- Hastings, A. B. & Buchanan, J. M. (1942). *Proc. nat. Acad. Sci., Wash.*, **28**, 478.
- Hele, M. P. (1950). *Nature, Lond.*, **166**, 786.
- Hemingway, A. (1939). *J. Physiol.* **95**, 3 P.
- Hers, H. G. & Duve, C. de (1950). *Bull. Soc. Chim. biol., Paris*, **32**, 20.
- Krebs, H. A. (1934). *Tabul. biol. Berl.* **9**, 209.
- Leut'skii, K. H. (1946). *Ukr. Biochem. J.* **18**, 87. Cited by *Chem. Abstr.* (1947), **41**, 6947.
- Meyerhof, O. & Geliazkova, N. (1947). *Arch. Biochem.* **12**, 405.
- Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
- Potter, V. R. & Elvejhem, C. A. (1936). *J. biol. Chem.* **114**, 495.
- Reiner, J. M. (1947). *Arch. Biochem.* **12**, 327.
- Sacks, J. (1951). *Arch. Biochem.* **30**, 423.
- Saxton, J. A. & Miller, M. L. (1944). *Arch. Path.* **37**, 34.
- Slein, M. W., Cori, G. T. & Cori, C. F. (1950). *J. biol. Chem.* **186**, 763.
- Somogyi, M. (1945). *J. biol. Chem.* **160**, 69.
- Stadie, W. C. & Haugaard, N. (1949). *J. biol. Chem.* **177**, 311.
- Stadie, W. C., Haugaard, N. & Hills, A. G. (1950). *J. biol. Chem.* **184**, 617.
- Swanson, M. A. (1950). *J. biol. Chem.* **184**, 647.
- Taylor, D. W. (1951). Private communication.
- Treadwell, C. R., Tidwell, H. C. & Grafa, B. G. jun. (1943). *J. biol. Chem.* **149**, 209.
- Utter, M. F. (1950). *J. biol. Chem.* **185**, 499.
- Vestling, C. S., Mylroie, A. K., Irish, U. & Grant, N. H. (1950). *J. biol. Chem.* **185**, 789.
- Wibelhaus, V. D. & Lardy, H. A. (1949). *Arch. Biochem.* **21**, 321.
- Youngburg, G. E. (1944). *Arch. Biochem.* **4**, 137.

Blood Clotting: the Function of Electrolytes and of Calcium

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(Received 10 May 1951)

The theory of blood clotting advanced by Morawitz (1904) and Fuld & Spiro (1904) postulated that the presence of free calcium ions was a necessary condition of coagulation. This was based on the evidence of Arthus & Pagés (1890) and Pekelharing (1892) that certain anions, namely, oxalate and citrate, whose calcium salts have low solubility products, inhibit the clotting of blood. Vines (1921), and Stewart & Percival (1928) have commented on the unsatisfactory nature of this evidence, and the recent work of Quick & Stephanini (1948) has cast further doubt on these accepted beliefs. They have suggested that calcium functions in blood clotting, not as a free ion, but in combination with prothrombin as an unionized complex. Also that the activity of citrate is not connected with the low solubility of its calcium salt. If these suggestions are accepted, it is of some interest to consider the alternative theories which can be put forward to explain the anticoagulant activity of oxalate and citrate anions.

(1) Oxalate and citrate ions form complexes with low solubility products with other elements, in particular, iron, cobalt and zinc. If it is assumed that some enzyme incorporating one of these elements is necessary for coagulation, then the anions might lower its activity by removal of an essential element from the enzyme.

(2) The process of coagulation involves the interaction of electrically charged colloidal particles. Ions, particularly multivalent ions, are known to

exert an influence on such processes and the activity of oxalate and citrate ions may be due to this effect.

The second theory, namely that ions such as oxalate and citrate might act as anticoagulants by a non-specific salt effect was first proposed by Schmidt (1895). Interest in this possibility was, however, largely dropped when Morawitz (1904) suggested that ionized calcium acted catalytically in the coagulation of blood, and when Sabbatani (1908) explained the action of citrate ions in terms of the low solubility product of its calcium salt.

