186. THE ESTIMATION OF PROTHROMBIN IN HUMAN PLASMA

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It is well known that a deficiency of vitamin K leads to a reduction in plasma prothrombin and a consequent tendency to haemorrhage. Estimations of plasma prothrombin are therefore needed in experimental studies on vitamin K deficiency, and in the assay of the vitamin and related compounds. Moreover, the estimation has an important clinical application, especially in estimating the tendency to haemorrhage in cases of biliary obstruction, and so giving warning of the risks of operation. There is abundant evidence that the tendency to haemorrhage in these cases is due to low plasma prothrombin, which results from deficient absorption of vitamin K in the absence of bile salts from the intestine.

A consideration of the methods available for estimating plasma prothrombin indicated the need for a technique which should combine quantitative accuracy with reasonable simplicity. Indeed it seemed doubtful whether any method could be regarded as truly quantitative. That which seemed most satisfactory in principle was the method first published by Warner *et al.* [1936] and subsequently modified by them [Smith *et al.* 1937]. It is a very elaborate method, and apparently has only been used by its authors. The present paper describes a study of the factors involved in a method of this type, with a view to securing quantitative results, and introduces a modified technique which is distinctly simpler than the original.

The principle of the original method was as follows. The plasma to be tested was freed from fibrinogen by treatment with a thrombin preparation, and the mixture kept until the added thrombin had been completely destroyed by the antithrombin of the plasma. The fibrinogen-free plasma was then suitably diluted with saline, treated with calcium and a tissue extract (prepared from beef lung), and allowed to incubate for the conversion of prothrombin into thrombin. The thrombin activity of the incubating mixture was estimated by determining the time taken to form a clot with added fibrinogen. It was found that during incubation the thrombin first increased, then reached a temporary steady level, and later decreased. The unit of thrombin adopted was the amount giving a clotting time of 15 sec. under the conditions of the method, and the estimation was based on determining the dilution of plasma required to give this standard clotting time during the period of maximal activity. For the plasma of a normal dog, which was the first species studied, the titre obtained was 325–400 units, and the incubation time required for thrombin development was 30–90 sec. Later the method was applied to various species, including man [Warner *et al.* 1939, 1, 2]. Human plasma gave a titre not much lower than that of the dog, but the rate of conversion of prothrombin into thrombin was much slower in human plasma.

Now the method of calculation here used implies that two samples of plasma, normal and abnormal, can be precisely compared on the basis of the different dilutions which give the same thrombin activity—for example, a plasma of “50 units” diluted 1 : 50 is compared with a plasma of “300 units” diluted 1 : 300. This will only be satisfactory if the antithrombin of the plasma is inactive at both dilutions. The authors believed this to be the case; they considered that under the conditions of their method prothrombin was completely converted into thrombin without loss. But some of their statements seem to cast doubt on this point. First, in their incubating mixtures they obtained only a temporary steady level of maximal thrombin activity followed by deterioration. This suggests that the temporary steady level may not represent complete conversion of prothrombin into thrombin but may result from a balance between the rates of formation and destruction of thrombin. If this is so, the antithrombin effect may be greater in the lower dilutions of plasma, and this would lead to an underestimate of the titre of samples of low prothrombin content. Secondly, they state that it is necessary to determine exactly the dilution required to give the standard clotting time; an interpolation curve could be used only to correct for deviations of 1 or 2 sec. from the standard 15 sec. This seems to imply that they obtained no regular relationship between dilution and thrombin activity applicable to all plasma samples. If a regular relationship had been obtained, it should have been possible to determine it for normal plasma over a wide range of dilutions and compare the normal with the abnormal at any convenient dilution. The method of calculation therefore seems to be arbitrary.

From the technical point of view, the method is complicated because it requires two reagents, thrombin and fibrinogen, which are laborious to prepare and which are only stable for short periods. In the new modification, the procedure is simplified in two respects. First, the preliminary removal of fibrinogen from the plasma is omitted, and this eliminates the need for thrombin as a reagent. As the plasma is used in high dilution, the amount of fibrinogen in the mixture incubated with calcium and thromboplastin is very small, and the slight clot which forms does not interfere with the measurement of samples for clotting tests with added fibrinogen. It was found, too, that the presence of this trace of fibrinogen caused no reduction in the thrombin activity developed in the mixture, as compared with samples from which fibrinogen had been removed.

Secondly, in the new modification, there is a perfectly regular relationship between plasma dilution and thrombin developed, and it is therefore possible to base the estimation on a short series of convenient dilutions, instead of discovering the precise dilution of the plasma which gives a standard thrombin activity. The new method differs also in other details. The source of thromboplastin is rabbit brain, chosen as being more easily pulped and extracted than lung. The concentrations of calcium and tissue extract used are those which have been determined to fall within the optimum range for the conversion of prothrombin into thrombin. Both must be in optimal concentration; an excess of either is inhibitory. Incubation for thrombin formation is carried out at 15–20°, because the thrombin is most stable in this range of temperature [Ferguson, 1938, 1, 2], and clotting tests with fibrinogen are made at 37–38°.

