ficed 24 hours after burning showed a marked increase in the elaboration of hormones compared with the values observed for adrenal tissue from normal dogs. Prior treatment of dogs with 7 U.S.P. units of corticotropin per kg also caused the production of more hormone than untreated controls, but the increase in Compound F was significantly less than in the tissue from burned dogs. Adrenal glands from dogs which were sacrificed 72 hours after burning showed a return to control levels of hormone production with the exception of Compound F which was significantly less than the normal value.

1. Brady, R. O., *Endocrinology,* 1953, v52, 49.
2. Minard, D., and Jensen, R. E., *Naval Med. Research Inst. Reports,* 1952, v10, 543.
3. Haynes, R., Savard, K., and Dorfman, R. I., *Science,* 1952, v116, 690.
4. Hofmann, F. G., and Davison, C., *J. Clin. Endocrinol. and Metabolism,* 1953, v13, 848.
5. Burton, R. B., Zaffaroni, A., and Keutmann, E. H., *J. Biol. Chem.,* 1951, v188, 763.
6. Selye, H., *Adrenal Cortex,* Transactions of the 4th Conf., Josiah Macy, Jr. Foundation, 1953, 29.
7. Liddle, G. W., Rinfret, A. P., Richard, J., and Forsham, P. H., *J. Clin. Endocrinol. and Metabolism,* 1953, v13, 842.
8. Weichselbaum, T. E., Margraf, H. W., and Elman, R., *Fed. Proc.,* 1953, v12, 287.
9. Fisher, R. A., *Statistical Methods for Research Workers,* Oliver and Boyd, London, 1941, 120.

Received October 22, 1953. P.S.E.B.M., 1953, v84.

A Simple Procedure for Separation of Prothrombin and Accelerator Globulin from Citrated Human Plasma.* (20737)

MERLE LOVELL LEWIS AND ARNOLD G. WARE.†

From the Department of Biochemistry and Nutrition, School of Medicine, University of Southern California and the Los Angeles County Hospital, Los Angeles.

In studies of the mechanism of blood coagulation, it is frequently desirable to make use of purified systems rather than plasma or serum from which one or more of the components have been removed. As more data become available on the various properties of the clotting components in different species, the advisability of working with systems derived from a single species become apparent. A number of practical difficulties are encountered in attempting to prepare purified fractions from human plasma by methods designed for use with bovine material. The relative lability of human accelerator globulin (Ac-G) and purified human prothrombin makes it necessary to use fresh plasma. This accentuates the difficulty of obtaining sufficient plasma to apply efficiently the methods previously used for large quantities of bovine plasma. In addition, many of the adsorption technics are applicable only to oxalated plasma, which precludes their direct use on citrated blood which is the most convenient source of large volumes of human plasma. The method described here makes it possible to prepare both purified prothrombin and partially purified plasma Ac-G from the same small (10-100 cc) samples of fresh citrated human plasma. The prothrombin is obtained in high yield and free of other known clotting factors. The procedure has the additional advantages of simplicity and rapidity; the preparation is easily completed in a single working day.

Assay methods. Prothrombin. Both the one-(1) and 2-stage(2) assays were used for measuring prothrombin. In the 2-stage method acacia was eliminated from the incubation mixture. With this modification nor-

* This investigation was supported by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army, and by the U. S. Public Health Service.

† We wish to acknowledge the technical assistance of Mrs. Irene M. Smyth. We wish also to express our appreciation to Dr. Fremont E. Davis, Miss Angelyn A. Konugres, and the staff of the Transfusion Laboratory, Los Angeles County Hospital, for their cooperation in obtaining the blood used in these experiments.