The only systematic studies of the action of neutral salts on coagulation are those of Glazko & Greenburg (1939), Astrup (1944) and Mommaerts (1945). These authors investigated the anticoagulant activity of anions, including oxalate and citrate ions, on the clotting of purified fibrinogen by thrombin. They showed that in this second phase of blood coagulation, the anticoagulant activity of the anions tested was connected with their valency.

The results reported in this paper were obtained by observing the effect of various anions on the coagulation of whole blood, and by determining the concentration and relative proportions of electrolytes needed to restore the coagulability of salt-free plasma. Many physiological processes are known to be highly sensitive to changes in the concentration and species of ions present in the suspending medium. The results indicate the process of coagulation to be similarly sensitive to disturbances in the electrolyte balance of the blood. In particular they

suggest that coagulation can only occur when the total ionic strength of the blood is within prescribed limits, and only when a certain proportion of the cations present are of a suitable species. They indicate that the anticoagulant activity of oxalate, citrate and fluoride ions is due not only to their ability to depress the calcium ion concentration of blood, but also to their ionic charge; with citrate ions the latter effect predominates, with fluoride ions the former. They also suggest there is no need to ascribe any special function, such as catalytic activity, to the element calcium in the coagulation process, or the formation of defined compounds with the elements of the blood, but instead suggest that calcium functions by maintaining an electrolyte balance suitable for the interaction of the various plasma colloids.

METHODS

Determination of the concentration of anions needed to prevent the coagulation of whole blood. Human blood was collected by venipuncture using a cold dry syringe and transferred immediately to a polyethylene beaker kept at 0°. Portions (0.2 ml.) were transferred by Pasteur pipettes to small glass tubes each containing 0.2 ml. of a solution of the Na or K salt of the test anion. These tubes were kept at 20° and examined after 12 hr. for clotting. The syringe and pipettes were treated before use with dimethyldichlorosilane to render their surfaces inert towards the blood (Jacques, Fidler, Feldstedt & Macdonald, 1946).

The preparation of calcium-free plasma. The decalcification of blood by cation-exchange resins was first described by Steinberg (1944) and developed into a laboratory method by Stephanini (1948). The method described below is similar

to that reported by Stephanini, and we can confirm that the use of cation-exchange resins provides a convenient and effective method of decalcifying blood without otherwise altering its properties.

Human blood was collected by the procedure described earlier, and transferred to a polyethylene flask containing the sodium form of the cation-exchange resin Zeo-Karb 225. For each 10 ml. of blood added 4 g. were used. The mixture of blood and resin was kept at 0° and gently stirred for a period of 15 min. The blood was then separated from the resin by filtration and the plasma from the blood by centrifugation. All glassware used in these operations was treated before use with dimethyldichlorosilane. The plasma produced by this method contained less than 0.2 µg. Ca/ml. and was stored until required in polyethylene flasks at 0°.

The preparation of salt-free plasma. Plasma decalcified by the method just described was dialysed for 24 hr. at 20° against running distilled water to remove salts. It was found necessary to take considerable precautions to prevent the access of Ca to the plasma during dialysis. When the ionic strength of plasma is 0.03 as little as 3 µg. Ca/ml. is sufficient to cause coagulation. The dialysis apparatus was therefore constructed of polyethylene and the cellophan membrane washed thoroughly in N-HCl before use to remove traces of Ca. In earlier experiments in which glass funnels were used to support the dialysis membrane clotting frequently occurred during dialysis. Experiments described later in this paper suggest this was probably due to the liberation of Ca from the glassware.

RESULTS

The inhibition of coagulation by anions. Table 1 shows the concentrations of various anions needed to prevent coagulation. Also shown are the solubility products of the calcium salts of the anions

Table 1. *The anticoagulant activity of anions with reference to their valency and their ability to remove calcium ions from solution*

(Measurements were made of the concentration of salt required to prevent the coagulation of human blood in Pyrex glass tubes at 20°.)