The rates of conversion of prothrombin into thrombin are slow. The slow conversion is partly due to the special properties of human prothrombin, and partly to the low incubation temperature.

When due allowance is made for the known differences (in volume relations and temperature) between the modified method and the original method of

Warner *et al.*, it is found that the modified method gives definitely lower activity than the original, but it seems to have the advantage of greater regularity.

It will be convenient to give first the description of the new technique and method of calculation, and then to proceed to a fuller consideration of the different reactions involved and of the grounds for regarding the method as giving quantitative results.

Reagents.

The estimation of plasma prothrombin

(1) Isotonic sodium chloride (0.9 %).

(2) Glyoxaline buffer.

Take 1.72 g. glyoxaline. Add 90 ml. N/10 HCl and make up to 100 ml. with water.

This solution is isotonic with blood and gives pH 7.25.

(3) Tissue extract.

(a) Stock tissue preparation. Take the brain of a freshly killed rabbit. Free it from blood vessels as completely as possible by stripping off the membranes. Grind the tissue in a mortar with acetone. Pour off the liquid and grind the tissue with fresh acetone. Repeat this several times, and then transfer the dry tissue to a Büchner filter and remove the remaining acetone by suction. If necessary, wash the tissue with acetone again and continue suction until the tissue is quite dry. The dry powdered brain is kept as a stock, and retains its activity for about 10 days.

(b) For use, an extract of the dried brain is made in a solution containing 0.05 g. anhydrous CaCl_2 and 0.855 g. NaCl per 100 ml.

Take 0.05 g. dried brain; grind it in a mortar with 5 ml. of the calcium-containing saline, adding the solution gradually so as to obtain a smooth emulsion. Centrifuge the mixture; take off the supernatant opalescent fluid, and dilute it with three times its volume of the calcium-containing saline. This extract, containing calcium and thromboplastin, is made up fresh as required.

(4) Fibrinogen.

For the preparation the following reagents are needed:

(a) Magnesium hydroxide suspension.

Take 100 ml. 20 % MgCl_2 and gradually add 25 ml. concentrated ammonia. Filter on a Büchner funnel and wash repeatedly with water. Suspend the magnesium hydroxide in 30 ml. isotonic sodium chloride.

(b) Buffered oxalated saline.

Take 5 ml. 1.85 % potassium oxalate; add 5 ml. glyoxaline buffer and make the volume to 250 ml. with isotonic sodium chloride.

(c) Buffered saline. Take 5 ml. glyoxaline buffer and dilute to 250 ml. with isotonic sodium chloride.

(d) Saturated ammonium sulphate solution.

(e) Oxalated plasma. In a clinical biochemical laboratory it is convenient to use mixed residues of human oxalated blood obtained in the routine work. The blood should not be more than a few days old. It is centrifuged and the plasma is separated.

Take 30 ml. plasma and add 6 ml. magnesium hydroxide suspension. Mix well and centrifuge off the magnesium hydroxide, which carries down a considerable proportion of the plasma prothrombin. Take 30 ml. of the supernatant plasma and gradually add 10 ml. saturated ammonium sulphate, mixing well. Fibrinogen is precipitated. Centrifuge, discard the supernatant fluid and dissolve the fibrinogen in 30 ml. buffered oxalated saline. When solution is complete, add 10 ml. saturated ammonium sulphate to precipitate the fibrinogen a second time. Centrifuge; discard the supernatant fluid, and dissolve the fibrinogen in 10 ml. buffered oxalated saline. Transfer the fibrinogen solution to a collodion sac and dialyse for at least an hour against about 400 ml. buffered oxalated saline. Discard the external fluid, replace it with buffered saline (without oxalate) and repeat the dialysis.

The fibrinogen preparation is now ready for use. It will keep one day at ordinary refrigerator temperature. It can be stored for about a week if sealed up in ampoules and placed in a vacuum flask with solid CO_2 on the refrigerator. If this method is adopted the preparation should be distributed in lots of about 2 ml. and each opened only as required.

The method of preparation of fibrinogen is essentially the same as that used by Warner *et al.*, but there are two differences in detail. They corrected the reaction of the plasma after adsorption with $\text{Mg}(\text{OH})_2$ to pH 7.4. This is unnecessary because the addition of the $(\text{NH}_4)_2\text{SO}_4$ itself brings the reaction of the alkaline plasma back to the desired range. Secondly, the pH is controlled throughout the preparation by including glyoxaline buffer in the saline used for solution and dialysis. Under these conditions satisfactory preparations were regularly obtained. The dialysis was usually done in the refrigerator, but no difficulty arose from carrying out the other manipulations at room temperature.

The necessity for using a freshly made fibrinogen preparation is the only inconvenient feature of the method. In laboratories equipped with apparatus for the rapid desiccation of protein solutions (e.g. sera) it would be possible to prepare permanent preparations of desiccated fibrinogen [Ferguson & Erickson, 1939].

