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The Quantity of Thrombin Required to Clot Heparin-Plasma Mixtures.*

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It has been shown[1] that approximately 2.5 units of thrombin are required to cause clotting of 1.0 cc of oxalated bovine plasma in 15 sec. If heparin be added, larger amounts of thrombin are needed to do this, and we shall show that, within certain limits, the amounts needed vary in direct proportion to the amounts of heparin added. This linear relationship between heparin and thrombin provides a simple and reliable basis for the assay of heparin.

Materials and Methods. Crystalline sodium salt of sheep heparin was obtained through the courtesy of Dr. L. B. Jaques of Toronto. On the basis of his analysis it contained 7% water of crystallization, and possessed 26 anticoagulant units[2] per mg (cat assay). It was dissolved in saline (0.9% NaCl).

Fresh beef blood was collected at the slaughter house. Seven parts of blood were mixed with 1 part of 1.85% solution of potassium oxalate, and then centrifuged to obtain the plasma. Such plasma can be stored at —40° for more than a week, without alteration in the properties for which it is used.

Thrombin, free of antithrombin, was prepared by methods previously described.[3] It was dissolved in 0.9% salt solution. Assay was carried out by a simplified technic which involves treating 0.9 cc quantities of oxalated plasma with 0.1 cc quantities of thrombin solution of varying dilution. The tube which clots in exactly 15 sec contains approximately 2.5 units of thrombin.[1] From this and from the known dilution, the unit strength of the original stock solution is readily derived.

Experiments. A series of clotting mixtures was prepared, containing oxalated plasma, together with variable amounts of heparin. These mixtures were then clotted with thrombin. When heparin was omitted altogether, 2.9 units of thrombin were required to cause clotting of 1 cc of the mixture in 15 sec (Fig. 1). On addition of progressively stronger solutions of heparin, it was necessary to add stronger thrombin solutions in order to maintain the standard 15-sec clotting interval. As shown in the chart, the balanced relationship between thrombin and heparin concentration is a straight line function, and can be expressed by the equation

$$Y = 2.89 + 280X$$

This experiment has also been carried out with oxalated plasma of man, dog and pig. In all cases, a linear relationship prevailed. The slope and the intercept on the Y axis were found to vary, however.

Discussion. It must be assumed that the addition of heparin to clotting mixtures brings about destruction of considerable amounts of thrombin long before clotting is complete. In several experiments we have added thrombin in smaller amounts. In one case a clotting interval of 20 sec was selected. The balanced relationship between heparin and thrombin concentration was then not a straight line. In such an experiment heparin and its co-factor have more time to destroy thrombin. It thus appears that the linear relationship is not valid throughout the entire range of experimentation. However, under restricted conditions, as shown in Fig. 1, the linear relationship does hold, and for many purposes it is of great value in the assay of heparin. In conducting such an assay, one prepares a stock thrombin solution

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1 Seegers, W. H., and Smith, H. P., *Am. J. Physiol.*, 1942, **137**, 348.

2 Jaques, L. B., and Charles, A. F., *Quart. J. Pharm.*, 1941, **14**, 1.

3 Seegers, W. H., *J. Biol. Chem.*, 1940, **136**, 103.

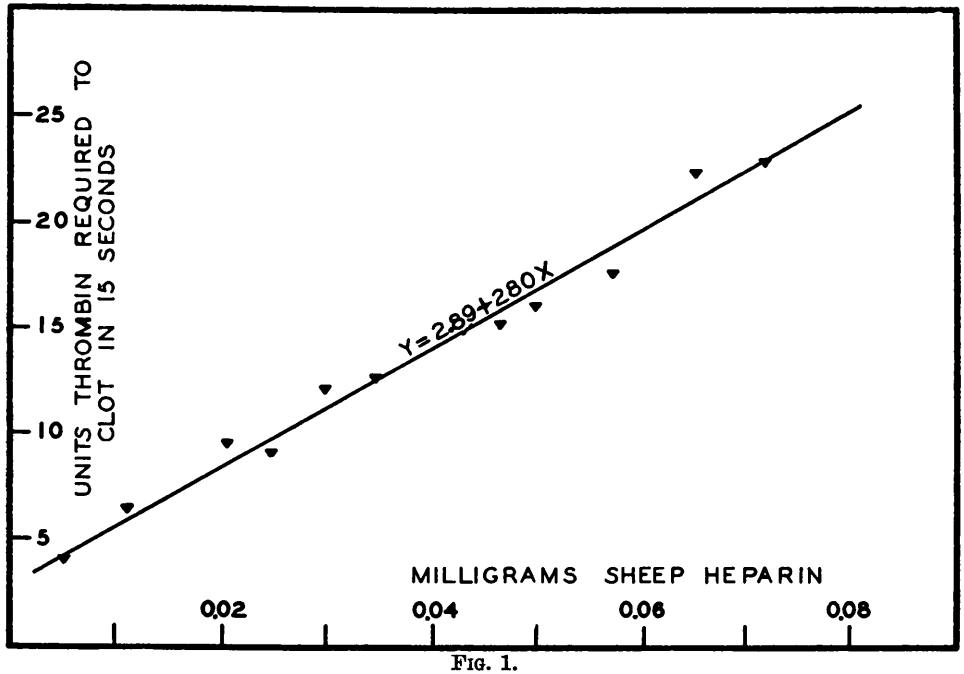

FIG. 1.

To a tube containing 0.8 cc of oxalated plasma were added 0.1 cc of saline (0.9% salt solution) and 0.1 cc of thrombin solution, dissolved in saline, and containing a total of 2.9 units of thrombin. Clotting occurred in exactly 15 seconds. To other tubes 0.8 cc of oxalated plasma was added, together with 0.1 cc of heparin solution of progressively increasing concentration. At each heparin level 0.1 cc of thrombin solution of variable concentration was added. By repeated trial, it was possible to determine the number of units of thrombin needed to cause clotting in exactly 15 seconds. This was done at a room temperature of 26-28°C. The amounts of thrombin in the final clotting mixture are plotted against the corresponding amounts of heparin.

of known titer, somewhat in excess of 200 units per cc. To 0.8 cc oxalated plasma 0.1 cc of the heparin in question is added. The mixture is then clotted with 0.1 cc of the thrombin solution at a room temperature of 26-28°C. By successive trial, and by interpolation, one then adjusts the dilution of the heparin or of the thrombin in such a way that a clotting time of exactly 15 sec is obtained. Knowing the number of thrombin units added, one can readily calculate the heparin concentration from a chart of the type shown in Fig. 1. As a control, a standard solution of heparin should also be analyzed. If necessary, slight corrections can then be made in the value obtained.

Summary. The amount of antithrombin-free purified thrombin required to clot plasma in 15 sec is, within limits, directly proportional to the quantity of heparin added to the plasma. This relationship can be used for the assay of heparin.