

THE EFFECT OF CITRATE ON EUGLOBULIN METHODS OF ESTIMATING FIBRINOLYTIC ACTIVITY

BY

MONAMY BUCKELL

From the Clinical Research Laboratories, Charing Cross Hospital, London

(RECEIVED FOR PUBLICATION MARCH 19, 1958)

The time taken for the spontaneous lysis of the calcium-clotted fibrinogen of the isolated euglobulin fraction of plasma was used as a method of estimating fibrinolysis by Kowarzyk and Buluk (1950) and further developed by Niewiarowski (1952). This method has the advantage of requiring simple apparatus and taking a relatively short time to perform. It does not in any sense measure "natural" fibrinolysis, but it seemed worth while investigating and comparing it with other methods to see if it could be usefully employed in following variations in fibrinolytic activity in an individual and detecting differences between groups. In the course of this investigation the influence of the anticoagulant used became apparent.

Methods

Preparation of Plasma Specimens.—To eliminate as far as possible the effects of lipaemia (which inhibits fibrinolysis) without taking fasting specimens, the subjects (members of the laboratory and medical staff) took no fat with their breakfast. Blood was taken, between 9 and 10 a.m., by clean venepuncture with minimum stasis, using a silicone-coated wide-bore (20 S.W.G.) needle and all-glass syringe lubricated with silicone fluid MS 550. Samples of blood, each of 4.5 ml., were distributed quickly into 15 ml. conical centrifuge tubes (cooled in melting ice) containing (1) for oxalated plasma, 0.5 ml. of 0.1 M ammonium oxalate; (2) for citrated plasma, 0.5 ml. of 3.8% sodium citrate; (3) for "true" plasma, silicone-coated tube, no anticoagulant.

The tubes were spun at approximately 3,000 r.p.m. for 10 minutes, the centrifuge buckets for the "true" plasma tubes being previously cooled at 0° C. All blood and plasma specimens were kept in melting ice and tests were put up within 20 minutes of obtaining the specimen.

Euglobulin Lysis Time.—The method of Niewiarowski (1952) was used.

Reagents.—The following are required: 0.025 M calcium chloride, 1% acetic acid, borate solution (9 g. sodium chloride + 1 g. sodium borate made up to 1 litre with distilled water to give pH 9.0 as found by a glass electrode), and 0.1 M ammonium oxalate.

Procedure.—The determinations were carried out in 15 ml. conical centrifuge tubes. The tubes were soaked in chromic acid cleaning mixture for a few hours, brushed out, and soaked overnight in tap water, rinsed with distilled water, and dried in the oven before use. Scrupulous care in cleaning is necessary to achieve good duplication and tubes must all be of the same dimensions.

Using each of the three plasma preparations, duplicate tests were put up as follows:

To 9 ml. of distilled water in a centrifuge tube add 0.5 ml. of plasma. The pH is brought up to 5.3 by adding 0.1 ml. of 1% acetic acid to each tube. The tubes are then stood for 30 min. in the refrigerator at 4° C. for the euglobulin fraction of the plasma to precipitate. The tubes were then centrifuged for five minutes, the supernatant decanted, and the tubes drained by inversion on filter paper, when 0.5 ml. of the borate solution was added, and the tubes were placed in the 37° C. water-bath and stirred gently with a glass rod. Of the 0.025 M calcium chloride solution, 0.5 ml. was added to the resulting solution of euglobulins in borate and the time at which the mixture clotted was recorded. The tubes were left at 37° C. and inspected at intervals, and the lysis time determined. When the completion of lysis seemed near the clots were inspected every five minutes.

Fibrin Plate Method.—Fibrinogen free from citrate was prepared by the method of Laki (1951) from Armour bovine plasma fraction I. Plates were prepared from a 0.1% solution of fibrinogen by the adaptation of Astrup's method described by Holburn (1955). Unheated plates, for estimation of enzyme and activator, and plates heated for 30 minutes at 85° C. for estimation of enzyme only, were used. Plasma samples were prepared for estimation on plates by precipitating the euglobulin fraction at pH 5.3, as described above, and dissolving the precipitate in 0.5 ml. of the sodium diethyl barbiturate buffer, pH 7.8, used in the preparation of the plates.

Results

Euglobulin Lysis Time.—It will be seen from Table I that in all eight experiments the greatest activity was found in the citrated specimen. Oxalate and silicone plasma gave less active prepara-

TABLE I
COMPARATIVE EUGLOBULIN LYSIS OF PLASMA
PREPARED IN VARIOUS WAYS

Sex	Age	Silicone Specimen		Oxalated Specimen		Citratated Specimen	
		Lysis Time	Rate	Lysis Time	Rate	Lysis Time	Rate
M	30	149	6.71	174	5.75	87	11.49
M	39	125	8.00	118	8.48	90	11.11
F	34	160	6.25	145	6.90	90	11.11
M	24	200	5.00	173	5.70	99	10.10
M	22	144	6.94	156	6.41	110	9.09
M	18	210	4.81	229	4.41	130	7.69
F	29	195	5.13	197	5.08	133	7.52
F	34	327	3.06	248	4.03	166	5.99
Average		189	5.74	180	5.85	113	9.26

Lysis time in minutes. Rate = $\frac{1,000}{\text{Lysis time}}$

tions, with no appreciable difference between them.

Citrate added in increasing amounts to a mixture of "true" plasma and distilled water before precipitating the euglobulin fraction by adding acetic acid to pH 5.3 gave the results shown in Fig. 1 where the increase in activity is proportional to the concentration of citrate in the mixture. Increasing quantities of oxalate also produced a slight activation but not to the same extent as citrate.