Procedure

Oxalated venous blood is collected as quickly as possible, with the minimum of oxalate necessary to prevent clotting. The plasma is separated. A series of dilutions of the plasma in isotonic sodium chloride is prepared, giving 1 vol. plasma in 25 vol., 1 : 50, 1 : 100 and 1 : 200.

From each of these, the following mixtures are made:

- 1 ml. diluted plasma.
- 1 ml. glyoxaline buffer.
- 2 ml. brain extract (containing Ca).

It will be noted that the dilutions of plasma at the stage of incubation are 1 : 100, 1 : 200, 1 : 400 and 1 : 800. These mixtures are incubated at 15–20° and at intervals samples are taken for clotting tests as follows. A sample of 0.2 ml. is measured into a test tube and placed in a water bath at 37°. Fibrinogen, 0.1 ml., is rapidly added from a pipette calibrated “to blow out”. A stop watch is started at the moment when the fibrinogen is introduced and the time taken when the mixture gels. The end point is determined by dipping into the mixture a capillary pipette provided with a teat, drawing the fluid up slightly into the capillary, and lifting the tip of the pipette just out of the fluid. This manipulation is rapidly repeated until a solid gel adheres to the capillary tip. With a little practice the end point can be read very precisely.

In repeated clotting tests on the incubating mixtures it is found that the times become shorter as thrombin increases, then remain steady for some time and later lengthen. The result to be recorded is the clotting time for each mixture during the period of steady activity. It is always necessary to take repeated tests to ensure that the readings are made at the right stage, as the time required for development of maximal thrombin activity varies with different plasma samples. But in practice only a few tests are necessary on each incubating mixture, because the period of maximal activity is heralded by the appearance of a slight fibrin clot in the mixture. When this occurs, the tube is shaken to cause the little clot to retract and the clotting tests are begun. Each mixture is tested at intervals of 2 or 3 min. The period of maximal activity is reached earliest in the 1 : 100 dilution, and later with increasing plasma dilution. For this reason it is possible to start all four mixtures incubating at the same time, fitting in the clotting tests on all as they reach their maximal activity. For a

normal plasma the usual times required for the development of maximal activity are:

Dilution 1 : 100:	5-10 min.
1 : 200:	10-20 "
1 : 400:	20-30 "
1 : 800:	30-40 "

The standard clotting times in the clotting tests with fibrinogen during the period of maximal activity, for an average normal plasma, are:

Dilution 1 : 100:	clotting time 26 sec.
1 : 200:	" 42 "
1 : 400:	" 67 "
1 : 800:	" 107 "

The dilutions mentioned are those existing in the incubating mixture. The further dilution of 2 to 3 in the clotting test is not included in the recorded figures.

When the method is applied to a plasma of low prothrombin content, the clotting times are longer and the times required for the development of maximal activity are also longer. It is therefore important to note the appearance of the slight fibrin clot in the incubating mixture; this is especially useful as a guide to the time tests and should be made when dealing with a plasma of unknown prothrombin titre.¹

Clotting times can be accurately read in the range 15-120 sec. For clotting times above 120 sec. it is difficult to obtain sharp end points. Therefore, when dealing with samples of plasma of low prothrombin titre, the higher dilutions cannot be used, but it will be possible to get readings with one or more of the dilutions mentioned in all cases.

Calculation

The titre of any plasma, expressed as a percentage of the normal average, is given by the formula $T/T' \times 100$, where T is the clotting time for the standard normal plasma at any given dilution, and T' the clotting time for the "unknown" plasma at the same dilution.

For example, a plasma from a case of obstructive jaundice gave the following readings:

Dilution	T' sec.	T sec.	$T/T' \times 100$
1 : 100	56	26	46
1 : 200	90	42	47
1 : 400	141	67	47½

In general, the values obtained from readings made at different dilutions agree well, though there is sometimes more variation than in the above example. The fact that the results are consistent for different dilutions shows that this method of calculation affords, at least, a practical means of comparing abnormal plasma with the standard normal. The question whether it also gives a truly quantitative scale of prothrombin titres requires an understanding of the different reactions involved. Three reactions need to be considered from the quantitative aspect, namely (1) the formation of fibrin from fibrinogen under the influence of thrombin, (2) the conversion of prothrombin into thrombin and (3) the effect of plasma antithrombin in high dilutions of plasma.

¹ The appearance of this slight fibrin clot, though generally a useful guide, should not be relied on absolutely. The clot is not noticeable in a 1 : 800 dilution of normal plasma, and may easily be missed in lower dilutions of a plasma poor in fibrinogen.

Factors involved in the estimation of prothrombin

(1) *The relation between thrombin concentration and the rate of clotting of fibrinogen.* For experiments on this reaction thrombin preparations were made as follows.

Oxalated plasma, 10 ml., is diluted to 100 ml. in cold distilled water; a drop of octyl alcohol is added, and CO_2 is passed through the mixture for about 5 min. to precipitate prothrombin. The precipitate is separated by centrifuging and dissolved in 10 ml. isotonic sodium chloride containing a little glyoxaline buffer; 0.5 ml. $M/10 \text{ CaCl}_2$ is then added and the mixture warmed to 37° . The clot which forms is removed by rolling it out on a glass rod. The excess Ca is removed either by dialysis or by precipitation with an equivalent amount of oxalate.

