VARIATIONS IN THE REACTION BETWEEN THROMBIN AND FIBRINOGEN AND THEIR EFFECT ON THE PROTHROMBIN TIME

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The appearance of fibrin provides the end-point in every clotting test, and the duration of the thrombin-fibrinogen reaction is thus a component of any measurement of clotting time. While many tests are designed to study the early stages of clotting, in interpreting the results it is necessary to consider to what extent the observed differences may be due simply to differences in the "indicator reaction" between thrombin and fibrinogen. This precaution particularly applies to one-stage tests in which comparisons are made between different bloods.

The reaction between thrombin and fibrinogen may be separately investigated, and fortunately the measurement of the thrombin clotting time of citrated plasma (Biggs and Macfarlane, 1953) is one of the simplest of all clotting tests. This paper reports a case in which the one-stage prothrombin time was spuriously lengthened by an abnormally slow thrombin-fibrinogen reaction; tests were then made in four other cases to investigate the processes involved.

TABLE I

PROTHROMBIN TIME AND VARIOUS THROMBIN CLOTTING TIMES WITH PLASMA FIBRINOGEN CONCENTRATIONS

Test	Mean Clotting Times (sec.)		Patient's Plasma Fibrinogen Concentration (g. %)
	Patient	Control	
One-stage prothrombin time:			
30/5/50 ..	28.5*	26.2	
20/6/51 ..	16.2	12.1	
Thrombin clotting time:			
30/5/50 ..	20	13	0.35
2/6/50 ..	19.8	11.0	
11/8/50 ..	9.6	Control 1 6.1	0.30
		" 2 5.2	
20/6/51† ..	15.3	8.0	

* The clotting entries carrying a decimal place are the means of two or three replicate readings.

† In the thrombin clotting time tests of 20/6/51, the clot appeared to form more gradually in the patient's plasma than in the control plasma.

Case Presentation

E. M., a man aged 29, had a malabsorption syndrome with ascites and tetany. The whole blood calcium was 5 mg.%, plasma albumin 1.25 g.%, plasma globulin 1.72 g.%, blood urea nitrogen 20 mg.%, blood fatty acids 405 mg.%, packed cell volume 34%, and cephalin flocculation + + +.

The one-stage prothrombin time with human brain reagent was prolonged. The thrombin clotting time was observed over a period of a year and was always greatly prolonged. These results are shown in Table I, with two readings of the plasma fibrinogen concentration. The first of these was made by the micro-Kjeldahl nitrogen method and the second by a clot-weight method (Ingram, 1952a): both are within normal limits (Ingram, 1952b).

The following experiments were made on June 20, 1951:

Equal Mixture of Control and Patient's Plasma.—

The prothrombin time and thrombin clotting time were intermediate between those of the control and the patient alone (Table II).

Isolation of Fibrinogen.—Equal volumes of control and patient's plasma were precipitated at $\frac{1}{2}$ -saturation with ammonium sulphate and the precipitates were taken up in 0.85% saline; the precipitation was once repeated and the solutions were dialysed for 20 hours against saline. It was noted that the patient's precipitates spun down more readily than those from the control plasma. Samples of these solutions were diluted 50:50 with veronal buffer pH 6.8 (Owren,

TABLE II

PROTHROMBIN TIME AND THROMBIN CLOTTING TIME IN PATIENT'S AND CONTROL PLASMAS AND IN EQUAL MIXTURES

Test Substance	One-stage Prothrombin Time (sec.)	Thrombin Clotting Time (sec.)		
		Thrombin 1	Thrombin 2	Thrombin 3
Patient's plasma	16	10	14	ca 22
Patient-control plasma mixture*	13.1	5.6	8.6	14.3
Control plasma*	12.1	4.8	7.1	11.0

* Each entry is the mean of three readings.

1949) and the thrombin clotting times repeated. The patient's fibrinogen clotted more slowly than that from the control (patient's mean, 7.5 sec.; control mean, 6.8 sec.; each is derived from five readings; for the difference between the means, $P < 0.05$).

