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The effect of calcium on fibrinolysis *in vitro*

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SYNOPSIS The influence of calcium on fibrinolysis *in vitro* has been studied. In the absence of inhibitors, fibrinolytic activity is enhanced by calcium. In whole blood the inhibitory effect of calcium on fibrinolysis can be explained by its effect on the inhibitors present. Anti-activator is dependent on the presence of calcium for its formation, although calcium has no further influence on its action. Antiplasmin activity is enhanced by the presence of calcium.

There have been many conflicting reports on the effect of calcium on the fibrinolytic system. Fearnley and Tweed (1953) first observed that ionic calcium caused some inhibition of diluted plasma clot lysis. This was later confirmed by Sherry, Lindemeyer, Fletcher, and Alkjaersig (1959), and Thomas (1958) reported the inhibition of streptokinase-induced clot lysis by calcium. Ratnoff (1952), however, noted an accelerating effect of calcium on the euglobulin lysis time, and this was confirmed by Kowalski, Kopec, and Niewiaroswki (1959).

Explanations of the inhibitory effect of calcium on plasma clot lysis were given by Bidwell (1953), who suggested that the type of fibrin influences the rate of fibrinolysis, and by Bickford and Sokolow (1961), who suggested that the urea-insoluble fibrin formed in the presence of calcium is less susceptible to fibrinolysis than the urea-soluble form. Medart (1958) proposed that calcium disturbs the equilibrium between two forms of fibrinogen, the one susceptible, the other inhibitory, to fibrinolysis. None of these workers took into account the conflicting results reported by Ratnoff (1952) in the euglobulin lysis time.

Ratnoff concluded that the acceleration of euglobulin clot lysis was due to the effect of calcium on the activation of plasminogen, and not on the action of plasmin. Although this fits in with the subsequent findings of Kowalski, Latallo, and Niewiarowski (1956) that the activity of plasmin is not influenced by calcium, Ratnoff could not demonstrate its effect on the activation of plasminogen by streptokinase or chloroform.

In the light of these reports it was thought that the influence of calcium on the fibrinolytic system should be more fully investigated, in order to discover whether calcium accelerates or inhibits fibrinolysis, and, if the latter, whether it acts directly on plasmin or indirectly through the inhibitors. The inhibitory components of the fibrinolytic system considered in this paper are the combined effect of the antiplasmins (Norman, 1960) and the anti-activator effect described by Flute (1960). In plasma the inactive precursor plasminogen is acted upon by blood activator to form the fibrinolytic enzyme plasmin. This activation of plasminogen to plasmin is inhibited by anti-activator, which is formed rapidly on incubation of blood in glass (Flute, 1960). The proteolytic action of plasmin on fibrin is inhibited by the antiplasmins which are present in circulating blood (Norman, 1960). The effect of calcium on the fibrinolytic system as a whole is measured in plasma. To separate the enzymes and their inhibitors use is made of the euglobulin precipitation method. The euglobulin fraction prepared at 4°C. immediately after collection of blood is used as a source of activator, plasminogen, and plasmin, free from inhibitors. The effect of calcium on anti-activator is tested in the euglobulin fraction prepared from incubated plasma or serum. This fraction will contain anti-activator but no antiplasmins. The role of calcium in the formation of anti-activator is studied by comparing euglobulin fractions of serum incubated both in the presence and absence of calcium. The combined influence of anti-activator and antiplasmin is measured in incubated serum. In this way the effect of calcium on the components of the fibrinolytic system has been studied.

REAGENTS

Human venous blood was used as a source of most of the components of the fibrinolytic system.

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DECALCIFYING AGENTS These are (1) 10% dipotassium ethylene diamine tetra-acetate (E.D.T.A.) (0·1 ml./10 ml. blood); (2) 3·8% trisodium citrate (1 ml./10 ml. blood); and (3) 0·1 M ammonium oxalate (1 ml./10 ml. blood).

The E.D.T.A. interfered to some extent with the subsequent clotting procedures, and, as citrate was found to be suitable for the removal of calcium in these experiments, this was used in the majority of the tests.

FIBRINOGEN Solution of bovine fibrinogen (bovine plasma fraction I, Armour Pharmaceutical Co.) containing 0·5 g. in 100 ml. buffer.