Thrombin preparations made in this way are very stable, retaining their activity for several weeks on the refrigerator with only slight loss.

Table 1

Dilution of thrombin	Clotting time sec.
Undiluted	$7\frac{1}{2}$
1 : 2	$11\frac{1}{2}$
1 : 3	14
1 : 4	20
1 : 6	$25\frac{1}{2}$
1 : 10	37
1 : 15	55
1 : 20	$74\frac{1}{2}$
1 : 30	116

Experiments were made in which a series of dilutions of such preparations was tested by performing clotting tests with fibrinogen. The clotting tests were made with 0.2 ml. of the diluted thrombin and 0.1 ml. fibrinogen in the manner

Fig. 1. Relation between dilution of a thrombin preparation and clotting time with added fibrinogen.

already described. A typical experiment is given in Table 1. The same result is shown graphically in Fig. 1, where the dilution of the thrombin (i.e. the volume to which one volume of the original preparation is diluted) is plotted against the clotting time obtained in tests with fibrinogen. The points in Fig. 1 lie very

¹ Here and subsequently the symbol D represents the volume to which 1 vol. of sample is diluted.

close to a straight line which cuts the time axis at 3 sec. As the shorter clotting times may easily be slightly overestimated, the true relation may well be a straight line passing through the origin—indeed, for clotting times above 25 sec. a straight line through the origin would fit the points equally well. That is to say, the dilution of the thrombin is directly proportional to the clotting time; or, the concentration of thrombin is inversely proportional to the clotting time. If the concentration of thrombin were plotted against the clotting time, the result would be a rectangular hyperbola. As it is obviously preferable to choose the method of plotting which gives the linear relation with thrombin, all the results to be described will be expressed in this way, as a relation between “dilution” (the volume to which one volume of a sample is diluted) and the clotting time in a test in which 0.2 ml. of the diluted sample is treated with 0.1 ml. fibrinogen.

These results with thrombin are in agreement with those obtained by Quick [1935] with similar preparations.

(2) *The conversion of prothrombin into thrombin.* Preparations of prothrombin were made as follows.

Oxalated plasma is first freed from fibrinogen by treatment with thrombin. A sample of 10 ml. plasma is treated with a few drops of thrombin (prepared as above) and the mixture warmed to 37°. When the clot has formed, the fibrin is removed by rolling it out on a glass rod. More thrombin is added, and any further fibrin clot removed in the same way. Repeated treatments with a drop of thrombin are continued until no further clot forms. The mixture is then kept for at least 15 min. to allow of complete destruction of the excess thrombin by the plasma antithrombin. The plasma is then diluted ten-fold with water, a drop of octyl alcohol is added and CO₂ is passed through the mixture for 5 min. Prothrombin is precipitated. The precipitate is separated by centrifuging and dissolved in 10 ml. buffered saline (using the glyoxaline buffer as already described).

From preparations made in this way, a series of dilutions was made in isotonic saline, and each mixture incubated with calcium and thromboplastin and tested at intervals for thrombin activity, exactly as in the method for estimating plasma prothrombin. It was found that the clotting times (with added fibrinogen) decreased rapidly during the early stages of incubation and then more gradually, until a maximal activity was reached. The time required to develop full activity was 30–90 min., and the thrombin then remained stable for at least an hour. It was also noticed that the time taken to reach maximal activity was the same for smaller as for larger amounts of prothrombin, and that the thrombin formed at any given stage of incubation was roughly proportional to the prothrombin present.

At maximal activity, the relation between the dilution of the preparation and the clotting time with fibrinogen was perfectly regular. Examples are given in Fig. 2, which show the results with four prothrombin preparations of different strength. For each preparation, the points fall on a straight line which cuts the time axis at some point between 0 and 4 sec. As with thrombin, the ratio D/T can be regarded as constant for clotting times above 25 sec. For any one prothrombin preparation, the prothrombin concentration (ϕ) in the incubating mixture is inversely proportional to the dilution (D). We know that the thrombin concentration (θ) is inversely proportional to the clotting time (T). For any one plasma the ratio D/T is constant, and this means that the ratio θ/ϕ is constant. This does not prove that the prothrombin is quantitatively converted into thrombin, but it does show that the thrombin formed is always proportional to the prothrombin present.

It is evident also that the value of the constant D/T for any preparation is proportional to the prothrombin titre of the preparation. The ratio of the titres of two preparations will be given either by the ratio T_s/T_u , where T_s and T_u are the clotting times for the "standard" and "unknown" respectively at the same dilution, or by the ratio D_u/D_s where D_s and D_u are the dilutions of the standard and unknown respectively which give the same clotting time.

Fig. 2. Relation between the dilution of a prothrombin preparation and the clotting time with added fibrinogen during the period of maximal thrombin activity.

