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Transactions of the
6th Congress of the European Society of Haematology
Copenhagen, 1957

BASEL (Switzerland) S. KARGER NEW YORK

Separatum Printed in Switzerland

University Institute for Thrombosis Research, Rikshospitalet, Oslo, Norway (Head: Prof. *P. A. Owren*)

Assay of Prothrombin

By H. Stormorken and P. Hjort

There are still technical and theoretical problems concerning the assay of prothrombin which are unsolved. At present, the methods for prothrombin assay can be divided into three groups: a) the two-stage assay measuring the peak activity, b) the two-stage assay measuring the area enclosed by the activity curve, and c) the one-stage assay. These methods offer different solutions to the main two problems: to eliminate the influence of both the prothrombin conversion factors, and of the antithrombins.

In this study we have investigated the influence of some nonprothrombin factors on the following methods: The modified two-stage method (three-stage method) used in our laboratory, the two-stage area method[1] and the Russell viper venom-cephalin method[3]. Based on these investigations, and on the results of other workers, we concluded that the set-up of the methods shown in tables I, II and III to be the most satisfactory.

I. The influence of fibrinogen: Fibrinogen affects the results of the area method[2]. About half of the thrombin formed is adsorbed onto the fibrin. The test plasma should therefore be defibrinated by a standardized procedure to get reliable results. The fibrinogen in the test plasma did not influence the results with the three-stage and Russell viper venom-cephalin method. In the last stage of the area and three-stage method we prefer to use bovine fibrinogen, because the end points are much easier to read than is the case with human fibrinogen due to the opacity which appears just before clotting.

Table I

Three-Stage Method

1.0 ml saline thromboplastin 1.0 ml purified proconvertin 1.0 ml purified proaccelerin (bovine) 1.0 ml calcium chlor., 10 mM	Step I Prothrombinase formation
0.2 ml test plasma dil. 1/50 0.2 ml calcium chlor., 20 mM 0.2 ml prothrombinase sol.	Step II Prothrombin conversion
0.4 ml bovine fibrinogen 0.2 ml conversion mixture	Step III Thrombin assay

Due to species specificity the thromboplastin and proconvertin should be homologous to the test plasma.

Table II

Two-Stage Area Method

0.4 ml thromboplastin dil. 1/20 0.4 ml defibrinated test plasma 0.4 ml calcium chlor., opt. conc.	Incubation mixture
0.4 ml purified bovine fibrinogen 0.2 ml incubation mixture	Thrombin assay

Evaluation: Measurement of the area enclosed by the thrombin curve.

Table III

Russell Viper Venom-Cephalin Method

0.2 ml adsorbed bovine plasma
0.2 ml test plasma diluted 1/50
0.2 ml Russell viper venom-cephalin mixture
0.2 ml calcium chloride containing a dilute adsorbate from prothrombin-free human serum

II. The influence of proaccelerin-accelerin: It was shown in the original publication of the RVV-cephalin method that the proaccelerin concentration of the test plasma did not influence the results, since it showed normal prothrombin concentration in proaccelerin-deficient plasma. The bovine substrate plasma supplies large amounts of proaccelerin which is converted to accelerin by the RVV during the incubation period before calcium is added (fig. 1). The three-stage method was shown also to be insensitive to the proaccelerin concentration in the test plasma. The area method, however, did not give normal values with proaccelerin-deficient

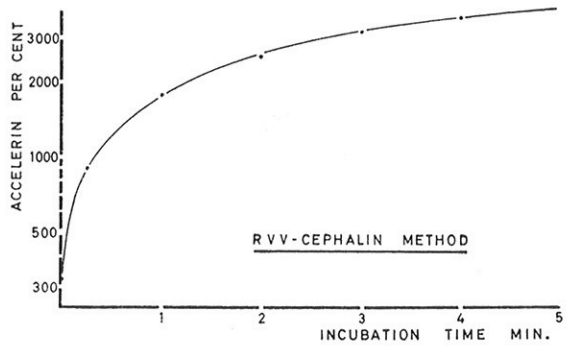
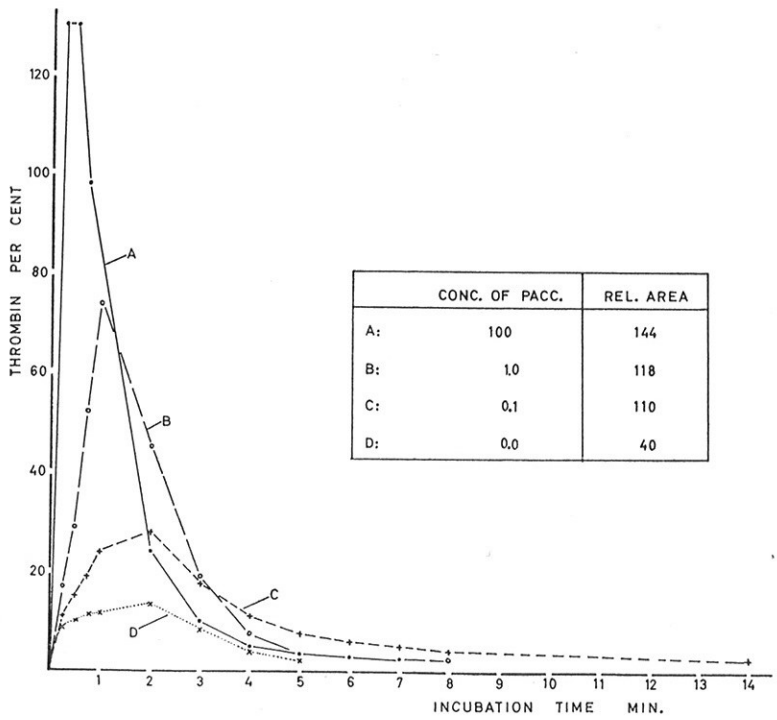


