A study of two methods for estimating plasma fibrinogen and the effect of epsilon aminocaproic acid and protamine

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SUMMARY Fibrinogen concentrations were determined in normal plasma and in plasma from patients with high and low levels. There was a good correlation between the results of a rapid semi-quantitative fibrinogen titre technique and those of a quantitative assay of coagulable fibrinogen. In normal subjects fibrinogen levels were not significantly influenced by taking blood into epsilon aminocaproic acid (EACA) or by the addition of protamine to plasma. In patients with the defibrination syndrome in whom increased plasma fibrinolysis was not detected, fibrinogen levels were not affected by taking blood into EACA but considerably increased levels were observed after the addition of protamine to plasma. In patients undergoing thrombolytic therapy the fibrinogen levels measured were increased both in blood taken into EACA and in plasma containing protamine. It is suggested that EACA acted by preventing lysis *in vitro* whilst protamine counteracted abnormal fibrin polymerization. The pattern of results may be of diagnostic importance.

The acute defibrination syndrome is an acquired haemorrhagic disorder associated with reduced levels of plasma fibrinogen. It may occur in a number of clinical conditions and is thought to be due to intravascular activation of the coagulation or fibrinolytic systems. Many patients have thrombocytopenia, and it seems likely that activation of coagulation is the primary event but in thrombolytic therapy hyperplasmaemia occurs. In either case fibrin or fibrinogen breakdown products may interfere with the polymerization of the fibrin monomer. This not only contributes to the bleeding tendency but may also affect coagulation tests, particularly the estimation of fibrinogen. Furthermore, lysis of fibrinogen may occur *in vitro*, in samples obtained for analysis, thus leading to erroneously low results. In order to prevent this, Nilsson and Olow (1962) took blood into EACA, a potent inhibitor of the fibrinolytic system.

Protamine sulphate accelerates the thrombin-fibrinogen reaction even in normal plasma. In the defibrination syndrome the addition of protamine to plasma tends to counteract the abnormal polymerization of fibrin. Fibrinogen values determined in the presence of protamine then tend to be higher than those observed in its absence (Bloom and Campbell,

1965; Bloom, Boyns, and Wingfield, 1968) and it is possible that this information may be of diagnostic value.

The fibrinogen titre method (Sharp, Howie, Biggs, and Methuen, 1958) has been widely used as a rapid semiquantitative method for the determination of plasma fibrinogen. Sharp and Eggleton (1963) modified this technique to provide evidence of activation of the fibrinolytic system *in vivo*. Little information is available, however, concerning the correlation of the fibrinogen titre technique with the conventional quantitative assay of coagulable fibrinogen. The object of this paper is to demonstrate this correlation and to determine the effect of the addition of EACA or protamine sulphate on the estimation of fibrinogen in normal and pathological plasma samples. Calcium has been reported to improve abnormal thrombin-fibrinogen reactions (Astrup, Brakman, and Nissen, 1965) and the effect of the addition of calcium chloride on fibrinogen determination was also studied. It will be shown that the results of the fibrinogen titre technique correlated with those of the assay of thrombin-coagulable fibrinogen and that with the addition of protamine or EACA a pattern of results emerged which may be of diagnostic importance.

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Materials and Methods

Nine parts of blood were taken into 1 part of 3.8% w/v trisodium citrate and plasma obtained by centrifuging at 3,000 rpm for 10 minutes.

FIBRINOGEN TITRE

This was performed by the method of Sharp *et al* (1958). Sevenfold doubling dilutions of 0.5 ml plasma were prepared in 0.9% saline to give a range of 1:2 to 1:128. The plasma dilutions were clotted at 37°C with 0.1 ml human thrombin, 25 units per ml (Fibrindex, Ortho), and the highest dilution of plasma containing clot (fibrinogen titre) was noted after 10 minutes.

DEMONSTRATION OF FIBRINOLYSIS

A second row of tubes was prepared as above except that the plasma was diluted in saline containing EACA at a concentration of 100 mg per 100 ml. Saline and EACA-saline dilutions of normal plasma and of equal volumes of patients' and normal plasma (to supply fibrinogen and plasminogen) were also set up. All tubes were incubated at 37°C and examined at intervals. Fibrinolysis was indicated by lysis of clots in plasma diluted with saline, in the absence of lysis in control normal plasma and in samples containing EACA.