Now if, in diluted plasma, the prothrombin were completely converted into thrombin without loss, the prothrombin titre of an abnormal plasma could be calculated as a percentage of the standard normal equally well by fixing the dilution and comparing clotting times, or by fixing the clotting time and comparing the corresponding dilutions. It will be shown that for plasma the two methods do not give the same results because the ratio D/T (or θ/ϕ) is not constant for all dilutions of the same plasma, but decreases with decreasing dilution. Before considering more fully the results obtained with plasma, it will be convenient to describe experiments on the antithrombin activity of diluted plasma.

(3) *The antithrombin of plasma.* It is well known that plasma contains a substance capable of destroying thrombin. Under natural conditions of blood coagulation this "antithrombin" has the function of destroying the excess thrombin formed at the site of injury and so preventing the spread of coagulation to the general circulation. It does not act as an anticoagulant, probably because the velocity of the reaction between thrombin and antithrombin is less than that of the reaction between thrombin and fibrinogen. Quick [1938, 1] considers that the antithrombin of normal plasma is closely associated with the albumin fraction.

In undiluted plasma, and in any dilution less than 1:50, the rate of destruction of thrombin is rapid. If such plasma mixtures are incubated with calcium and thromboplastin, and the thrombin activity followed by performing clotting tests with added fibrinogen, an initial increase in thrombin is obtained, followed by a rapid fall, with no steady intermediate period of maximal activity.

At dilutions greater than 50, a steady period occurs, but it is evident that this may be due to temporary balance between the rates of formation and destruction of thrombin. It has already been mentioned that in the estimation of plasma prothrombin it is necessary to use the range of dilutions from 100 to 800, and that for samples low in prothrombin the lower dilutions of this range must be used. Experiments have therefore been made to determine the rate of destruction of thrombin by diluted plasma in this range.

Thrombin was prepared by the method already described, and the titre of the preparation standardized by clotting tests with added fibrinogen. Mixtures were made of diluted thrombin and diluted plasma, using the range of plasma dilutions from 1 : 100 to 1 : 800, and varying amounts of thrombin. These were incubated at 15–20° and at intervals samples were taken for clotting tests with fibrinogen. The thrombin present at any given stage, expressed as a percentage of that originally present, was calculated from the formula $T/T' \times 100$, where T is the clotting time in a control test for the thrombin without plasma, and T' the clotting time for the incubated mixture. The results are summarized in Table 2. Different samples of plasma varied somewhat in antithrombin activity,

Table 2. *Destruction of thrombin by diluted plasma*

Dilution of plasma	Percentage of the original thrombin remaining after incubation for			
	5 min.	10 min.	20 min.	30 min.
1 : 100	75–95	65–80	60–75	50–70
1 : 200	—	85–100	75–90	70–85
1 : 400	—	95–100	90–100	85–90

and Table 2 shows the range of variation. Some destruction of thrombin occurred in dilutions from 1 : 100 to 1 : 400, and the effect diminished with increasing dilution. It is small at dilution 1 : 400. At dilution 1 : 800 there is probably still some slight effect. For each individual plasma the decrease in the effect with increasing dilution was observed.

Fig. 3. Rate of destruction of added thrombin by plasma diluted 1 : 100. × Mixture 1. • Mixture 2. The original thrombin concentration in mixture 1 was twice that in mixture 2.

From experiments in which the dilution of the plasma was fixed and the amount of thrombin varied, it was found that the percentage of the original thrombin remaining at a given time was approximately the same whatever the original amount of thrombin. That is to say, when varying amounts of thrombin are incubated with the same amount of antithrombin, the thrombin lost in a given time is not a constant absolute amount, but a constant proportion of that originally present. This is illustrated in Fig. 3, which shows the loss of thrombin in two mixtures. Both contained the same plasma diluted to 100 vol., but the thrombin in mixture 1 was double that in mixture 2. The rate of loss, expressed as a percentage of the original value, is much the same for the two.

When one considers the times required for the development of maximal thrombin activity when diluted plasma is incubated with thromboplastin and calcium, it is evident that some antithrombin action may occur during this period, and although one cannot predict the quantitative effect in a system in which thrombin is being gradually formed, one would expect that the loss of thrombin would progressively increase with decreasing dilution of the plasma.

(4) *Thrombin formation from prothrombin in diluted plasma.* In the light of the preceding results, we can now consider the interpretation of the readings obtained in the estimation of plasma prothrombin. In Table 3 are given the figures on which the normal standards for the method are based. The samples of plasma were obtained from hospital patients, none of whom suffered from biliary obstruction or any other disease associated with a tendency to haemorrhage.

It is evident that here the ratio D/T is not constant but as the dilution decreases the clotting times become progressively longer than they would be if the ratio were constant. That is to say, the ratio θ/ϕ , which measures the thrombin formed relatively to prothrombin present, decreases as the concentration of antithrombin increases. The loss of thrombin is however quite regularly related to the plasma dilution, and it is possible to find an empirical formula expressing this relation for any given plasma.