Tests on Defibrinated Plasma.—Equal volumes of plasma from control and patient were heated at 56° C. for 3 min. and cooled rapidly. The precipitated fibrinogens were removed by centrifugation and a solution of purified human fibrinogen (Lister Institute) was added equally to the supernatants, to a concentration of about 0.3 g.%. Samples of these plasma-fibrinogen mixtures were then mixed in equal proportions and the thrombin clotting time determined on all three mixtures. There was no significant difference between the mean results.

In conclusion, this patient appeared to have an abnormality of the fibrinogen which delayed the thrombin-fibrinogen reaction and thus apparently the one-stage prothrombin time.

Investigations in Other Cases

Effect on Prothrombin Time of More Rapid Thrombin-Fibrinogen Reaction.—The following experiments were made on citrated plasma taken from a patient showing a short thrombin clotting time (Table III).

TABLE III
MEAN THROMBIN CLOTTING TIMES IN PLASMA

Test Substance	Thrombin Clotting Time (sec.)			Fibrinogen Concentration in Citrated Plasma (g.%)
	Thrombin 1	Thrombin 2	Thrombin 3	
Patient's plasma ..	6.1*	24.2	70.8	0.39
Control ..	6.4	26.1	74.4	0.32
Ratio:				
Patient's clotting time/Control clotting time† ..	0.95	0.93	0.95	

* Each mean is derived from four readings, and in each case the difference between patient and control means is significant ($P < 0.05$).

† The ratios suggest that the magnitude of the effect is independent of the clotting time.

(1) Equal volumes of patient's and control plasma were precipitated at $\frac{1}{4}$ -saturation with ammonium sulphate. The precipitates were drained, dissolved in saline containing one-tenth volume of veronal buffer pH 6.8, and made up to the volumes of the original plasmas. The procedure was once repeated and the solutions dialysed overnight against the same solvent.

Plasma obtained from two other persons was pooled and defibrinated with a trace of thrombin.

Thrombin clotting times were then measured on each fibrinogen, both with and without the addition of defibrinated plasma, to confirm that the relative reactivity of the samples had not been greatly altered by the isolation (Table IV).

One-stage prothrombin times with human brain extract in four dilutions were then measured on each fibrinogen in clotting mixtures containing 0.1 ml. each

TABLE IV
MEAN THROMBIN CLOTTING TIMES IN SOLUTIONS OF FIBRINOGEN ISOLATED BY AMMONIUM SULPHATE PRECIPITATION

Test Substance	Thrombin Clotting Time (sec.)		
	Thrombin 1	Thrombin 2	Thrombin 3
Without defibrinated plasma:			
Patient's fibrinogen ..	5.9*	17.7	38.0
Control ..	6.9	20.9	42.9
Ratio Patient's clotting time/Control clotting time ..	0.85†	0.85	0.89
With defibrinated plasma:			
Patient's fibrinogen ..	6.8	28.0	
Control ..	7.7	32.2	
Ratio Patient's clotting time/Control clotting time ..	0.87†	0.90	

* Each mean is derived from three readings, and in each case the difference between patient and control means is significant ($P < 0.01$).

† The ratios suggest that the magnitude of the effect is again independent of the clotting time.

of the defibrinated plasma, one of the fibrinogen solutions, brain extract, and M/40 calcium chloride. The results are given in Table V, which shows that the proportional difference between the clotting times from the two fibrinogens increased as the clotting times lengthened. Now, Table IV showed that the proportional differences between the thrombin clotting times of these fibrinogens did not increase as the clotting time lengthened, so Table V suggests that, as the prothrombin time lengthens, the time required for the interaction between thrombin and fibrinogen represents

TABLE V
MEAN PROTHROMBIN TIMES IN MIXTURES OF DEFIBRINATED PLASMA, FIBRINOGEN SOLUTION, BRAIN EXTRACT, AND M/40 CALCIUM CHLORIDE SOLUTION

Test Substance	1-Stage Prothrombin Time (sec.)			
	Brain 1/1	Brain 1/20	Brain 1/80	Brain 1/300
Patient's fibrinogen ..	12.2*	21.0	31.6	45.7
Control ..	12.6	21.8	34.0	51.8
Ratio Patient's clotting time/Control clotting time	0.97	0.96	0.93	0.88

* Each mean is derived from three readings, and the increasing difference between the pairs of means, as shown by the falling ratios, is highly significant ($P < 0.01$: tested on the interaction between brain concentrations and fibrinogens in an analysis of variance made on log. clotting times).

an increasing fraction of the interval elapsing between recalcification and the appearance of clot. A hypothetical example will make this clear.