ESTIMATION OF THROMBIN-COAGULABLE FIBRINOGEN

This was estimated by a modification of the method of Ratnoff and Menzie (1951). Plasma, 0.2 ml, was diluted in 0.5 ml saline and 0.1 ml thrombin, 25 units per ml (Fibrindex, Ortho), was added. The tube was incubated at 37°C for 30 minutes. The fibrin clot was harvested, washed, and the protein content estimated with Folin and Ciocalteu's reagent, after alkaline digestion as described by Varley (1967). The values were not corrected for dilution of the plasma sample with citrate. In this paper this technique is referred to as 'fibrinogen assay'.

EFFECT OF ADDING EACA, PROTAMINE, OR CALCIUM

To test the effect of EACA on the estimation of fibrinogen, blood was taken into trisodium citrate containing EACA at a concentration of 1 g per 100 ml. Nine parts of blood were added to 1 part citrate-EACA so that the final concentration of EACA in blood was 1 mg per ml.

Protamine sulphate (Evans Medical Ltd) was diluted to 1 mg per 100 ml in normal saline and 0.1 ml was added to the tubes containing diluted plasma in the fibrinogen titre tests and fibrinogen assays. Calcium was introduced by dissolving the

dried thrombin in 0.025 M calcium chloride and substituting this for the saline solution of thrombin in the fibrinogen titre and assay methods.

Results

Fibrinogen titres and assays were performed on 88 normal plasma samples. The fibrinogen titres ranged from 1:32 to 1:128 and the assays ranged from 185 to 463 mg per 100 ml (mean 307, SD 77). Fibrinogen titres and assays were also performed on 39 samples from patients with increased plasma fibrinogen levels, and on 43 samples from eight patients with reduced levels. Six of these patients had the acute defibrination syndrome. Four of these had abruptio placentae, one had amniotic fluid embolus, and one had suffered multiple injuries. The diagnosis of the defibrination syndrome was based on the presence of a bleeding disorder with low fibrinogen levels, prolonged thrombin clotting time, thrombocytopenia, defective thromboplastin generation, and reduced levels of factor VIII.

Of the other patients in this group, one had received Arvin and one had been treated with streptokinase. The fibrinogen titres of these eight patients varied from nil to 1:32 and the assays from nil to 150 mg per 100 ml.

When the results of fibrinogen titres of all samples were compared with those of fibrinogen assays a general correlation was apparent (Table I). Thus

No. of Plasma Samples	Fibrinogen Titre	Fibrinogen Assay (mg/100 ml)		
		Range	Mean	SD
62	1 : 128	300-824	534	142
34	1 : 64	270-409	342	51
33	1 : 32	130-280	195	41
13	1 : 16	77-198	104	48
7	1 : 8	41-70	57	11
8	1 : 4	25-50	40	12
6	1 : 2	9-40	21	10
7	0	0-10	3	5

Table I Distribution of fibrinogen titres with corresponding fibrinogen assays

samples with low fibrinogen titres had low fibrinogen assays and the degree of overlap of fibrinogen titres with assays was never more than 1 dilution. The mean assay values plotted on logarithmic scale against corresponding titres produced a straight line, except at the very lowest level (0-10 mg per 100 ml) where the estimation of fibrinogen is probably very inaccurate (Fig.).

The results of the presence of EACA, protamine or calcium on the fibrinogen titres and assays of 10

Fig. Fibrinogen titres of 170 normal and pathological plasma samples plotted against the mean fibrinogen levels (mg per 100 ml) at each titre.

normal plasma samples are shown in Table II. Slightly higher results were observed in the fibrinogen assay with added protamine but none of the differences were statistically significant.

The results of taking blood into EACA or the

addition of protamine or calcium to samples with low fibrinogen levels due to the defibrination syndrome or Arvin therapy are shown in Table III. Epsilon aminocaproic acid had no significant effect and the presence of calcium resulted in only small and insignificant elevation of fibrinogen assays. The addition of protamine, however, caused consistent elevation of the fibrinogen titres and highly significant elevation of the fibrinogen assays ($P < 0.001$). In none of these samples was fibrinolytic activity detected in the plasma.

The results of taking blood into EACA or the addition of protamine or calcium to plasma samples from two patients undergoing streptokinase therapy are shown in Table IV. Addition of calcium had little effect except in one sample (MN₃) where the fibrinogen level was apparently increased. Taking blood into EACA or the addition of protamine, however, resulted in consistently increased fibrinogen titres and much higher assays of coagulable fibrinogen. All these samples showed evidence of increased fibrinolysis. The addition of EACA to plain citrated plasma in the test for fibrinolysis, ie, after separation of the plasma and during its dilution, did not significantly affect the fibrinogen titres but prevented subsequent lysis. Epsilon aminocaproic acid thus prevented fibrinogenolysis and fibrinolysis *in vitro* but did not promote the formation of fibrin in a manner similar to that of protamine.