Anion	Valency	Concn. required to prevent coagulation (mm)	Ca salt	
			Solubility product	Calcium ion concn. of saturated aqueous solution (mm)
Azide	1	300	Soluble	—
Chloride		300	Soluble	—
Fluoride		80	3.4×10^{-11}	0.2
Glycollate		300	Soluble	—
Iodate		150	2.2×10^{-7}	6.0
Iodoacetate		300	Soluble	—
Mandelate		300	Soluble	—
Malate	2	100	2.5×10^{-3}	50
Malonate		100	6.25×10^{-4}	25
Oxalate		5	1.8×10^{-9}	0.06
Succinate		100	6.4×10^{-3}	80
Sulphate		100	6.1×10^{-5}	8
Thiosulphate		100	Soluble	1
Tartrate		30	7.7×10^{-7}	0.9
Citrate	3	5	1.7×10^{-8}	0.4
Ferricyanide		30	Soluble	—
Methanetrisulphonate		30	Soluble	—
Naphthalenetrisulphonate		20	Soluble	—

tested and the calcium ion concentration of their saturated aqueous solutions. The values of their solubility products are those given in the International Critical Tables (1929) except the value for calcium citrate which was calculated from the data of Schubert & Lindenbaum (1950).

The results show a close connexion between anti-coagulant activity and valency for the anions tested. The concentrations of uni-, bi-, and tri-valent ions needed to prevent clotting fell in the order 10 : 3 : 1 respectively. There appears to be no direct connexion between anticoagulant activity and ability to remove calcium ions from solution; the concentration of fluoride ions needed to prevent clotting is sixteen times that of citrate ions, although the former are more active in removing calcium from solution. A close examination of the table reveals, however, that all of the anions tested having calcium salts with solubility products less than 10^{-7} were more active in preventing coagulation than were other ions of the same valency.

The concentration of electrolytes and calcium required to cause coagulation of salt-free blood. The results shown in Fig. 1 were obtained by successively adding salts and calcium ions to plasma decalcified and dialysed as described previously. The figure shows that coagulation will not occur if the ionic strength of blood is less than 0.01. As the salt concentration is raised the quantity of calcium required for coagulation falls to a minimum value at an ionic strength of 0.03. When the salt concentration is increased beyond this point there is an approximately proportional increase in the quantity of calcium needed for coagulation until an ionic strength of 0.3 is reached. Beyond this there is an upper limit of ionic strength, varying with salt added, at which coagulation will no longer occur.

For the most part the data from which Fig. 1 was constructed were obtained by adding sodium and calcium chlorides to salt-free plasma. It was found, however, as the diagram shows, that for ionic strengths below 0.10 closely similar results could be obtained by adding sodium sulphate, or potassium

ferricyanide in place of sodium chloride. The difference in behaviour of these salts at ionic strengths above 0.10 is to be expected, since their solutions can then no longer be regarded as dilute.

Fig. 1. The calcium concentration required for the coagulation of human plasma of varying ionic strength. Decalcified salt-free human plasma was treated with CaCl_2 solution, and a solution of a neutral salt NaCl , Na_2SO_4 or K_3FeCN_6 . Tests were made in Pyrex glass tubes at 20° and the calcium concentration needed for coagulation within 12 hr. observed.

The effect of dilution upon the concentration of calcium required for coagulation. The results so far given suggest that the presence of free calcium ions exerts a considerable influence on the coagulation process. They do not, however, provide direct evidence concerning the condition of the element in coagulating plasma, in particular to what extent the element functions in the adsorbed, combined and ionized states. In view of the importance attached by Quick & Stephanini (1948) to the non-ionized calcium in coagulating plasma, the following experiment was made to determine if calcium did function solely in the non-ionized state as Quick & Stephanini suggest.

Table 2. *The concentration of calcium ions required for the coagulation of plasma diluted with varying volumes of salt solution*

(Decalcified, salt-free, human plasma was diluted with 0.15 and 0.03M-NaCl solution containing Ca ions at the concentrations listed in the table. The coagulation time at 20° in Pyrex tubes was observed.)

Dilution of plasma	Ionic strength of plasma	Coagulation time (hr.) at calcium concentrations (mm)				
		2.0	1.0	0.5	0.2	0.1
1:2	0.15	0.5	0.5	4.0	>24	>24
1:5	0.15	0.5	0.5	4.0	>24	>24
1:10	0.15	0.5	0.5	4.0	>24	>24
1:20	0.15	0.5	4.0	4.0	>24	>24
1:2	0.03	0.5	0.5	1	4	12
1:5	0.03	0.5	0.5	1	4	12
1:10	0.03	0.5	0.5	1	4	12
1:20	0.03	0.5	0.5	4	4	12

Table 3. *The coagulation of decalcified plasma by various substances*

(Measurements were made of the quantity of test material required to cause the coagulation of decalcified human plasma within 12 hr. at 20° in Pyrex glass tubes.)