THROMBIN Solution of bovine thrombin (Thrombin Reagent, Leo Pharmaceutical Products) containing 50 N.I.H. units/ml. saline. This has been found to be free from significant contamination by components of the fibrinolytic system.

STREPTOKINASE Streptokinase-streptodornase Varidase (Lederle Laboratories).

BUFFERS Veronal buffer, *p*H 7·4, ionic strength 0·154, was prepared from the following formula: sodium diethyl barbiturate, 11·745 g., sodium chloride 14·67 g., 0·1 N hydrochloric acid 430 ml., distilled water to 2 litres.

Sodium borate solution, *p*H 9·0, was prepared from sodium chloride 9 g., sodium borate 1 g., distilled water to 1 litre.

METHODS

SEPARATION OF THE ENZYMES FROM THEIR INHIBITORS Inhibitor-free euglobulin fraction was prepared by the following method. Venous blood was collected into a plastic syringe which had been cooled to 4°C. and placed in a precooled plastic tube containing anticoagulant and immediately centrifuged at 1,710 *g* (3,250 r.p.m.) for 10 minutes at 4°C. The platelet-poor plasma was transferred to 18 times its volume of ice-cold distilled water and the *p*H adjusted to 5·3 by the addition of 1% acetic acid (usually one-fifth of the plasma volume). This was allowed to stand at 4°C. for 30 minutes, then centrifuged at 1,710 *g* (3,250 r.p.m.) for 10 minutes at 4°C. and the supernatant discarded. The resulting euglobulin precipitate was free from significant antiplasmin activity; meticulous observance of the cold handling technique and use of plastic apparatus prevented the formation of anti-activator.

Antiplasmin-free euglobulin fraction was prepared from plasma or serum which had been incubated in a glass container at 37°C. for 30 minutes to allow formation of anti-activator. Once formed, this is precipitated with the euglobulin fraction (Flute, 1960).

MEASUREMENT OF FIBRINOLYTIC ACTIVITY The fibrin plate (Astrup and Müllertz, 1952) was prepared using bovine fibrinogen (0·5 g. Armour plasma fraction I/100 ml. buffer) and thrombin, modified by the incorporation of 1 ml. 0·01 M $CaCl_2$ or 0·12 M NaCl in the plate. The results are expressed as the product of two diameters of the area of lysis in square millimetres. In this paper all results quoted are the means of three readings.

The heated fibrin plate (Lassen, 1953) was prepared using bovine fibrinogen and thrombin, again modified by the inclusion of calcium or sodium chloride in the plate.

The euglobulin lysis time was determined as follows:— The euglobulin precipitate derived from 0·5 ml. plasma is dissolved in 0·5 ml. buffer. To 0·4 ml. of this solution 0·5 ml. 0·01 M $CaCl_2$, 0·025 M $CaCl_2$ or 0·12 M NaCl is added, and the mixture clotted with 0·1 ml. thrombin. The time taken for complete dissolution of the clot at 37°C. is called the euglobulin lysis time.

RESULTS

EFFECT OF CALCIUM ON FIBRINOLYSIS AS MEASURED IN PLASMA Citrated platelet-poor plasma was activated by adding streptokinase, 100 units/ml. plasma, and aliquots were applied immediately to unheated fibrin plates, containing calcium or sodium chloride. As shown in Table I, there is significantly less lysis in the presence of calcium. It must be borne in mind that these results were influenced by activation by streptokinase of the plasminogen contaminating commercial fibrinogen, and also by any contaminant inhibitors which may be present.

TABLE I

LYSIS PRODUCED BY STREPTOKINASE-ACTIVATED PLASMA ON FIBRIN PLATES IN PRESENCE AND ABSENCE OF CALCIUM

	Test Plasma	*1*	*2*	*3*	*4*	*5*	*6*
Standard plates	Without calcium	662[1]	252	142	20	664	613
	Calcium present	352	107	48	trace	482	559
Heated plates	Without calcium	49	46	42	30	78	36
	Calcium present	25	23	29	20	69	9

[1]These figures represent the areas of lysis in square millimetres, and are the means of three readings.