Table 3. *Clotting times obtained at the period of maximum thrombin development with serial dilutions of normal plasma*

Case	Results of clotting tests on mixtures in which the volume to which 1 vol. of plasma was diluted at the stage of incubation was:					Mean prothrombin titre
	50	100 sec.	200 sec.	400 sec.	800 sec.	
1	—	22	35	61	90	116
2	—	28	45	66	104	97
3	—	33	44	78	123	87
4	—	25½	39	64	100	105
5	—	30	46	76	133	86
6	—	(22½)	36	60	88	116
7	—	25	38	60	110	105
8	—	28½	45	67	112	95
9	—	30	41	65	(106)	97
10	—	27	39	60	103	105
11	—	35	47	66	125	89
12	14	24	38	58	90	113
13	15	24	36½	55	93	115
14	20	31	47½	73	123	88
15	—	32	46½	77	132	85
Average normal	—	28	41½	66	109	(100)
Standard normal	16	26	42	67	107	—

Consider the series of dilutions (D) in decreasing order. They form a geometric progression with the factor $\frac{1}{2}$. It is found that the corresponding clotting times are in geometric progression with the factor $1/1.6$. The "standard normal" clotting times at the foot of Table 3 form a series in which this relation applies strictly, and they fall close to the actual average normal results.

Table 4

Dilution (D)	Clotting time (T)	D/T	n
800	107	$\frac{800}{107}$	0
$400 = \frac{800}{2}$	$\frac{107}{1.6} = 67$	$\frac{800}{107} \times 0.8$	1
$200 = \frac{800}{2^2}$	$\frac{107}{1.6^2} = 42$	$\frac{800}{107} \times 0.8^2$	2
$100 = \frac{800}{2^3}$	$\frac{107}{1.6^3} = 26$	$\frac{800}{107} \times 0.8^3$	3
$50 = \frac{800}{2^4}$	$\frac{107}{1.6^4} = 16$	$\frac{800}{107} \times 0.8^4$	4

The corresponding values of D , T and the ratio D/T which accord with the empirical formula are set out in Table 4. Here the dilution 1 : 800, with its corresponding clotting time, 107, is taken as a starting point, but any other starting point would serve equally well. Whenever the plasma dilution is halved (antithrombin concentration doubled), the ratio D/T , which is proportional to θ/ϕ , is reduced to 0.8 of its value at the higher dilution. Doubling the anti-thrombin involves at each stage a loss of 20 % of the thrombin.

Fig. 4.

It is evident that if $\log D$ is plotted against $\log T$, the resulting graph will be a straight line, and if D is plotted against T , the result is a logarithmic curve. It is possible to plot the full curve for all dilutions, and this has been done in Fig. 4.

The results for any individual sample of plasma agree closely with the empirical formula. For each, an "idealized" series of clotting times can be found which agrees with the observed series as well as could be expected in view

Table 5

	Dilution (<i>D</i>)				
	800	400	200	100	50
Case 12. Clotting time (<i>T</i>):					
Observed	90	58	38	24	14
Idealized	94	59	37	23	14
Case 13. Clotting time (<i>T</i>):					
Observed	93	55	36½	24	15
Idealized	94	59	37	23	14
Case 14. Clotting time (<i>T</i>):					
Observed	123	73	47½	31	20
Idealized	123	77	48	30	19

of the degree of accuracy of reading clotting times. The curve fitting was done by calculating from the readings at each dilution, according to the empirical formula, the clotting time which should correspond to dilution 1 : 800. The mean of these results was taken as the "idealized" clotting time at dilution 1 : 800, and this point was used to calculate the complete "idealized" series according to the empirical formula. In Table 5 the actual and idealized clotting times for three samples are set out. They were chosen because they included dilution 1 : 50 as well as the other four, and are quite typical.

Now in dealing with samples of plasma of low prothrombin titre, the same general relation applies. A geometric series of dilutions with the factor $\frac{1}{2}$ corresponds to a geometric series of clotting times with the factor 1/1.6. Take any standard dilution D_s as the starting point, and let T_s and T'_s be the clotting times for the normal and abnormal samples of plasma respectively. Then at dilution $D_s/2$, the clotting times for the two samples will be $T_s/1.6$ and $T'_s/1.6$, and so on—the clotting times for the two vary always in the same proportion, and the ratio T/T' is constant for any dilution.

But the empirical formula is symmetrical in respect of D and T , and it can be shown that the ratio D'/D , where D and D' are the dilutions of the normal and abnormal which give the same clotting time, is constant for any clotting time. It gives a lower value than the ratio of clotting times at the same dilution.

The following example shows the results in a case of obstructive jaundice. At dilutions 1 : 400, 1 : 200 and 1 : 100 the clotting times were 104, 68 and 41 sec. respectively. The values of the ratio T/T' at each of the three dilutions are 0.64, 0.63 and 0.63. From the full standard normal curve in Fig. 4 it is found that the clotting times 104, 68 and 41 sec. are given by dilutions 1 : 770, 1 : 410 and 1 : 195 respectively, and the values of the ratio D'/D for the three clotting times as 0.52, 0.49 and 0.51. The discrepancy between the two ratios increases for lower prothrombin titres.