Suppose, for instance, that the thrombin-fibrinogen reaction occupies one-tenth of the whole prothrombin time when this is 20 sec., and two-tenths when, by dilution of the brain reagent, the prothrombin time is increased to 50 sec.; then, in the one case the thrombin-fibrinogen reaction will constitute the final two seconds and in the other, the final 10 seconds. Suppose, further, that in two plasmas the thrombin-fibrinogen reactions proceed at differing rates in the

ratio 7 : 10. Other things being equal, the prothrombin times of these plasmas will follow the values given in Table VI. The differences shown in the tests will be relatively smaller with concentrated brain and

TABLE VI
HYPOTHETICAL EXAMPLE

Test Substance	1-Stage Prothrombin Time (sec.)	
	Concentrated Brain	Dilute Brain
"Fast" plasma ..	$18 + 2 \times 0.7 = 19.4$	$40 + 10 \times 0.7 = 47$
"Slow" " " ..	$18 + 2 = 20.0$	$40 + 10 = 50$
Ratio "Fast" plasma clotting time "Slow" plasma clotting time	0.97	0.94

relatively greater with dilute brain. If it is alternatively assumed that the thrombin-fibrinogen reaction occupies a constant fraction of the prothrombin time, this effect does not appear.

(2) Two weeks later further samples of citrated blood were obtained from this patient and from a different control subject. Fibrinogen was isolated by the method of Jaques (1943), employing phosphate buffers at pH 6.6, and dialysed against saline overnight. The results were similar to those in the previous experiment: the mean thrombin clotting times of the patient and control fibrinogen solutions alone (each derived from four readings) were 7.9 sec. and 10.0 sec. respectively (ratio, 0.79); and the mean prothrombin times with diluted brain extract on defibrinated plasma were 36.6 sec. and 42.1 sec. respectively (each derived from four readings: ratio, 0.86). These and the previous findings were broadly in agreement.

In conclusion, the effect on the one-stage prothrombin time suggested that, as the clotting time was lengthened by dilution of the brain reagent, the thrombin-fibrinogen reaction came to occupy an increasing fraction of the interval recorded as the prothrombin time.

The Influence of Other Factors.—Citrated plasma was obtained from another patient showing a rapid thrombin clotting time relative to a normal control.

Effect of Differently Reacting Fibrinogens upon Earlier Processes Involved in Prothrombin Time.—Fibrinogens were isolated by Jaques's method from

TABLE VII
MEAN CLOTING TIMES ON ISOLATED FIBRINOGENS

Test Substance	Thrombin Clotting Time (sec.) on Fibrinogen Alone	1-Stage Prothrombin Time (sec.) with Defibrinated Plasma Added*
Patient's fibrinogen	7.6 (3)†	47.6 (2)
Control " " ..	9.5 (3)	50.7 (2)
Patient's clotting time Control clotting time	0.81	0.94

* The brain extract was diluted.

† The number of readings contributing to each mean is shown in parenthesis.

the patient's and control plasmas as before. Table VII gives the thrombin clotting times on the fibrinogen solutions alone and the prothrombin times with defibrinated plasma from another source, as in the previous experiments. The results are similar to those from the previous case.

These samples were then used to test the hypothesis that the "fast" fibrinogen sample contained an accelerator of thrombin generation, whose activity might explain the difference in the prothrombin times when the fibrinogens were tested with defibrinated plasma. Two-stage prothrombin time curves were obtained at 23° C. from mixtures containing 67% defibrinated plasma, 28% fibrinogen solution, and 1.7% molar calcium chloride solution. Duplicate curves were obtained from each fibrinogen with brain extract in final concentrations of 2.8% and of 0.28%. The mixtures clotted on the rising limbs of the curves at about 1 min. with the concentrated brain extract and at about 1.5 min. with the dilute extract. With each concentration of brain extract, the rising limbs of the four curves could be closely superimposed.