Streptokinase-activated plasma was also tested on heated fibrin plates. Although the results do not indicate such significant differences (Table I) as those on the unheated plates, there is consistently less lysis when calcium is present. Heating destroys the contaminating plasminogen and inhibitors in the plate, and these results therefore depend on the activity of the plasmin formed by activation by streptokinase of the plasminogen in the plasma sample: they are also influenced by the inhibitors in the plasma.

These results indicate that calcium inhibits fibrinolysis in plasma.

EFFECT OF CALCIUM ON FIBRINOLYSIS AS MEASURED IN INHIBITOR-FREE EUGLOBULIN FRACTION Euglobulin lysis times were carried out on blood taken from stressed and unstressed individuals, in all cases preparing the euglobulin fraction at 4°C. As seen in Table II lysis times are much shorter when calcium

TABLE II

EFFECT OF CALCIUM ON FIBRINOLYSIS IN THE ABSENCE OF INHIBITORS[1]

Plasma Sample	*Anticoagulant*	*Buffer*	*Without Calcium*	*Calcium Present*
1	Oxalate	Veronal	425	205
	Citrate	Veronal	350	180
	Oxalate	Borate	550	170
	Citrate	Borate	440	160
2	Citrate	Borate	320	120
3	Citrate	Veronal	101	96
4	Citrate	Veronal	135	123
5	Citrate	Veronal	360	290
6	Citrate	Veronal	255	180
7	Citrate	Veronal	240	190
8 Post-cardiac surgery	Citrate	Veronal	16	13

[1]The euglobulin lysis times are expressed in minutes in the presence and absence of calcium using inhibitor-free euglobulin fraction,

is present. This is independent of the anticoagulant or buffer used. The euglobulin lysis time under these conditions is a measure of the activity of activator and plasmin; it is not influenced by anti-activator or anti-plasmin activity. From these results it appears that fibrinolysis, in the absence of inhibitors, is accelerated by calcium. It is not possible to tell whether calcium acts on the activator or on plasmin.

Inhibitor-free euglobulin fraction was applied to unheated fibrin plates. Lysis was greater in the plates containing calcium (Table III). These results also indicate that calcium enhances fibrinolysis in the absence of inhibitors.

TABLE III

EFFECT OF CALCIUM ON FIBRINOLYSIS IN THE ABSENCE OF INHIBITORS[1]

Test Sample	*1*	*2*	*3*	*4*	*5*
Without calcium	90	40	340	110	60
Calcium present	330	120	370	160	90

[1]Lysis was produced by inhibitor-free euglobulin fraction on unheated fibrin plates, in the presence and absence of calcium. Areas of lysis in square millimetres representing the mean of three readings.

Fibrinolysis, as measured in the inhibitor-free euglobulin fraction, is enhanced by calcium. It was thought that the inhibitory action of calcium on fibrinolysis as measured in plasma might be explained by its effect on the inhibitors.

INFLUENCE OF CALCIUM ON PREFORMED ANTI-ACTIVATOR Inhibitor-free euglobulin fraction and antiplasmin-free euglobulin fraction were prepared from the same citrated plasma samples. Euglobulin lysis times were carried out on both fractions, with and without the addition of calcium, and the results are shown in Table IV. The longer lysis times recorded

TABLE IV

INFLUENCE OF CALCIUM ON PREFORMED ANTI-ACTIVATOR[1]

Test No.	*Inhibitor-free Euglobulin*		*Antiplasmin-free Euglobulin*	
	Without Calcium	*Calcium Present*	*Without Calcium*	*Calcium Present*
1	253	178	400	240
2	238	193	400	219
3	261	177	400	240
4	360	250	500	370
5	160	123	240	190

[1]Euglobulin lysis times (min.) in the presence and absence of calcium, using inhibitor-free euglobulin fraction and antiplasmin-free euglobulin fraction. Antiplasmin-free euglobulin fraction is prepared from citrated plasma after incubation to allow development of antiactivator.

using the antiplasmin-free euglobulin fraction indicate the presence of anti-activator in this fraction. Calcium accelerates the rate of lysis in both fractions to a comparable degree, indicating that the inhibition caused by anti-activator is not enhanced by calcium.