The effect of combinations of EACA, protamine, and calcium on the estimation of fibrinogen was not tested.

Discussion

In the defibrination syndrome or during thrombolytic therapy it may be necessary to perform estimations of plasma fibrinogen urgently. Chemical pre-

Plasma Sample	Fibrinogen Titre				Fibrinogen Assay (mg/100 ml)			
	Citrated Plasma	EACA-citrated Plasma	Citrated Plasma with Calcium	Citrated Plasma with Protamine	Citrated Plasma	EACA-citrated Plasma	Citrated Plasma with Calcium	Citrated Plasma with Protamine
1	1:64	1:64	1:64	1:64	380	372	368	425
2	1:32	1:32	1:32	1:32	223	228	231	240
3	1:128	1:128	1:128	1:128	390	384	395	450
4	1:64	1:64	1:64	1:64	300	310	286	335
5	1:32	1:64	1:64	1:32	287	280	280	280
6	1:64	1:32	1:32	1:32	320	320	335	340
7	1:64	1:32	1:64	1:64	276	280	250	292
8	1:64	1:64	1:64	1:32	328	328	320	285
9	1:128	1:128	1:128	1:128	360	380	360	360
10	1:32	1:32	1:32	1:64	225	220	220	240
					Mean 308.9 (A)	310.2 (B)	304.5 (C)	324.7 (D)
					SD 58.4	58.9	60.5	71.7

Table II Fibrinogen titres and corresponding fibrinogen assays of normal plasma samples

Statistical significance: A vs B, $P > 0.5$; A vs C, $P > 0.5$; A vs D, $P > 0.3$.

Plasma Sample ¹	Fibrinogen Titre				Fibrinogen Assay (mg/100 ml)			
	Citrated Plasma	EACA-citrated Plasma	Citrated Plasma with Calcium	Citrated Plasma with Protamine	Citrated Plasma	EACA-citrated Plasma	Citrated Plasma with Calcium	Citrated Plasma with Protamine
MW ₁	0	0	0	1:4	0	0	0	40
MW ₂	0	0	0	1:8	0	0	9	60
MW ₃	0	0	1:2	1:16	0	0	9	85
IK ₁	1:8	1:16	1:16	1:32	67	53	80	120
IK ₂	0	0	0	1:8	0	0	0	70
BP ₁	0	0	0	1:16	0	10	10	80
BP ₂	1:16	1:16	1:16	1:32	150	150	150	200
CE ₁	1:2	1:2	1:4	1:8	40	45	60	90
CE ₂	1:2	1:2	1:2	1:16	25	20	40	105
P.B.	1:2	1:2	1:2	1:32	9	9	20	140
L.G.	1:4	1:4	1:8	1:16	40	40	70	95
B.H.	1:4	1:4	1:4	1:32	25	40	35	100
				Mean	29.7 (A)	30.6 (B)	40.3 (C)	98.8 (D)
				SD	43.7	42.5	44.2	41.4

Table III Fibrinogen titres and corresponding fibrinogen assays on plasma samples obtained from patients with the defibrination syndrome

¹MW₁, MW₂, and MW₃ on Arvin therapy.

Statistical significance: A vs B, $P > 0.5$; A vs C, $P > 0.5$; A vs D, $P < 0.001$.

Plasma Sample	Fibrinogen Titre				Fibrinogen Assay (mg/100 ml)			
	Citrated Plasma	EACA-citrated Plasma	Citrated Plasma with Calcium	Citrated Plasma with Protamine	Citrated Plasma	EACA-citrated Plasma	Citrated Plasma with Calcium	Citrated Plasma with Protamine
MN ₁	1:4	1:32	1:4	1:32	20	225	25	250
MN ₂	1:8	1:32	1:8	1:32	50	220	60	250
MN ₃	0	1:32	1:8	1:64	10	220	95	300
NM ₁	1:8	1:128	1:4	1:128	63	460	48	490
NM ₂	1:4	1:64	1:4	1:128	40	384	36	390
NM ₃	1:8	1:32	1:8	1:64	55	260	45	285

Table IV Fibrinogen titres and corresponding fibrinogen assays on plasma samples obtained from patients on streptokinase therapy

cipitation (Parfentjev, Johnson, and Clifton, 1953) and immunological methods (eg, FI test, Hyland) can be performed rapidly, but the results may correlate poorly with those of tests which measure coagulable fibrinogen (Sharp *et al*, 1958; Bloom and Campbell, 1965). It is probable that both the precipitation and immunological methods measure breakdown products of fibrin and fibrinogen or soluble fibrin polymers which do not coagulate. On the other hand these derivatives may interfere with the polymerization of normal fibrin monomer and so reduce the apparent levels of coagulable fibrinogen. It seems likely, however, that the concentration of normally coagulable fibrinogen is most relevant to haemostasis.