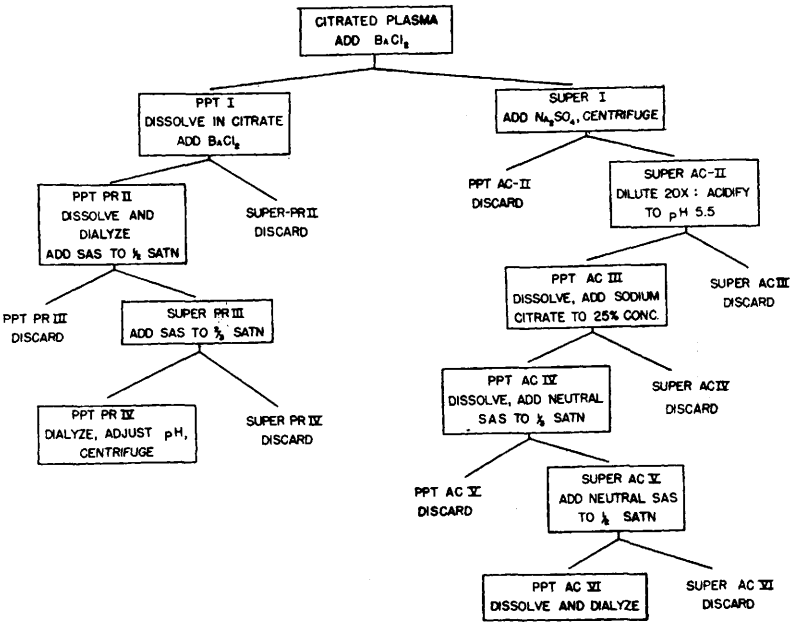

FIG. 1. Flow-sheet for preparation of prothrombin and accelerator globulin from citrated human plasma. Ppt = precipitate; Pr = prothrombin; Super = supernatant; Ac = accelerator globulin; SAS = saturated ammonium sulfate.

mal plasma was found to contain about 150 units of prothrombin per ml. *Accelerator globulin.* The one-stage assay of Lewis and Ware(3) was used for the determination of Ac-G activity.

Procedure. Prothrombin is adsorbed on a barium citrate precipitate formed by addition of barium chloride to fresh citrated plasma; accelerator globulin remains in the plasma. The barium citrate precipitate is dissolved in dilute citrate, and the prothrombin further purified by an additional adsorption, followed by ammonium sulfate fractionation. The accelerator globulin is separated from the plasma by acid precipitation, followed by precipitation in strong citrate and finally ammonium sulfate fractionation. The overall procedure is shown in the flow sheet in Fig. 1. *Preparation of prothrombin.* 100 ml cold, non-lipemic citrated plasma (citrate concentration approximately 0.02 M) are placed in a centrifuge cup of 125 ml capacity. Ten ml of 1 M barium chloride are added dropwise, with shaking, and the mixture inverted once or twice to mix. The tube is allowed to stand in the cold for 10-15 minutes and then centrifuged in a refrigerated centrifuge at 3000 g for 25-30 minutes. The supernatant fluid is decanted and saved for the isolation of Ac-G. The precipitate is dissolved at room temperature in sufficient 0.02 M sodium citrate in saline to give a volume of 100 ml. A small amount of insoluble residue remains, which is removed by centrifugation for a few minutes at low speed. The dissolved Precipitate I (Fig. 1) is decanted into another centrifuge cup, and 10 ml of 1 M barium chloride are added dropwise. After standing in the cold for 10-15 minutes, the tube is centrifuged for 25-30 minutes at top speed. The supernatant is discarded, and the precipitate (Pr-II) is suspended in sufficient 0.02 M sodium citrate in saline at room temperature to give a volume of 50 ml. The suspension is immediately divided into 2 portions of 25 ml each, and each portion is transferred to a dialysis tube about 24 inches in length and having a folded diameter of at least 2 inches.‡ The tubes are attached to some device which will keep the contents in motion during dialysis, and dialysis is carried out at room temperature

‡ It is important that thin dialysis membranes be used and that the volume of suspension be kept small enough to permit efficient dialysis.

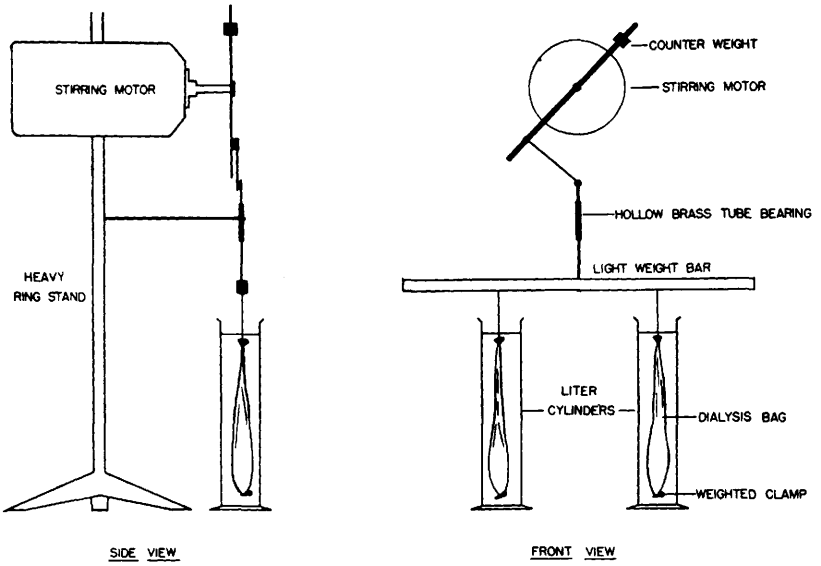

FIG. 2. Schematic drawing of device for rapid dialysis of multiple samples.