Inhibitor-free euglobulin fraction was prepared from citrated plasma. Antiplasmin-free euglobulin fraction was prepared from incubated serum, E.D.T.A. having been added to the serum after incubation (0·01 ml. 10% E.D.T.A. per ml. serum). Euglobulin lysis times were then set up, using 0·4 ml. inhibitor-free euglobulin fraction and varying amounts of antiplasmin-free euglobulin fraction in a clot of volume 1·1 ml. Calcium or sodium chloride was added before clotting with thrombin. As seen in Table V, the addition of antiplasmin-free euglobulin fraction prolongs the euglobulin lysis time, due to the presence of anti-activator. Calcium, however, does not increase this inhibition. Thus anti-activator, once formed, is not influenced by calcium, and the inhibitory action of calcium on whole blood lysis cannot be explained by its effect on anti-activator.

EFFECT OF CALCIUM ON DEVELOPMENT OF ANTI-ACTIVATOR *in vitro* There remains the effect of calcium on the formation of anti-activator, and Flute (1962) has suggested that calcium is indeed necessary for its formation. Anti-activator is not identifiable as such in circulating blood, but is formed after incubation in contact with glass (Flute, 1960). In order to test the effect of calcium on its formation, 3 ml. platelet-poor E.D.T.A. plasma (0·1 ml. 10% E.D.T.A. per 100 ml. plasma) and 3 ml. native plasma were clotted with 0·1 ml. thrombin (500 N.I.H. units/ml.), incubated in glass for 30 minutes at 37°C. The clots were then removed, E.D.T.A. added to the serum containing none, and euglobulin precipitates prepared from each. The anti-activator activity of each of these fractions was tested by adding varying amounts to 0·4 ml. inhibitor-free euglobulin fraction, and carrying

TABLE V

INFLUENCE OF CALCIUM ON PREFORMED ANTI-ACTIVATOR[1]

	Added Antiplasmin-free Euglobulin					
	0	*0·1 ml.*	*0·2 ml.*	*0·3 ml.*	*0·4 ml.*	*0·5 ml.*
Without calcium	135	154	225	255	345	345
Calcium present	125	149	195	225	285	285

[1]Euglobulin lysis times (min.) in the presence and absence of calcium, with the addition of increasing amounts of antiplasmin-free euglobulin fraction containing preformed anti-activator. Antiplasmin-free euglobulin fraction prepared from incubated serum and added to constant amounts of inhibitor-free euglobulin and euglobulin lysis times carried out.

out euglobulin lysis times as described in the previous experiment. Calcium was added to all tubes before clotting with thrombin. The E.D.T.A. was used in this experiment to obtain more complete removal of calcium. It can be seen from the results in the previous experiments (Table V) that citrate in concentrations sufficient to prevent clotting does not prevent the formation of anti-activator.

Prolongation of the euglobulin lysis above that of the control, containing inhibitor-free euglobulin alone, was taken as a measure of the anti-activator activity present. The results (Table VI)indicate that

TABLE VI

EFFECT OF CALCIUM ON DEVELOPMENT OF ANTI-ACTIVATOR[1] *in Vitro*

	Amount of Added Serum (antiplasmin-free) Euglobulin					
	0	*0·1 ml.*	*0·2 ml.*	*0·3 ml.*	*0·4 ml.*	*0·5 ml.*
Serum incubated without calcium	130	135	135	135	135	135
Serum incubated with calcium	125	150	195	225	285	285

[1]Euglobulin lysis times (min.) with the addition of antiplasmin-free euglobulin prepared after incubation of serum in the presence and absence of calcium.

very little anti-activator activity developed in the E.D.T.A. specimen during incubation. Quite considerable anti-activator activity was developed in the serum when calcium was not removed. These results substantially confirm Flute's findings. Calcium thus seems to enhance the development of anti-activator *in vitro*. This may account for the inhibitory action of calcium on fibrinolysis as measured in plasma.

EFFECT OF CALCIUM ON ANTIPLASMIN ACTIVITY In assessing antiplasmin activity the influence of anti-activator must be borne in mind. Whole blood was incubated in glass at 37°C. for 30 minutes to permit the complete development of anti-activator activity, the serum then removed and citrate added.

