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George Washington University	February 4, 1943
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14070

Antithrombic Activity of Plasma: Quantitative Interrelationships.*

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In a previous publication[1] it was shown that the capacity of plasma to destroy thrombin depends fundamentally upon the heparin co-factor which is present. The role of heparin is essentially that of a catalyst. In the present paper we shall show that, within wide limits, the ability of plasma to destroy thrombin can be expressed by a relatively simple equation. The relationships thus established form a sound basis for the assay of antithrombin.

Materials and Methods. Seven parts of bovine blood, freshly drawn, were mixed with 1 part of 1.85% $K_2C_2O_4 \cdot 2H_2O$, and then centrifuged. Each cubic centimeter of the plasma contained 0.8 cc plasma plus 0.2 cc of the oxalate solution. Between experiments, the plasma was stored at −40°.

Heparin was purchased from Roche-Organon, Inc., Nutley, New Jersey. It was diluted with oxalated saline (0.85% NaCl + 0.09% $K_2C_2O_4 \cdot 2H_2O$) to make a concentration of 40 Toronto[2] units per cc.

The thrombin used in these experiments was prepared by methods previously

* Aided by a grant from the John and Mary R. Markle Foundation. Funds were also supplied by the Graduate College, State University of Iowa.

1 Seegers, W. H., *Science*, 1942, **96**, 300.

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

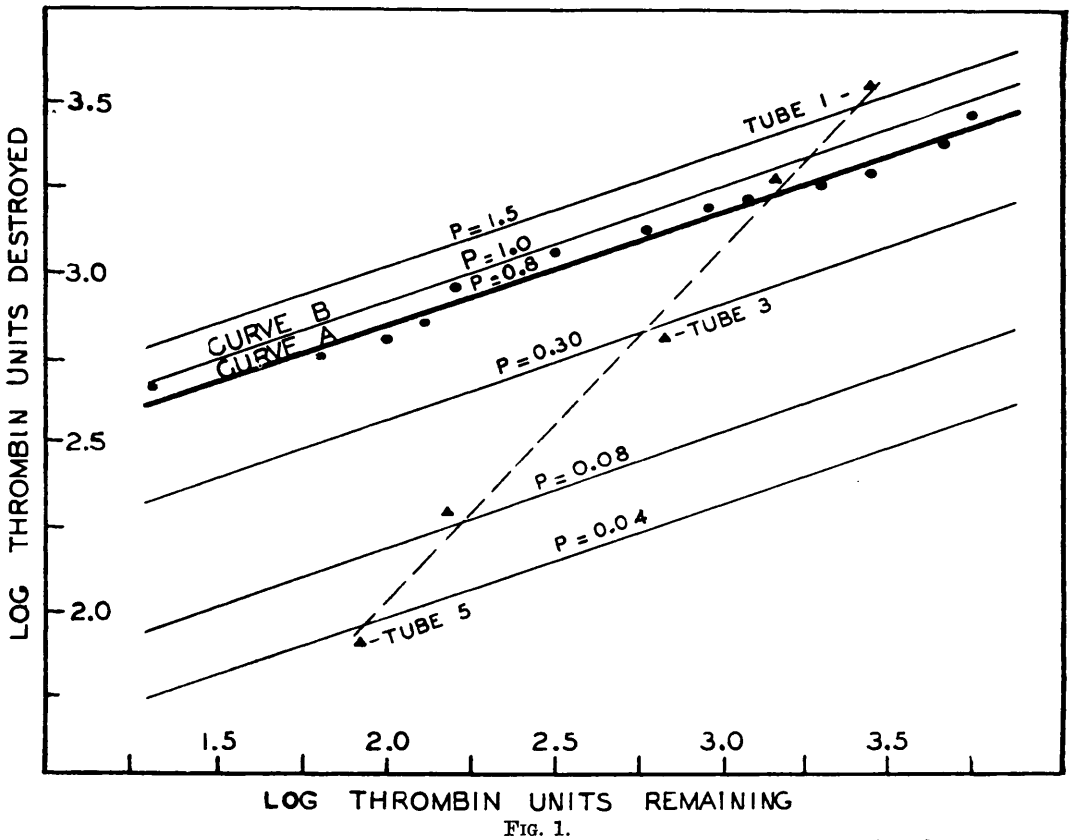

FIG. 1.