against 0.02 M sodium citrate in saline until the citrate is almost free of barium. Solution of the suspension should be complete within 25-35 minutes; an additional 1-2 hours may be required to dialyze out the barium. The dialyzed Precipitate Pr-II will be opalescent or even faintly milky in appearance. It is very important that the dialysis of this fraction be carried out at room temperature as rapidly as possible, and that the contents of the tubes be kept in constant motion during the dialysis period. Otherwise a gelatinous mass may be obtained, resulting in considerable loss of prothrombin and giving rise to difficulties in the subsequent sulfate fractionation. Good results have been obtained by using an apparatus similar to that shown in Fig. 2. The weighted dialysis tubes are suspended in one liter graduated cylinders, and the motor and bar adjusted to give a 9-inch sweep at a rate of about one per second. The dissolved, dialyzed precipitate (Pr-II) is transferred to a beaker set in an ice-salt bath, and cold saturated ammonium sulfate solution is added dropwise, with constant stirring, to a concentration of half-saturation. The mixture is centrifuged in the cold for 30 minutes at about 3000 g. The supernatant (Pr-III) is decanted into a beaker set in an ice-salt bath, and cold saturated ammonium sulfate is added dropwise, with constant stirring, to a concentration of two-thirds saturation. After centrifugation in the cold at top speed for 30 minutes, the supernatant (Pr-IV) is discarded. The precipitate (Pr-IV) is dissolved in about 5 ml of cold distilled water and dialyzed immediately against cold distilled water until free of sulfate, using some device for rapid dialysis such as described above. The dialyzed precipitate (Pr-IV) is carefully adjusted to pH 6.5 with 0.01% sodium hydroxide, and centrifuged in the cold at high speed to remove any precipitate. The supernatant fluid contains the prothrombin. The product should be clear or only very slightly opalescent.

Preparation of plasma accelerator globulin. To 10 ml of the cold Supernatant I from above, 0.75 ml of 1 M sodium sulfate is added dropwise. The barium sulfate precipitate is removed by centrifugation in the cold using high speed to secure complete sedimentation. The clear supernatant (Ac-II) is decanted into a beaker, diluted to 200 ml with distilled water, and rapidly but carefully adjusted to pH 5.5 with 1% acetic acid. The precipitate is removed by centrifugation in the cold and the supernatant (Ac-III) is discarded. The precipitate (Ac-III) is dissolved in 4.5 ml saline, and 7.5 ml of 40% sodium

TABLE I. Preparation of Prothrombin.

Fraction	Vol (cc)	One-stage assay: % of normal plasma	One-stage assay: Yield (%)	2-stage assay: Units/cc	2-stage assay: Units/mg protein*	2-stage assay: Yield (%)
Normal plasma	—	100	—	155	—	—
Starting "	800	97	—	150	2	—
Ppt Pr-I	800	120	124	195	145	130
II	400	260	133	390	860	130
III	20	160	4	460	180	8
IV	13	4000	67	6300	2210	68

* Protein measured by method of Daughaday, *et al.*(4).

citrate are added dropwise (room temperature). Centrifugation is carried out in the cold, using high speed to obtain clean separation. The supernatant (Ac-IV) is discarded. The precipitate (Ac-IV) is dissolved in sufficient chilled saline to give a volume of 2.0 ml, and transferred to a tube in an ice-salt bath; one ml of cold saturated ammonium sulfate, neutralized to pH 7 with strong ammonium hydroxide, is added dropwise with constant stirring. After centrifuging in the cold, the supernatant (Ac-V) is decanted into a tube set in a ice-salt bath, and 1.0 ml cold, neutralized saturated ammonium sulfate is added dropwise with constant stirring. Following centrifugation in the cold for 15-20 minutes at high speed, the supernatant (Ac-VI) is discarded. The precipitate (Ac-VI) is dissolved in 2 ml cold saline, and dialyzed immediately in the cold against 0.02 M sodium citrate in saline until free of sulfate.

Results. In Table I are shown the results of a large scale preparation of prothrombin which was subsequently used for electrophoresis. It will be noted that both one and 2-stage prothrombin assays indicate a yield in excess of 100% for Precipitates Pr-I and Pr-II. This phenomenon was encountered only occasionally, the usual yield at this stage of the preparation being around 80-90%. One-stage and 2-stage assays usually agree fairly well at the various stages of the procedure, but occasionally there are significant differences between the 2 assays. The final product contains a trace of thrombin but no fibrinogen or accelerator globulin by the assays used. Electrophoresis for 2 hours at pH 8.5 (sodium chloride-sodium barbital buffer, ionic strength 0.1) showed 2 components.