Material	Previous treatment	Quantity needed for coagulation in 12 hr. or less (mg./ml.)	Calcium removed by boiling with N-HNO ₃ (μg./g.)
Powdered soft glass	Washed with distilled water	20	230.0
Powdered Pyrex glass	Washed with distilled water	200	10.0
Powdered silica glass	Washed with distilled water	200	8.0
Powdered soft glass	Washed with N-HNO ₃	No coagulation with 1 g./ml.	—
Powdered Pyrex glass	Washed with N-HNO ₃	No coagulation with 1 g./ml.	—
Powdered silica glass	Washed with N-HNO ₃	No coagulation with 1 g./ml.	—
Calcium oxalate	Washed with distilled water	0.1	—

Plasma, free of calcium and salts, was prepared as described earlier in this paper. A series of dilutions of this plasma was made in sodium chloride solution containing various amounts of calcium chloride. The coagulation time of the diluted plasma was observed and is recorded in Table 2. The table clearly shows that the coagulation time is dependent upon the concentration of calcium, and not upon the quantity present. This is the reverse of what would be expected if the calcium necessary for coagulation were present solely in the non-ionized condition.

The coagulation of decalcified blood by powdered glass, calcium oxalate and other substances. In experiments described previously (Lovelock & Burch, 1951), it was found that decalcified plasma would often coagulate if kept in soda-glass vessels, or when in contact with powdered glass or silica. Similar observations were reported by Overman (1949), who found that diluted oxalated blood would clot when in contact with powdered glass and other similar materials. At the time it seemed that these observations indicated that coagulation could occur in the absence of calcium. The possibility that sufficient calcium was liberated from the glass, etc., to cause coagulation was not seriously considered, since it was then believed that the minimum concentration of calcium at which coagulation could normally occur was 0.4 mM. It was suggested that the function of calcium and of powdered glass in the coagulation of blood was respectively to combine with, or inactivate, some inhibitor of coagulation. Reference to Fig. 1, however, shows that in blood diluted to an ionic strength of 0.03 the concentration of calcium required for coagulation is only 0.04 mM (1.6 μg./ml.). There seemed a distinct possibility that a quantity of calcium as small as this could be liberated from glassware or powdered glass.

Table 3 shows the quantities of various materials needed to cause the coagulation of decalcified plasma, and the quantity of calcium which could be removed from the same quantity of material by boiling with N-nitric acid for 2 hr. It is evident from the results that sufficient calcium is available from

powdered glass and probably also from powdered silica to cause coagulation. That glass and silica washed with acid will not cause coagulation, further confirms that these materials cause the coagulation of decalcified blood by liberating calcium adsorbed upon their surfaces.

Fig. 2. The concentrations of barium, magnesium and calcium ions required for the coagulation of human plasma of varying ionic strength. Decalcified salt-free human plasma was treated with NaCl solution, and with a solution of CaCl₂, BaCl₂ or MgCl₂. Tests were made in Pyrex glass tubes at 20° and the divalent cation concentration needed for coagulation within 12 hr. observed.

The results shown in Table 3 also include the observation that coagulation can occur when calcium oxalate is added to decalcified blood. This experiment has been repeated successfully on several occasions and with blood from different persons. The calcium oxalate used was precipitated from a solution containing an excess of oxalate ions and washed with dilute potassium oxalate solution before use. It was unlikely, therefore, to have calcium ions adsorbed upon its surface. This result illustrates clearly the fallacy of assuming, as is frequently done, that the precipitated calcium oxalate in oxalated blood is inert.

The coagulation of decalcified plasma by cations other than calcium. Fig. 3 shows the relationship

between the electrolyte content of plasma and the concentration of calcium, magnesium and barium, required for coagulation. The concentration of each of these cations needed for coagulation varied with the ionic strength of the plasma in a closely similar manner. The most favourable ionic strength for coagulation was the same for all three elements, namely 0.03 and above this the concentration of each cation needed for coagulation increased proportionately with increasing ionic strength. The concentrations of calcium, magnesium and barium needed for coagulation rose in the order 1 : 8 : 60 respectively.