The present study has confirmed that the fibrinogen titre technique described by Sharp *et al* (1958) is a reliable, rapid semi-quantitative method for the estimation of coagulable fibrinogen. The results with this method were comparable, within the limits of the technique, with those observed using the method of Ratnoff and Menzie (1951), a technique which is

too time consuming for emergency use.

In normal plasma the presence of EACA, protamine, or calcium had little effect on the estimation of fibrinogen. In patients with the defibrination syndrome and those undergoing thrombolytic therapy, however, the addition of protamine caused considerable increase in the measured levels of coagulable fibrinogen. In these conditions it is likely that the polymerization of fibrin is abnormal due to the presence of fibrin or fibrinogen split products (Fletcher, Alkjaersig, and Sherry, 1962) or soluble intermediary fibrin polymer (Shainoff and Page, 1962). In the present study it is probable that protamine more or less corrected the abnormal polymerization and that this accounted for the observed increase of fibrinogen levels.

Epsilon aminocaproic acid is a potent inhibitor of the fibrinolytic system. Higher fibrinogen levels were observed when blood from patients treated with streptokinase was taken into EACA than when it was taken into plain citrate. Unlike protamine,

EACA did not promote the formation of fibrin but seemed to act by preventing fibrinogenolysis or fibrinolysis *in vitro* before and during the estimations. Epsilon aminocaproic acid did not affect the fibrinogen levels in patients with the defibrination syndrome but in these patients increased fibrinolysis was not detected. It seems desirable, however, that blood samples for fibrinogen estimation should be taken into anticoagulant containing EACA or some other fibrinolytic inhibitor when increased fibrinolysis is suspected, in order to prevent lysis *in vitro*.

The addition of calcium had no significant effect on fibrinogen titres or assays in the present study but its presence rendered the clot more opaque and less friable. It was then easier to harvest in the Ratnoff and Menzie method. In this manner calcium could possibly increase the accuracy of the technique, particularly at low fibrinogen levels. The thrombin-calcium chloride mixture thus constituted a convenient reagent for fibrinogen assays.

Although it is probable that the level of fibrinogen measured in the absence of protamine is most relevant to haemostasis a certain pattern of results emerged in the present study. In normal plasma the addition of protamine, EACA, or calcium had no significant effect on the measurement of fibrinogen. In the defibrination syndrome, when increased plasma fibrinolysis was not detected, the presence of EACA or calcium had no significant effect but higher fibrinogen levels were observed after the addition of protamine. In patients undergoing thrombolytic therapy the presence of calcium also had little effect but higher fibrinogen levels were observed in blood taken into EACA and after the addition of protamine.

In the acute defibrination syndrome it is necessary to distinguish rapidly between disseminated intravascular coagulation and increased fibrinolysis because inappropriate treatment may be harmful. As a result of this work we suggest that the following simple tests should be initially performed.

Blood is taken into EACA-citrate and into plain citrate and the fibrinogen titre performed on each sample. The fibrinogen titre with protamine is performed on the plain citrated sample. Whilst these tests are developing, a thrombin clotting time is

determined and a platelet count set up, the former giving rapid information concerning the presence or absence of defibrination.

If the fibrinogen titre in plain citrated plasma is lower than in EACA-citrated plasma increased fibrinolysis is suspected and is confirmed appropriately (see Methods, and Hardisty, Ingram, and Sharp, 1964). If, on the other hand, the fibrinogen titre is reduced in both EACA-citrated and plain citrated plasma but is higher in the presence of protamine the defect is more likely to be due to intravascular coagulation. This is confirmed in the presence of thrombocytopenia. We do not claim that this scheme is exhaustive and if time permits other investigations (Hardisty *et al*, 1964) should be performed. The scheme does, however, allow a reasonably rapid assessment which can easily be introduced into laboratory routine.

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