6. Wagner, R. R., *J. Immunol.*, 1955, v74, 446.
7. Anacker, R. L., Munoz, J., *Bact. Proc.*, Abstracts of 60th Annual Meeting, 1960, p88.

Received May 17, 1962. P.S.E.B.M., 1962, v111.

Observations on the Course of Fibrinolysis.* (27724)

MARGARET J. WHICHELOW† AND ALFRED JAY BOLLET
University of Virginia School of Medicine, Charlottesville

The existing methods for measuring various components of the fibrinolytic system suffer from uncontrollable variables and effects of multiple factors. For example, timed clot lysis methods of measuring activator are affected by the amounts of plasminogen and fibrinogen. Fibrin plate methods are qualitative rather than quantitative. Where casein is used as a substrate for fibrinolytic enzymes, care must be taken in the interpretation of the results since the physical properties of fibrin differ from those of casein, and plasmin is known to have different affinity for different substrates(1). The studies by Sherry and his co-workers(2) using I^{131} labelled fibrinogen give the most clearcut results, but preparation of the labelled fibrin is tedious.

The method described below has many of the features of the I^{131} labelled fibrinogen method without the necessity of labelling the fibrinogen, and gives information about the various phases of clot digestion, whether the time period under investigation be minutes or hours.

Methods. Five-ml samples of 0.3% human fibrinogen (Pentex) in borate buffer (0.18 M, pH 7.4) containing 0.04 M NaCl were used, and a suspension of human plasminogen‡ was added in most determinations. The mixture was clotted for 30 minutes at room temperature with 1.0 ml of serum, used immediately after preparation from recalcified fresh human plasma, as a source of thrombin since commercial thrombin preparations, bovine in origin, contain large quantities of bovine plasminogen. During clotting the flask was gently shaken to form a discreet, tight clot. The liquid was decanted and the clot washed twice by shaking for 5-10-minute periods in buffer, then incubated in a total volume of 5.0 ml of borate buffer, containing activator (in the studies to be reported human urokinase‡ was used). Lysis of the clot was followed by taking serial 0.5-ml aliquots, which were diluted to 5.0 ml with borate buffer and filtered immediately to remove insoluble fibrin. The amount of solubilized protein was then determined by reading the optical density at 280 mμ. The change in optical density was converted to amount of fibrin lysed by comparison with a fibrin preparation standardized using the Folin-Ciocalteu reagent by the method of Ratnoff & Menzie(3). The data reported are expressed as mg fibrin lysed per 100 ml of incubation solution.

Results and discussion. The plot of fibrin lysed with time is S-shaped (Fig. 1). The initial lag period was found to be due to the physical properties of the clot. Clots which are formed by allowing the mixture to gel without shaking were found to lyse without the lag period, but were not satisfactory because it was impossible to wash out contaminating protein. Flattening of the top of the curve occurred as fibrin dissolution neared completion. Only traces of ultraviolet absorbing material leaked out of the washed clots during incubation in the absence of added activator. On replicate observations, individual points on the curve differed from the mean by an average of 7.6% (± SEM 1.4%).

The maximum rate of lysis (the slope of the straight part of the curve) was found to be

* Supported in part by U. S. Public Health Service Research Grant.

† Present address: Dept. of Experimental Med., Guy's Hospital, London.

‡ Kindly supplied by Dr. J. L. Fahey, Parke, Davis & Co., Detroit. The contents of each vial of plasminogen, which on lytic assay contained 394 Loomis units were suspended in 40 ml of borate-saline buffer.

