. M.: Influence of the Contacting Surface on the Coagulability and Anticephalin Activity of Normal and Hemophilic Plasmas, Am. J. Physiol. **143**: 67-76, 1945.
10. Tocantins, L. M., Holburn, R. R., and Carroll, R. T.: Response of Plasma to Excess of Thromboplastin as a Measure of Prothrombin Activity, Proc. Soc. Exper. Biol. & Med. **76**: 623-630, 1951.
11. Rapaport, S. I., Aas, K., and Owren, P. A.: The Effect of Glass Upon the Activity of the Various Plasma Clotting Factors, J. Clin. Invest. **34**: 9-19, 1955.
12. Ware, A. G., and Stragnell, R.: An Improved One-Stage Prothrombin Method, Am. J. Clin. Path. **22**: 791-797, 1952.