Fig. 1. The formation of accelerin from proaccelerin by the Russell viper venom in the substrate plasma of the RVV-cephalin method before addition of calcium.

plasma (fig. 2). This method, therefore, is sensitive to proaccelerin, and we found that between 15–20 % of proaccelerin was necessary to give normal area. This implies that a defibrinated plasma must be assayed within a certain time due to the lability of accelerin.



	CONC. OF PACC.	REL. AREA
A:	100	144
B:	1.0	118
C:	0.1	110
D:	0.0	40

Fig. 2. The sensitivity of the two-stage area method to the concentration of proaccelerin in the test plasma.

III. The influence of proconvertin: Neither the RVV-cephalin method nor the three-stage method is sensitive to proconvertin in the test plasma since they give normal prothrombin values with proconvertin-deficient plasmas. Concerning the area method, most of our results indicate that the area is diminished when the proconvertin is greatly reduced or absent, while other workers have found normal area with this plasma. This might be explained by a variation in the ability of different thromboplastins to act as "partial thromboplastin", since the conversion in this case is mediated through the intrinsic system.

IV. The influence of the Stuart factor: The three-stage method gave a normal value for prothrombin with a plasma deficient in Stuart factor. Because of the small amounts of Stuart plasma available, we have not been able to investigate the influence of the Stuart factor on the area method, but since this factor affects both the extrinsic and intrinsic system, it is reasonable to believe that the area method is sensitive to the Stuart factor. Concerning the RVV-cephalin method we know that the Stuart factor affects the RVV-cephalin time[4]. Accordingly, the original RVV-cephalin method showed less than 5 % of prothrombin in Stuart plasma. To make the method insensitive to this factor we modified the method by adding a dilute adsorbate from prothrombin-"free" human serum to the calcium solution.

Earlier investigations in our laboratory have shown that serum shortens the RVV-cephalin time, and we have studied the factor responsible for this shortening. We believe that this factor, which we provisionally named RVV-cofactor, probably is identical with the Stuart factor, because serum from a patient with the Stuart defect did not shorten the RVV-cephalin time more than what should be expected from its high prothrombin concentration. The physicochemical properties of these two factors also agree closely.

V. The influence of antithrombin: Variations of antithrombin in the test plasma play little if any role in the one-stage RVV-cephalin method, because of the great antithrombin content in the substrate plasma. Whereas, in the three-stage and the area method, such variations may be important.

The model experiment shown in fig. 3 was performed to study the effect of the rate of thrombin formation on the neutralization by antithrombin. The same amount of thrombin was added to aliquots of serum at varying speeds and the neutralization curves followed. The area enclosed by this curve was greater when the addition was slow than when it was instantaneous. This indicates that the area method might be influenced by the rate of prothrombin conversion. Variations in the conversion time is largely avoided in the three-stage method by the introduction of the first stage with the production of an effective prothrombinase.

In the experiment shown in table IV we studied the amount of thrombin evolved in a two-stage method on serial dilutions of the test plasma. We confirmed the earlier finding that relatively more thrombin appears with increasing dilution. We interpret this finding to mean that the effect of antithrombin is reduced by dilution. With the area method, which uses undiluted plasma, one probably does not measure more than 1/5–1/10 of the thrombin evolved due to the great influence of antithrombin.

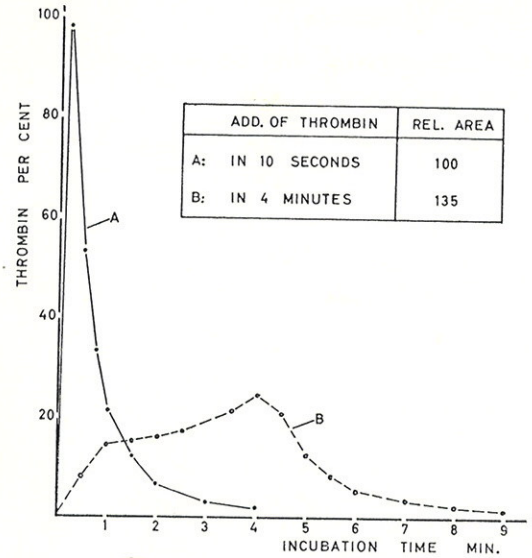


Fig. 3. Inactivation of thrombin in serum: The effect on the area of rapid versus slow addition of the thrombin.

Table IV

The effect on the thrombin yield of diluting the test plasma as estimated from the area and peak values in a two-stage method without addition of conversion factors

Plasma dilution	Area	%	Peak	%
Undiluted	90	100	130	100
1/2	78	168	92	113
1/10	92	1020	33	218
1/20	72	1630	16	246

In the three-stage method the concentration of antithrombin is minimized by preparing the prothrombinase from purified reagents, and by using a high dilution of the test plasma (1/150 in the conversion mixture).

VI. Comparison of the results with the three-stage method and the RVV-cephalin method: By comparison of the results with these two methods on several normal and pathological plasmas, closely parallel results were obtained.

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