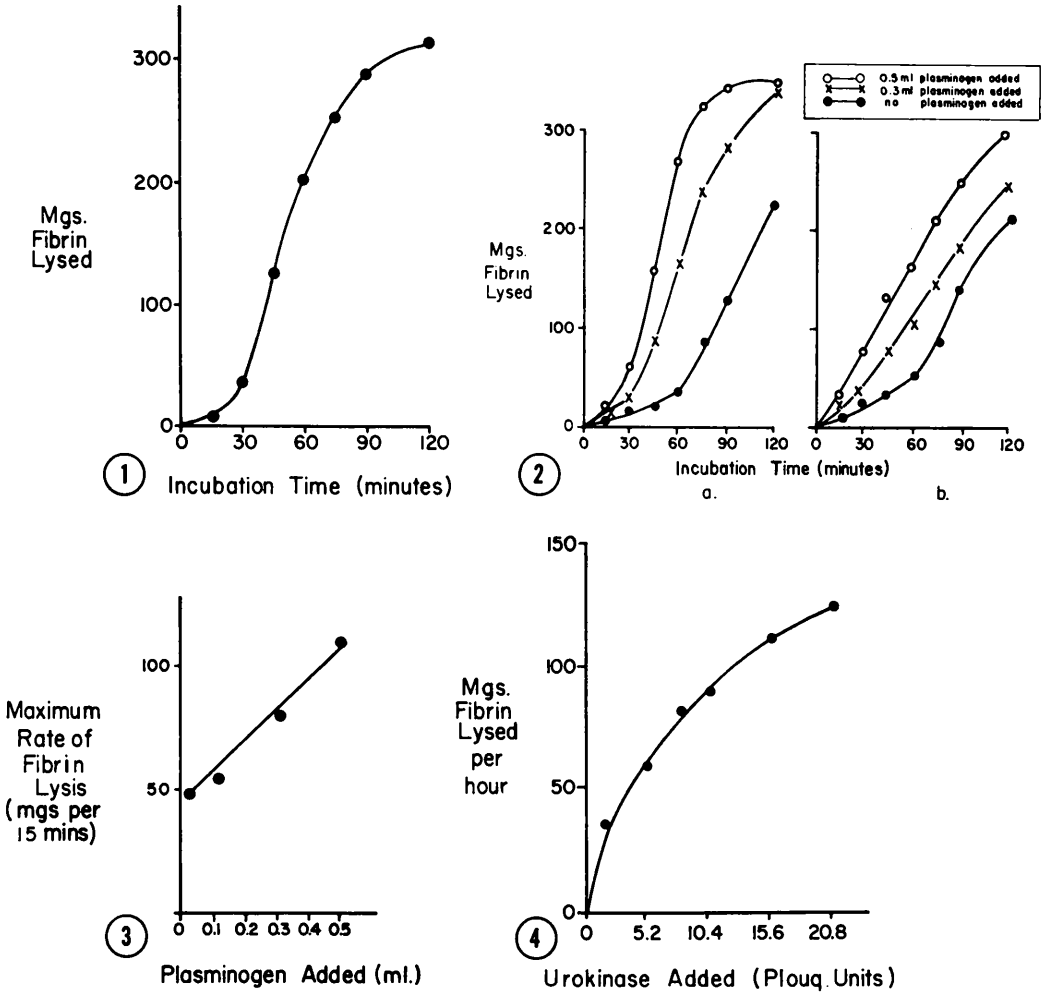

FIG. 1. Time course of lysis of a fibrin clot made in the presence of 0.3-ml plasminogen suspension and activated with 52 Ploug units of urokinase.

FIG. 2. a. Lysis of fibrin clots with various amounts of plasminogen added before clotting. Fifty-two Ploug units of urokinase used as activator. Maximum rate of lysis increases as plasminogen content increases. b. Lysis of fibrin clots with various amounts of plasminogen added after clotting. There is no significant difference between maximum rates of lysis of the clots, although onset of clot lysis occurs earlier with more plasminogen in incubation mixture.

FIG. 3. Relationship between amount of plasminogen added before clotting and maximum rate of fibrin lysis.