It has already been established that calcium does not further alter anti-activator activity once it has been formed and so the antiplasmin activity of the serum could be assessed directly, in the presence and absence of calcium. Serum (0·1 ml.) was added to 0·4 ml. inhibitor-free euglobulin fraction and euglobulin lysis times set up. The results (Table VII) show that the antiplasmin activity of the serum is increased in the presence of calcium.

TABLE VII

EFFECT OF CALCIUM ON ANTIPLASMIN ACTIVITY[1]

Test No.	*Without Calcium (hr.)*	*Calcium Present (hr.)*
1	13	24
2	4	10
3	5	11
4	7	13

[1]Euglobulin lysis times (hr.) in the presence and absence of calcium with the addition of 0·1 ml. incubated serum.

DISCUSSION

The inhibitory effect of calcium on lysis in plasma (Sherry *et al.*, 1959), and its accelerating effect on euglobulin clot lysis (Ratnoff, 1952) have been confirmed.

It is well known that metallic ions acting as cofactors can play an important role in enzymatic reactions. It seems that the fibrinolytic system offers no exception: in the absence of inhibitors, the lysis of fibrin proceeds more rapidly when calcium is present. It has not been determined whether calcium accelerates the proteolytic activity of plasmin itself, or influences the activation of plasminogen to plasmin by activator. Ratnoff (1952) believed that activator is affected directly, but could not demonstrate this using streptokinase or chloroform. The elucidation of this problem is made difficult by the fact that any test ultimately depends on the proteolytic activity of plasmin, and thus it is hard to distinguish between activator and plasminogen on the one hand and plasmin on the other.

In the light of the results using the euglobulin fraction, it was thought that the inhibitory effect of calcium in clot lysis could not be due merely to the different type of fibrin formed in the presence of calcium as suggested by Bickford and Sokolow (1961). They pointed out that the sulphydryl-bonded fibrin formed in the presence of calcium is insoluble in urea, and put forward the hypothesis that this sulphydryl-bonded fibrin might also be more resistant to the fibrinolytic enzymes. This difference in solubility of fibrin in urea is dependent on both calcium and the fibrin stabilizing factor (Laki and Lóránd, 1948) and it may be that this factor plays some part in making the clot more resistant to the fibrinolytic enzymes. The failure of calcium to

inhibit lysis of fibrin clots formed from the euglobulin fraction of plasma points to a more complex mechanism of inhibition.

The hypothesis put forward in this paper is that calcium inhibits fibrinolysis by acting through the inhibitors present in whole blood or plasma.

According to Flute (1960), anti-activator is formed when blood is incubated in contact with glass, and is precipitated with the euglobulin fraction. The inhibition it caused was not enhanced by calcium. However, as reported by Flute (1962), it was found that the formation *in vitro* of anti-activator requires calcium. This would explain the importance (Fearnley, Balmforth, and Fearnley, 1957) of the rapid dilution of whole blood at 4°C. in phosphate buffer in the 1 in 10 clot lysis time, so that calcium is removed before anti-activator can be formed.

The antiplasmin activity of plasma was found to be enhanced by calcium and this would contribute to the inhibition by calcium of fibrinolysis in whole blood or plasma. It is not known how calcium influences these inhibitors, or whether its effect is fully explained by this mechanism.

The finding that calcium prevents the spontaneous lysis of unheated fibrin plates prepared from some batches of Armour bovine plasma fraction I is difficult to explain in the light of these results. Fraction I is contaminated by plasminogen and traces of activator; antiplasmins are also present but it is doubtful whether they are sufficient in quantity to account fully for the inhibition of lysis when calcium is added. In such instances it is possible that the effect of calcium on the type of fibrin formed is of predominating importance, due to the lower concentration of the enzymes and their inhibitors.

It is apparent that the mechanism of inhibition of fibrinolysis by calcium *in vitro* is complex. It is partly explained by the effect of calcium on the formation of anti-activator and on antiplasmin activity, and is possibly influenced to some extent by the type of fibrin formed when calcium is present. In whole blood, these inhibitory influences must modify the accelerating effect of calcium observed when inhibitor-free euglobulin fraction is used.

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