In each case, the initial thrombin, and also the mixtures of plasma and of thrombin, were assayed for thrombin titer as follows: A stock solution, A, is prepared by mixing 1 volume of isotonic imidazole buffer[5] with 2 volumes of 15% acacia (in 0.9% NaCl) + 6 volumes of saline (0.9% NaCl). Solution B is prepared by mixing 1.5 cc of solution A with 0.5 cc of the thrombin solution to be analyzed, the latter diluted suitably with oxalated saline (0.85% NaCl + 0.09% $K_2C_2O_4 \cdot 2H_2O$). To a 3 cc tube, add 0.1 cc fibrinogen solution[4] + 0.4 cc of solution B. The clotting time is noted. By variable dilution and by interpolation one determines to what extent the thrombin solution must be diluted, in preparing solution B, in order that the final clotting mixture will coagulate in exactly 15 seconds. The product of all the various dilutions involved in the titration, multiplied by the correction factor, 1.5 (see text), represents the number of thrombin units in the specimen being analyzed.

described;[3] it was free of antithrombin, and it had a potency of 8000 units per mg N. It was dissolved in oxalated saline (0.85% NaCl + 0.09% $K_2C_2O_4 \cdot 2H_2O$).

In making the assay of thrombin, the thrombin unit is defined[4] as the amount of activity required to clot 1 cc of standard fibrinogen solution in 15 seconds. This standard fibrinogen solution contains buffer, a supplement of colloid, and an optimum amount of calcium chloride. In the present experiments, however, it was necessary to have oxalate present in order to prevent conversion of prothrombin into thrombin. Under these conditions, thrombin reacts more sluggishly with fibrinogen than when free calcium is present.[4] It has been found possible to correct this deviation from standard units by the use of a multiplication correction factor, 1.5. The results recorded in the present paper have been subjected to this correction, and the units are therefore the same as those previously used in this laboratory.

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

[4] Seegers, W. H., and Smith, H. P., *Am. J. Physiol.*, 1942, **137**, 348.

[5] Mertz, E. T., and Owen, C. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 204.

Results. To each of a series of tubes were added 1 cc of oxalated plasma, 0.1 cc of heparin solution, and variable amounts of thrombin. Oxalated saline was added in amounts sufficient to give a total volume of 4.0 cc. Under these conditions, the total amount of thrombin eventually destroyed is a function of the amounts of thrombin and of plasma (co-factor) used. The heparin added merely accelerates the reaction.[1] In the present experiments, heparin was present in sufficient amounts to complete the reaction within a very few minutes; nevertheless, an entire hour was allowed before the remaining thrombin was measured. During that time the fibrin was allowed to remain in the mixture.

In plotting thrombin destruction (Fig. 1) it was discovered that a linear relationship exists (Curve A) when the logarithm of thrombin destruction (Y) is plotted against the logarithm of thrombin remaining (X). The equation expressing this relationship may be written as follows:

$$(1) \qquad \log Y = K + n \log X$$

This equation also applies if heparin is not added to the reaction mixture. Under those circumstances much more time is required before equilibrium is reached.

In the above experiment, the individual tubes contained variable initial amounts of thrombin, but constant amounts of plasma. In each case the plasma (1.0 cc) contained oxalate solution; in reality each tube contained only 0.8 cc of plasma. A number of experiments were then made, using several fixed amounts of thrombin, but variable amounts of plasma with each. At each thrombin level tested, thrombin destruction was always more pronounced when larger amounts of plasma were added. Empirically it was determined that the equation, already given, could be modified to include variable amounts of plasma (P):

$$(2) \qquad \log \frac{Y}{P} = K + n \log \frac{X}{P}$$

In using the data already given in Curve A, Fig. 1, the constants for this equation can be calculated. The equation can then be written in the following form:

$$(3) \qquad \log \frac{Y}{P} = 2.235 + 0.336 \log \frac{X}{P}$$

With the aid of this equation, we have plotted, in Fig. 1, a series of parallel lines representing theoretical values with different amounts of plasma. The values plotted represent plasma volumes varying from 0.04 cc to 1.5 cc.