In conclusion, there was no evidence that the fibrinogen isolate contained an abnormal accelerator of thrombin generation.

Effect of Fibrinogen Concentration upon Differences in Thrombin Clotting Time.—Citrated plasma was obtained from a patient in whom the fibrinogen concentration was raised and the thrombin-fibrinogen reaction was delayed, relative to normal blood.

The fibrinogen concentrations were determined in the patient's (0.51 g.%) and the control (0.29 g.%) plasmas and a portion of the patient's plasma was diluted with saline to reduce the fibrinogen concentration to that of the control. Fibrinogen solutions were prepared from equal volumes of each sample by Jaques's method, as before. The fibrinogen concentration was determined on the three samples (patient, control, and diluted patient) and serial dilutions in saline were prepared from them. Two to four replicate readings of the thrombin clotting time

FIG. 1.—Thrombin clotting times of fibrinogens isolated from patient's and control plasma obtained from various concentrations of fibrinogen with one thrombin concentration. The ordinate is reciprocal, the abscissa logarithmic.

were then recorded from each dilution with a single thrombin concentration. The results are shown in Fig. 1, where the mean clotting times are plotted against the fibrinogen concentrations in the clotting reactions.

The graph shows three things. First, the relationship between fibrinogen concentration and thrombin clotting time (found by some observers and reviewed by Owren, 1947; confirmed by Shinowara, 1948) is clearly shown, with the optimum concentration being approached at about the normal plasma level. Second, the relative reactivity of the patient's and the control fibrinogen is independent of the concentration tested. Third, this relationship is also independent of the concentration of fibrinogen in the plasma samples from which the isolates were prepared.

In conclusion, the fibrinogen of this patient's plasma appeared to react with thrombin abnormally slowly. In a purified system, the effect was independent of the fibrinogen concentration and it was not a function of the difference between the concentrations of fibrinogen in the original plasmas.

Effect of Other Plasma Constituents upon Differences in Thrombin Clotting Time.—The thrombin clotting time was found to be more rapid in citrated plasma from another patient than in citrated plasma from either of two controls (patient, 9.0 sec.; control A, 9.7 sec.; control B, 10.8 sec.; each derived from four readings).

To determine whether the effect was related to differences between the fibrinogens or between other plasma constituents, equal samples of plasma from the patient and from control A (from whom the patient differed the less) were treated as follows:

One pair of samples was defibrinated with a trace of thrombin, and from another pair the fibrinogens were precipitated by $\frac{1}{2}$ -saturation with ammonium sulphate; these precipitates were drained with filter papers and taken up in distilled water to twice the volumes of the original plasma but were not dialysed. Equal mixtures of each plasma with each fibrinogen were prepared and thrombin clotting times obtained (Table VIII). In the results differences between the fibrinogens are apparent, but not between the plasmas.

TABLE VIII

MEAN THROMBIN CLOTTING TIMES (SEC., EACH DERIVED FROM FOUR READINGS) ON MIXTURES OF DEFIBRINATED PLASMA AND ISOLATED FIBRINOGEN FROM PATIENT AND CONTROL A

Plasma	Fibrinogen	
	Patient	Control A
Patient	11.2	14.6
Control A	11.6	14.7

The differences between the results from the two plasmas are not significant; but between the fibrinogens, $P < 0.01$.

In conclusion, the rapid thrombin clotting time of this patient's plasma was a property of the fibrinogen and not of other plasma constituents.

Discussion

When blood clots spontaneously there is a latent period of some minutes before thrombin appears in the system, but thereafter the concentration rises sharply to about 10 units/ml.; the liberation of thrombin with brain extract follows a similar course, but the latent interval is much reduced (Macfarlane and Biggs, 1953). It is commonly observed in two-stage tests (as in the experiment reported above) that clotting occurs at a fairly low thrombin concentration but while the concentration of thrombin is still rising rapidly, so that under physiological conditions the rate of the thrombin-fibrinogen reaction may be partly determined by the rate of liberation of thrombin.