Now in view of the antithrombin effect it is highly improbable that the ratio D'/D is proportional to the prothrombin titre. Take an extreme example; a plasma giving a clotting time of 107 at dilution 100. The time of incubation required to reach maximal activity in such a case is about the same as for a normal plasma at dilution 1 : 800 giving the same activity. It is evident that there must be greater loss of thrombin at dilution 1 : 100 than at dilution 1 : 800.

What then of the ratio T/T' for samples of plasma compared at the same dilution? Obviously the fact that the ratio is constant for all dilutions does not prove that it is proportional to the prothrombin titre (as it is in the case of antithrombin-free preparations). It is quite possible that the antithrombin effect might be relatively greater throughout for the abnormal plasma, while still

conforming to the general relation which determines the form of the curve. If the formula $T/T' \times 100$ is taken as measuring the prothrombin titre as a percentage of the normal, the assumption is that the proportion of thrombin lost at a given dilution is the same for all prothrombin titres. If this is not so, the titres will be measured on a distorted scale.

An attempt has been made to determine whether the scale of titres is regular by experiments on the recovery of prothrombin added to plasma. This presents difficulties. If the method is adopted of adding a given quantity of a standardized preparation to a standardized plasma and determining the titre of the mixture, the difficulty arises of expressing the titre of the prothrombin preparation and that of the plasma in comparable units. Since the plotting of dilution against clotting time gives for prothrombin preparations a straight line, and for plasma a logarithmic curve, the titres of the two cannot be simply compared. The best approximation to a comparable standardization is to assume the absence of appreciable antithrombin activity in plasma at dilution 1 : 800, and to suppose that at this dilution the thrombin formed from plasma prothrombin is comparable with that formed from the separated prothrombin preparations. Such a calculation implies, not only that plasma antithrombin has negligible effect at dilution 1 : 800, but also that in the prothrombin preparations the conversion into thrombin is as complete as in plasma. The prothrombin preparations are very slowly converted, and since it has been shown by Mertz *et al.* [1939] that prothrombin is gradually inactivated by thrombin, it is quite possible that in these long incubations the thrombin formed early may prevent complete conversion of the remaining prothrombin.

Nevertheless, some experiments have been made with the addition of standardized prothrombin to plasma (Table 6). In these the titre of the prothrombin preparation has been expressed as a percentage of standard normal plasma on the assumption that the plasma and the preparation are comparable at dilution 1 : 800; and the titre of the plasma, with and without added prothrombin, has been calculated from the formula $T/T' \times 100$, where T and T' are the clotting times of standard and "unknown" at the same dilution of plasma. Some results show good agreement, and most are not grossly aberrant if the errors are considered in relation to the total prothrombin estimated. The general tendency is to high recoveries, and in two experiments (5 and 6) the recoveries are much too high. These discrepancies may possibly be accounted for by an underestimate of the titre of the prothrombin preparation. It is noteworthy that if the calculations had been based on the ratio of dilutions giving the same clotting time, instead of the ratio of clotting times at the same dilution, the discrepancies would have been very much greater.

In order to overcome the difficulty of expressing titres of plasma and separated prothrombin in comparable units, another method of approach has been used, with a view to discovering whether the method of calculating titres from the ratio of clotting times gives a regular, or a distorted scale. The question is whether the same increment of prothrombin has the same effect in different regions of the scale. In three experiments (nos. 5, 6 and 7 in Table 6) successive increments of prothrombin were added to a plasma of low titre. In exp. 8, the same increment of prothrombin was added to two samples of plasma, one of normal and the other of rather low titre.

This method of comparing increments is only capable of detecting fairly gross distortion of the scale, but with this reservation the results are extremely good. The range of prothrombin titres covered by the whole set of experiments is 16.5–137 % of the normal average, and for each experiment the two increments

Table 6. *Prothrombin titres expressed as percentage of standard normal plasma*

Exp.	Plasma alone	Plasma + x	Plasma + $2x$	Prothrombin added $x =$	Prothrombin recovered	
					1st increment of x	2nd increment of x
1	86	180	—	(80)	94	—
2	70	167	—	(76)	97	—
3	42	87	—	(46)	45	—
4	32	91	—	(51)	59	—
5	16½	40½	63½	(13½)	24	23
6	45	93	137	(32½)	48	44
7	48	79	116	(37½)	31	37
8 (a)	63	85	—	(18)	22	—
(b)	85	111	—	(18)	26	—

agree reasonably well. In view of the fact that the determination of the increment is subject to the risk of summation of errors on two estimations, the results are suprisingly regular. It seems therefore that the method gives not only a practical index for comparing normal with abnormal plasma, but also a reasonably accurate quantitative estimation.