In addition to the experimental results centering about curve A, Fig. 1 also shows one group of our experimental points, obtained with variable amounts of plasma. Thus, tube 1 contained 1.52 cc; No. 3, 0.32 cc; No. 4, 0.08 cc; No. 5, 0.04 cc. It will be seen that the experimental points lie quite close to the theoretical thrombin levels indicated by the parallel lines which had been constructed on the basis of equation 3.

It might be added that the constants in equation 3 are somewhat different when highly impure thrombin is used. Evidently, certain impurities affect the reaction to some extent.

Discussion. The equation, expressing the logarithmic relationship between thrombin and co-factor, is at present entirely empirical. A preliminary attempt by Doctor George Glockler of the Department of Chemistry to reconcile the equation with traditional reaction mechanics has given encouraging results, which are, nevertheless, still incomplete.

The results which we have presented provide a sound basis for assay of antithrombin (*i.e.*, of the co-factor). By determining the values of X and of Y one can readily determine the value of P. From the standpoint of precision, the relative amounts of thrombin and of plasma which one mixes should be so adjusted that approximately 50% of the thrombin will be destroyed. From the equation, or from the chart, the co-factor titer is readily calculated, and expressed in percentage of the normal plasma, upon which the equation and the chart are based. If, instead of these relative values for the co-factor, one wishes to express results as units, one can select a unit standard based either upon a definite amount of plasma, or upon definite percentage destruction of a fixed amount of thrombin.

Summary. In the destruction of thrombin

by plasma "antithrombin," heparin serves merely as a catalyst. The amount of thrombin eventually destroyed by a given amount of plasma is dependent fundamentally upon the amount of co-factor present. The concentrations may be expressed by the following equation:

$$\log \frac{Y}{P} = 2.235 + 0.336 \log \frac{X}{P}$$

where Y is the amount of thrombin destroyed, X is the amount of thrombin remaining, and P is the amount of plasma (bovine) present in the reaction mixture.

14071

Reduction of Blood Pressure of Hypertensive Rats by Administration of Certain Marine Oils.*

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Our studies on the nature of the substance present in renal extracts which is effective in reducing the blood pressure in experimental hypertension have demonstrated that this active principle is of relatively small molecular weight.[1] This is evident from its chemical and physical properties as well as its effectiveness when administered orally. It was hoped, therefore, that sources other than mammalian kidney, in which the active principle is present in only small amounts, might be found as a more practical source of the material. Attempts to obtain active extracts from the kidney of fishes and amphibia have up to the present been unsuccessful. However, we have not been able to obtain large amounts of fresh tissue from these sources. Since these experiments have been in progress, there has appeared a recent report of Friedman, Soloway, Marrus and Oppenheimer,[2] in which certain quinones were demonstrated to have the capacity to reduce the blood pressure. We have been able to confirm these results. There has also appeared the report of the effectiveness of vitamin A,[3] which we have found to be relatively inert. It suggested itself, therefore, that the active material might be a derivative of the vitamin itself or one of the contaminants present in fish oils from which vitamin concentrates are prepared.

Methods. Rats have been used throughout in the present study. They were rendered hypertensive by fixation of the kidney by looping a stout cotton thread over the poles in the shape of a figure 8.† The blood pressures were determined by the plethysmographic method.[4]

The materials used in the present study consisted of pure provitamin A in the form of a mixture consisting of 90% β-carotene and 10% α-carotene and preparations and concentrates of various fish oils. These were administered orally by admixture with the animal's food. In addition to the preparations used in their natural form, some of the preparations were subjected to the following procedures which resulted in the destruction of vitamin A.

(1) Aeration by means of a current of air

* Aided by grants from the John and Mary R. Markle Foundation, from the National Life and Accident Insurance Company and from Mr. Joe Werthan.

1 Grollman, A., Harrison, T. R., and Williams, J. R., *Fed. Proc.*, 1934, **1**, 34; in press.

2 Friedman, B., Soloway, S., Marrus, J., and Oppenheimer, B. S., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 195.

3 Pena, J. G., and Villaverde, M., *Rev. Cubana Cardiol.*, 1940, **2**, 322.

† This simplified procedure has proved to be superior to that in which silk is applied to the kidney, as originally suggested by Page.

4 Williams, J. R., Grollman, A., and Harrison, T. R., *J. Clin. Invest.*, 1939, **18**, 373.