The rate of thrombin liberation was found to be reduced by dilution of the brain extract in two-stage tests on defibrinated plasma (Ingram, 1955). This reduction was comparable to the low rate of liberation of thrombin observed in thrombocytopenic and haemophilic blood (Macfarlane and Biggs, 1953). The supposition of a prolonged thrombin clotting time under such conditions agrees well with the results of the prothrombin time experiment in the second case studied.

In the thrombin clotting time test the concentration of thrombin is brought almost instantaneously to the test level, so that the conditions do not closely simulate the physiological clotting of fibrinogen. A rising concentration of thrombin would be difficult to reproduce in a simple test, but the ordinary method of measuring thrombin clotting time seems, from the above observations, to reflect at least some features of the normal process.

The biochemistry of the reaction between thrombin and fibrinogen has been extensively studied (Laki, 1953; Lóránd, 1954; Bailey and Bettelheim, 1955), and the experimental error of the measurement of thrombin clotting time is among the lowest of "one-stage" clotting tests (Ingram and Armitage, 1952; further details of experimental error are given in the Appendix). The reaction is believed to occupy the final two to five seconds in the clotting of normal blood in a glass tube (Biggs, 1955). Proceeding at this rapidity the reaction is unlikely to be affected by variations in "progressive antithrombin," and this has been confirmed by Lyttleton (1949).

Clinical Use of the Test.—It is convenient to mix 0.1 ml. of citrated plasma and 0.1 ml. of saline and then to time the reaction after adding 0.1 ml. of thrombin solution diluted so that the resulting clotting time is about 10–20 sec. These quantities

are economical in reagents and provide the same concentration of plasma as in the one-stage prothrombin time test.

The reaction between thrombin and fibrinogen is affected in various ways by ionized calcium (Robbins, 1944; Laki and Lóránd, 1948; Burstein, 1951; Ferry, Miller, and Shulman, 1951; Rosenfeld and Jánszky, 1952; Burstein and Guinand, 1954), so that for clinical investigation the reaction should be measured in recalcified plasma; this is conveniently done either by replacing the saline with M/40 calcium chloride solution or by adding to the diluted thrombin one-fortieth of its volume of molar calcium chloride solution.

Thrombin may be prepared by the method of Douglas and Biggs (1953). Prepared thrombin is available from various sources (e.g., the bovine thrombin, "topical," of Parke, Davis & Co.). In a comparative study of the thrombin clotting times with human and bovine thrombins, five out of six human plasmas reacted somewhat differently to the two thrombins, and it is therefore thought that human thrombin should be used for clinical investigations.

The thrombin clotting time might advantageously be measured more often in clinical work than current publications suggest to be the case. The test is affected by heparin, but may be returned to normal by adding toluidine blue (Biggs and Macfarlane, 1953). This therefore provides a simple indication of heparinaemia. The test becomes progressively more sensitive to heparin as the thrombin concentration is reduced (Biggs, 1955). It is also possible that clinical abnormalities of blood coagulation may be associated with disturbances of the thrombin-fibrinogen reaction, but little evidence has yet been presented.

Summary

A case is described in which a lengthening of the prothrombin time seemed to be due to an abnormally slow reaction between thrombin and fibrinogen.

Investigations in other patients suggested that, as the prothrombin time lengthened, the thrombin-fibrinogen reaction occupied an increasing fraction of the interval between recalcification and the appearance of the clot; and that differences observed between the thrombin clotting times in normal and abnormal plasmas depended upon a property of the fibrinogens and were independent of fibrinogen concentration, thrombin concentration, or other plasma constituents.

The measurement of the thrombin clotting time is a simple test and might advantageously be used more often. Technical details are discussed.