FIG. 4. Relationship between amount of urokinase added and maximum rate of clot lysis.

proportional to the amount of plasminogen incorporated into the clot, for a given amount of activator (Fig. 2a and 3). The activity without added plasminogen is due to the inherent amount of plasminogen contaminating the fibrinogen and the thrombin preparations used. In contrast, clots into which no extra plasminogen has been incorporated during clotting, but with increasing amounts of plasminogen and a standard amount of urokinase added after clotting to the incubation medium, there is no increase in the maximum rate of lysis (Fig. 2b). These results are similar to those obtained with I^{131} labelled fibrinogen(2). These data support the view that the most likely agent to be helpful in the treatment of pre-formed thrombi is activator, rather than preparations of plasminogen plus activator, or plasmin.

With this method, a relationship is found

between amount of urokinase (or other activator) added and maximum rate of fibrinolysis for clots with a given amount of incorporated plasminogen (Fig. 4). The method was useful for quantitating activator in human urine, and may be useful for other body fluids.

Summary. The time course of fibrinolysis was observed by release of solubilized protein from washed clots. The slope of the resulting curve was proportional to the amount of plasminogen incorporated into the clots; plasminogen added after clotting did not alter the maximum rate of lysis. With a constant amount of plasminogen, the rate of fibrin lysis was a measure of the amount of activator added.

1. Sherry, S., Fletcher, A. P., Alkjaersig, N., *Physiol. Rev.*, 1959, v39, 343.

2. Alkjaersig, N., Fletcher, A. P., Sherry, S., *J. Clin. Invest.*, 1959, v38, 1086.

3. Ratnoff, O. D., Menzie, C., *J. Lab. and Clin. Med.*, 1951, v37, 316.

Received May 25, 1962. P.S.E.B.M., 1962, v111.

Changes in Plasma Proteins of Mice During Rejection of Skin Homografts and Heterografts.* (27725)

ERLE E. PEACOCK, JR. AND W. PAUL BIGGERS
Department of Surgery, University of North Carolina School of Medicine, Chapel Hill

Hardin reported an increased concentration of alpha globulin in the serum of C3H mice which were rejecting full thickness skin homografts, which he interpreted as being a non-specific reaction to injury and inflammation(1). Because skin homografts produce circulating antibodies of several types and gamma globulins apparently are not increased during antibody formation, we have questioned whether increase in the alpha globulin fraction is not the result of specific antibody production. We have reasoned that, if increased alpha globulin is the result of antibody formation, repeated grafting should cause a continued rise in this fraction as the immune state is increased, and heterografts should produce a greater rise in alpha globulin than homografts of the same size. In addition, if, as Pressman has hypothesized, concentration of tissue rejection antibodies in the graft or graft bed is responsible for previous failures to demonstrate circulating antibodies, alpha globulins should rise following removal of the graft if tissue rejection antibodies are formed in this fraction of the plasma proteins (3). The following experiments were performed to determine whether an increasing state of foreign tissue immunity is accompanied by increased alpha globulin and whether excision of a foreign tissue graft has any effect upon the alpha globulin fraction.

* Supported by grants from Nat. Inst. of Arthritis and Metabolic Dis., U. S. P H S., Bethesda, Md.

Material and methods. C57 mice were used for skin donors and C3H mice for recipients in the homograft experiments. Skin from the ear of New Zealand rabbits was transferred to C3H mice in the heterograft experiments. The same donor animal was used repeatedly when multiple grafts were transferred. The skin grafts were 2 × 2 cm full thickness grafts sutured into a similar size defect on the posterior thorax. Just before the skin grafts were performed, the tail was excised from the recipient animal and 1 cc of whole blood was allowed to drop into a microcentrifuge tube. At the conclusion of the experiment, the mice were beheaded and blood was collected from the carotid system in the same manner. After clot retraction had occurred and centrifugation had been performed, batches of serum were frozen and stored at 0°C. Paper electrophoresis of the serum proteins was determined with a Spiñco Model R paper electrophoresis apparatus; electrophoresis patterns were not affected by freezing, as was shown also by Hardin(1).

Exp. 1. Eighteen mice were autografted