TABLE II. Preparation of Accelerator Globulin.

Fraction	Conc. of Ac-G (% of standard)	Yield (%)	Protein conc.(4) (g %)
Starting plasma	82	—	
Super I	74	90	
Ppt Ac-III	80	57	1.30
IV	48	29	.45
VI	37	12	.09

In Table II are shown the results of a typical preparation of plasma accelerator globulin. The preparation contained no prothrombin or fibrinogen, and could be converted to the more active serum Ac-G by thrombin. The final product represents an overall purification of approximately 35 times. As yet no preparations have been attempted which would give sufficient material for electrophoretic analysis.

Discussion. The procedure as described provides a rapid method for obtaining human prothrombin in high yield from citrated plasma. Although not electrophoretically homogeneous, the product is highly active and appears to be free of other known clotting factors. The major portion of the product prepared in this manner appears to be prothrombin. The contaminant has the properties of lipoprotein, and the concentration in the final product appears to be a function of the lipid content of the original plasma. Each milligram of protein in the products prepared in this manner from nonlipemic plasma contains the prothrombin activity of at least 10 ml of normal plasma. This is comparable to the activity of the purified human prothrombin prepared by other technics(5). It would appear, in agreement with Seegers(5), that the concentration of prothrombin in normal human plasma is

somewhat lower than 10 mg %.

The preparation of accelerator globulin is obtained in much lower yield, as might be expected from the greater lability of this substance. The yield may be increased somewhat by omitting the precipitation with citrate, but the product so obtained is not so stable.

The stabilizing effect of citrate during the fractionation procedures is seen with prothrombin as well as Ac-G. The partially purified prothrombin product, Precipitate Pr-II, when dialyzed against saline and stored overnight at 4°C retains only about one-third the activity of a portion of the same preparation kept in 0.02 M citrate.

The stability of the purified prothrombin is also materially influenced by the age of the starting plasma. Preparations made from plasma which had been refrigerated or frozen for a period of several weeks have lower yields and lost one-third to one-half of their activity on overnight storage in the refrigerator. However, even when fresh plasma is used as a starting material, the stability of purified human prothrombin appears to be less than that reported for bovine prothrombin. On storage at —10°C for 3 months, solutions of human prothrombin show a steady decrease to about 20% of their original activity. Stability is not appreciably increased by drying the product from the frozen state followed by storage in the deep freeze.

Summary. A simple and rapid procedure is described for the separation of prothrombin of high specific activity and partially purified accelerator globulin from fresh citrated human plasma. Each product appears to be free from other known clotting factors.

1. Ware, Arnold G., and Stragnell, Robert, *Am. J. Clin. Path.*, 1952, v22, 791.

2. Ware, Arnold G., and Seegers, Walter H., *ibid*, 1949, v19, 471.

3. Lewis, Merle Lovell and Ware, Arnold G., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 640.

4. Daughaday, William H., Lowry, O. H., Rosebrough, N. J., and Fields, W. S., *J. Lab. Clin. Med.*, 1952, v39, 663.

5. Seegers, Walter H., and Andrews, E. B., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 112.

Received October 22, 1953. P.S.E.B.M., 1953, v84.

A One-Stage Method for the Determination of Accelerator Globulin.* (20738)

MERLE LOVELL LEWIS AND ARNOLD G. WARE.†

From the Department of Biochemistry and Nutrition, School of Medicine, University of Southern California, and the Los Angeles County Hospital, Los Angeles.

An assay designed to measure human accelerator globulin (Ac-G) activity should meet certain criteria if dependable results are to be obtained. Of primary importance, the procedure should be simple and rapid because of the lability of human Ac-G. It should also be specific for Ac-G and unaffected by fluctuations in other clotting components in the test material. If possible, all components supplied to the test should be of human origin in order to avoid difficulty due to species differences.

Such a test system has been achieved by using as a substrate aged human plasma containing an excess of prothrombin and fibrinogen, but nearly devoid of Ac-G. Increased sensitivity to small amounts of Ac-G is obtained by using a low concentration of thromboplastin. To assure reproducible results, and to serve as a constant check on the activity of the thromboplastin, a standardized beef plasma is run with each group of tests.

Preparation of reagents. Substrate. One

* This investigation was supported by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army, and by the U. S. Public Health Service.

† We wish to acknowledge the technical assistance of Mrs. Irene M. Smyth. We also wish to express our appreciation to Dr. Fremont E. Davis, Miss Angelyn A. Konugres, and the staff of the Transfusion Laboratory, Los Angeles County Hospital, for their cooperation in obtaining the blood used in these experiments.