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APPENDIX

Error of the Test

The thrombin clotting time was measured by one observer with various thrombin solutions in plasmas from 19 subjects (seven controls and 12 patients) over the range 8–30 sec.; 41 observations were obtained, each based on three to six replicate readings. The data were analysed to investigate the scatter among the replicates, as a measure of the experimental error of the test. It was found that the later replicates in a series tended to give a longer clotting time than the earlier, which was thought to reflect a deterioration in reagents. When this effect had been allowed for, the residual scatter was proportional to the mean clotting time, and the average coefficient of variation (the standard deviation expressed as a percentage of the mean) was about 3%. (This value agrees well with that of 5% previously published (Ingram and Armitage, 1952), which was derived only from the range of replicate readings without elimination of the trend and should thus be larger than the present estimate.) The coefficient of variation appeared to vary more among the patients than among the control subjects.

Despite the tendency for the clotting time to lengthen during the period of testing, an unbiased comparison between two plasmas A and B can be obtained if the readings are made in such an order that the temporal effect is eliminated, as in Table IX (Cox, 1951). With

TABLE IX
SEQUENCE OF REPLICATE READINGS FROM TWO SAMPLES

No. of Replicates	Order of Testing Plasmas A and B
2	AB, BA
4	AB, BA, BA, AB
6	AB, AB, BB, AA, AB, AB
8	AB, BA, BA, AB, BA, AB, AB, BA

These sequences are designed to eliminate systematic trends related to order of testing (such as progressive changes in reagents or observer facilitation or fatigue). The first sequence eliminates a linear trend (a steady drift throughout the period of observation); the second and third sequences eliminate also a quadratic trend (a steadily increasing drift); and the fourth sequence eliminates also a cubic trend (a more rapidly increasing drift). Clearly, balanced sequences cannot be obtained with odd numbers of replicates.

These sequences are generally applicable to all tests in which the subjects or treatments are compared by means of consecutive replicate readings and in which systematic trends might intrude.

this precaution, it was possible to devise a significance test for the difference between the mean readings from any two plasmas, using the estimate of sampling error from the analysis reported above. The procedure was to calculate the mean of all the readings from the two plasmas, the mean readings from each plasma, and the difference between the plasma means; the standard error of this difference was then a given percentage

(Table X) of the mean of all the readings. The significance of the difference between the means was tested by regarding the ratio of the difference to its standard error as a normal deviate: if the ratio exceeded 1.96, the probability of a greater difference arising by

TABLE X
STANDARD ERROR OF THE DIFFERENCE BETWEEN TWO MEANS EXPRESSED AS A PERCENTAGE OF THE MEAN OF ALL THE READINGS

No. of Readings Contributing to Each Mean	Percentage Factor for Determining Standard Error of Difference between the Means
2	3.0
4	2.1
6	1.7
8	1.5

Example:

Patient's readings: 10.4, 11.2, 10.7, 11.0, mean 10.83 sec.

Control readings: 12.1, 11.6, 11.8, 12.2, mean 11.93 sec.

Grand mean: 11.38 sec. Difference between patient and control, 1.10 sec.

S.E. difference = $11.38 \times \frac{2.1}{100} = 0.24$; $\frac{1.10}{0.24} = 4.6$. The difference is significant ($P < 0.01$).

A more accurate estimate, and also values for unequal number of replicates and other instances not covered by this table, may be obtained from the formula:

$$S.E. = (0.03)\bar{y}\sqrt{\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}$$

Where n_1 and n_2 are the numbers of replicates making up the two series of readings (ordinarily, of course, $n_1 = n_2$), $\bar{y}$ is the grand mean and S.E. is the required standard error of the difference between the means of the two series.

chance was less than 1 in 20 ($P < 0.05$); if the ratio exceeded 2.58, the probability was less than 1 in 100 ($P < 0.01$).

A significant difference does not necessarily imply a pathological difference, for significant differences are

to be expected within the normal range. The value of the significance test is that it shows when apparent differences may be discounted on the grounds of experimental error alone.

Coefficients of variation which have been obtained from replicate readings of one-stage prothrombin times made by the same observer (for the experiment shown in Table V, for instance, the value was 1.78%) and by different observers have agreed well with the values obtained from this analysis for the thrombin clotting time. Thus the values given in Table X probably indicate the order of magnitude that might be obtained by other workers both for the thrombin clotting time and for the one-stage prothrombin time.

Further details of the investigation into the variability of the test results will be published elsewhere.

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