Heparin and "sequestrene" (di-sodium salt of ethylene diamine tetra acetic acid, E.D.T.A.) were also tried as anticoagulants. Neither was satis-

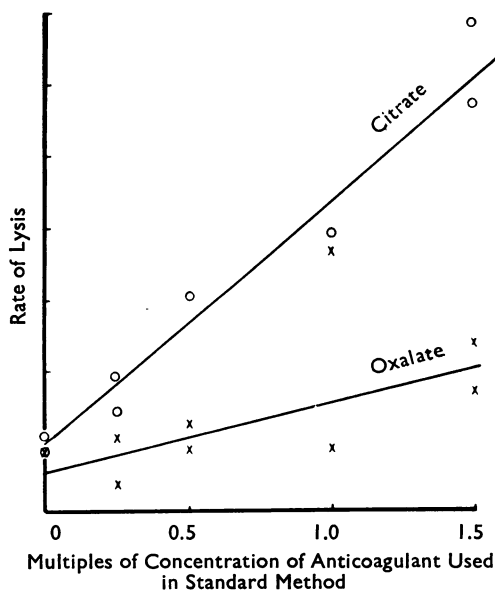


FIG. 1.—Effect of concentration of anticoagulant on euglobulin lysis rate.

factory; heparin plasma yielded a euglobulin solution that did not clot on the addition of calcium, and the euglobulin clots made from sequestrene plasma left a sludge in the bottom of the tube so that the completion of lysis was difficult to determine.

Paper electrophoresis of euglobulin solutions gave no detectable albumin and showed the lipoprotein content to be reduced to about a third of that of the parent plasma.

Fibrin Plate Method.—Table II gives the comparative fibrinolytic activity by the fibrin plate method for five sets of plasma samples, and both the heated and unheated plates give the same

TABLE II
COMPARATIVE FIBRINOLYTIC ACTIVITY BY FIBRIN
PLATE METHOD (AREA LYSED IN MM.²)

Sex	Age	Silicone Specimen		Oxalated Specimen		Citratated Specimen	
		Heated Plate	Un-heated Plate	Heated Plate	Un-heated Plate	Heated Plate	Un-heated Plate
F	34	6	46	6	30	20	125
F	34	No lysis	50	No lysis	45	39	79
M	24	Trace	20	14	25	35	68
M	24	16	49	12	52	45	64
M	24	No lysis	47	No lysis	42	60	145
Average		4.4	42.2	6.4	38.8	39.8	96.2

order of activity as was found by the euglobulin lysis time method on a different series of specimens. No lysis was produced by 3.8% sodium citrate solution on heated or unheated plates.

Discussion

Müllertz (1952) found that citrate added to the fibrinogen from which plates were prepared increased the activity of plasmin upon them. The results reported here show that citrate consistently increases the fibrinolytic activity in the isolated euglobulin fraction of human plasma as measured by two different methods.

The area lysed on a heated fibrin plate is a measure of the amount of proteolytic enzyme in the test solution. In the unheated plates plasminogen, destroyed by heating, is present and is converted to plasmin by activator in the specimen and the resulting lysis represents the sum of enzyme plus activator. The difference between the areas lysed on the unheated and heated plates gives the amount of activator and was about the same for oxalated and true plasma, 32.2 and 37.8 respectively, but was greater, 56.4, for the citrated samples. Thus there was an increase of activator as well as of pre-formed plasmin. This is not due

to citrate being carried over in the separation of the euglobulin fraction. No lysis was produced by six times as much citrate as was present in the plasma treated. Citrate may therefore be active in the transformation of proactivator to activator, which in turn activates plasminogen to plasmin, this increased plasmin content being shown on both the unheated and the heated plates and by the increased speed of lysis. Further work needs to be done to determine the precise site of action of citrate, but it appears that citrate should be avoided in the preparation of specimens for the estimation of fibrinolysis either by the euglobulin lysis time method or by the fibrin plate method, and that oxalate is suitable as it gives a result very close to that of true plasma.

The euglobulin methods deal with a simplified system in which the albumin anti-plasmin and much of the lipoprotein has been removed and do not represent the state of affairs in whole blood. It is doubtful how much of the activity in the euglobulin lysis time method is pre-formed plasmin and how much is formed from plasminogen during the test. Certainly when citrate is used this would appear to be possible. After several days' storage at 4° C. both oxalated and citrated plasma gave euglobulin clots that still lysed, although more slowly. No lysis was obtained by Bidwell's (1953) method on these stored samples.

The citrate effect found with euglobulin was not obtained when Bidwell's method was performed simultaneously with the work shown in Table I. Oxalated plasma was the most active in six out

of eight tests, but the differences were not great enough for any conclusions regarding anticoagulant to be drawn. These variations of activity with anticoagulant make it imperative to stick to the same substance throughout any comparative series and to state what anticoagulant was used when recording results.

Summary

Citrate increases the fibrinolytic activity of the isolated euglobulin fraction of human plasma.

An increase in plasminogen activator as well as of active enzyme is produced.

Citrate should not be used in the preparation of specimens for fibrinolysis assay by the fibrin plate or the euglobulin lysis time methods.

Oxalate is a suitable anticoagulant for use in the above method.

I wish to thank Dr. F. A. Elliott, who initiated this work, for his help and encouragement, and the Research Subcommittee of Charing Cross Hospital for supporting the project; also Dr. A. L. Copley for calling our attention to the euglobulin lysis time method, Mrs. J. Pearson, B.Sc., for assistance in the earlier part of the work, and Dr. P. G. Bond for his help in the later part.

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