Other methods of prothrombin estimation

In the method of Warner *et al.*, and in the modification of it described in the present paper, the two essential stages in coagulation, namely, the formation of thrombin and the coagulation of fibrinogen by thrombin, are separated. Other methods have been based on the coagulation of plasma in the presence of thromboplastin and calcium under controlled conditions. In such coagulation systems the two reactions are proceeding in the same mixture, and during the short time required for clotting only part of the plasma prothrombin is converted into thrombin. What is measured is essentially the rate of thrombin formation.

One group of methods [Schönheyder, 1936; 1938; Dam & Glavind, 1938] depends on working with a concentration of tissue extract below the optimum, and determining the concentration of a standardized extract required to produce a standard clotting time with the plasma. The method requires precise standardization of the extract against normal plasma, and also requires that the plasma should be obtained free of thromboplastin.

Another group of methods is derived from that of Quick [Quick *et al.* 1935; Quick, 1938, 2]. The principle of the method as originally described was that when oxalated plasma was treated with calcium salts and an excess of thromboplastin, the only variable affecting the coagulation time was the prothrombin titre of the plasma. The relation between clotting times and prothrombin titres was worked out on the basis of results with serial dilutions of normal plasma. This calibration curve was not a convenient one to work with, as large differences in prothrombin concentration produced relatively small differences in clotting time. On Quick's scale, a prothrombin titre of 100 % of normal gives a clotting time of 12 sec.; 50 % of normal gives 15 sec. Moreover, various subsequent reports from other authors have shown that it is necessary to standardize the tissue extract; thromboplastin is required not in simple excess but in optimal concentration. The writer has been unable to reproduce Quick's clotting times even with optimal amounts of tissue extracts; the times found for undiluted plasma varied from 22 to 40 sec., and this agrees with the findings of Illingworth [1939]. Moreover, it was found that variations in the tissue extract affected not

only the clotting time for undiluted plasma, but the relation between clotting time and plasma dilution in serial dilutions of plasma. In fact it is very difficult to apply the method in a strictly quantitative fashion, though it gives a convenient means of detecting gross deficiencies of prothrombin.

Methods both of Schönheyder's type and Quick's type require standardization of the thromboplastin against normal plasma. Such a standardization makes no allowance for the range of normal variation, and it is impracticable to cover the full normal range whenever a standardization is required.

Another objection to any method which depends on the rate of thrombin formation is that other factors, beside the concentrations of prothrombin and thromboplastin, may affect the results. The differences in the rate of conversion of prothrombin into thrombin in different species have already been mentioned, and there is evidence that in human plasma under different conditions there may be variations in the rate of prothrombin conversion [Ziffren *et al.* 1939; Smith *et al.* 1939; Owen *et al.* 1939]. These authors recommend a modification of Quick's method as a useful practical measure of the tendency to haemorrhage, but do not regard this type of method as giving a true measure of prothrombin titres.

It is evident therefore that a method of the "two-stage" type is to be preferred, and it is hoped that the technique here described will meet the requirements of workers who need greater accuracy than can be obtained in methods of Quick's type.

SUMMARY

1. The estimation of plasma prothrombin, by a modification of the method of Warner *et al.*, is described.
2. The quantitative aspects of the different reactions involved are considered.
3. Evidence is presented that the method gives a quantitative measure of prothrombin titres, or at least that there is no serious departure from a genuinely quantitative scale.
4. The principles underlying other methods are briefly considered.

REFERENCES

- Dam & Glavind (1938). *Biochem. J.* **32**, 1018.
 Ferguson (1938, 1). *J. Lab. clin. Med.* **24**, 273.
 — (1938, 2). *Amer. J. Physiol.* **123**, 341.
 — & Erickson (1939). *Proc. Soc. exp. Biol., N.Y.*, **40**, 425.
 Illingworth (1939). *Lancet*, **1**, 1031.
 Mertz, Seegers & Smith (1939). *Proc. Soc. exp. Biol., N.Y.*, **41**, 657.
 Owen, Hoffman, Ziffren & Smith (1939). *Proc. Soc. exp. Biol., N.Y.*, **41**, 181.
 Quick (1935). *Amer. J. Physiol.* **115**, 317.
 — (1938, 1). *Amer. J. Physiol.* **123**, 712.
 — (1938, 2). *J. Amer. med. Ass.* **110**, 1658.
 — Stanley Brown & Bancroft (1935). *Amer. J. med. Sci.* **190**, 501.
 Schönheyder (1936). *Biochem. J.* **30**, 890.
 — (1938). *Amer. J. Physiol.* **123**, 349.
 Smith, Warner & Brinkhous (1937). *J. exp. Med.* **66**, 801.
 — Ziffren, Owen & Hoffman (1939). *J. Amer. med. Ass.* **113**, 380.
 Warner, Brinkhous & Smith (1936). *Amer. J. Physiol.* **114**, 667.
 — — — (1939, 1). *Proc. Soc. exp. Biol., N.Y.*, **40**, 197.
 — — — (1939, 2). *Amer. J. Physiol.* **125**, 296.
 Ziffren, Owen, Hoffman & Smith (1939). *Proc. Soc. exp. Biol., N.Y.*, **40**, 595.