The values and sequence of activity of calcium, magnesium and barium ions in the coagulation of plasma are similar to those required to neutralize the charge of certain phosphate colloids. For example, the charge on sphingomyelin is neutralized by calcium, magnesium and barium ions at concentrations of 0.9, 5.0 and 20 mM respectively (Bungenberg de Jong, Teunissen-Van Zijp & Teunissen, 1940). Bungenberg de Jong (1949) has further shown that the activity of bivalent cations in neutralizing the charge on phosphatide colloids is reduced when the ionic strength of the suspending medium is increased.

DISCUSSION

The experimental observations indicate that the coagulation process requires the concentration and proportion of simple electrolytes in plasma to be within a prescribed range. In particular, the process is sensitive to changes in the proportion of the total ionic strength contributed by calcium ions. Coagulation occurs most readily when the proportion is approximately 1 : 16 for calcium and for other ions respectively. The experimental establishment of the idea that a certain electrolyte balance is necessary for normal coagulation has proved of use in resolving some of the discrepancies concerning the function of calcium in coagulation and of oxalate, citrate and fluoride ions in preventing it. For example, the discrepancy between the concentrations of oxalate, citrate and fluoride ions needed for coagulation and their ability to decalcify plasma can be resolved without recourse to complex theories concerning the function of calcium in coagulation. The addition of these ions to plasma disturbs the electrolyte balance in a twofold manner; first by lowering the calcium ion concentration, and secondly by raising the total ionic strength. That citrate ions are more effective than fluoride ions in preventing coagulation then follows as a natural consequence of their higher valency. With oxalate ions their superior decalcifying activity compensates for their lower valency to give them approximately the same anticoagulant activity as citrate ions. That the decalcifying activity of these ions

alone is insufficient to prevent coagulation is further illustrated by the observation that decalcified blood can be coagulated by the addition of calcium oxalate, citrate or fluoride. It should be noted that the solubilities of the calcium salts listed in Table 1 refer to their solutions in water only. They are of use in illustrating qualitatively the ability of the anions tested to decalcify blood, but are almost certainly different quantitatively from the solubilities of the same salts in blood, especially in the presence of an anticoagulant concentration of the test anion. Norbdo (1936) has shown, for example, that the calcium ion content of a saturated solution of calcium oxalate in serum ultrafiltrate is 0.18 mM. The calcium ion content of a saturated solution of the same salt in water is 0.06 mM. When precise values of the solubilities of calcium salts in whole blood become available it should be possible, using also the data in Fig. 1, to calculate the concentration of a given anion needed to inhibit the coagulation of blood. The comparison of the calculated and the observed anticoagulant activity of salts such as those listed in Table 1 would provide a critical means of checking the validity of the theories outlined above.

It is frequently stated that the coagulation of citrated or oxalated blood following the addition of calcium is further proof of the necessity of that element for coagulation. In spite of the obvious illogicality of this statement, in that the addition of calcium removes the oxalate and citrate ions, it is of some interest to compare it with the observation illustrated in Fig. 1. Here the addition of calcium ions to plasma, prevented from coagulating by the addition of sodium chloride, also causes coagulation. In both of these examples it is the restoration of the electrolyte balance which restores the coagulability of the plasma.

The experiments show that even with the smallest amount of calcium at which coagulation will occur, namely 0.08 mM, it is the concentration and not the quantity of calcium in plasma which is important. This is not in accord with those theories which postulate that some non-ionized calcium compound is necessary for coagulation. Neither is the evidence that the concentration of calcium required for coagulation varies with the ionic strength of the plasma, nor the observation that the concentrations of other bivalent elements needed for coagulation are different from those of calcium. An acid radical giving a firm non-ionized compound with 1 part of calcium, 8 of magnesium or 60 of barium, seems very improbable. In general terms the experimental evidence does not support the idea that a defined compound of calcium and one of the macromolecules of plasma, presumably analogous to iron in haemoglobin, is necessary for coagulation.

Calcium is distributed in plasma in the form of free ions, ions adsorbed upon the many colloid species present and probably also in non-ionized combination with the simpler anions of plasma. The equilibria controlling the proportion of the element distributed in these various forms are likely to be complex and easily disturbed by changes of the electrolyte and calcium ion content of plasma. In these circumstances the discovery of the precise function, or functions, of calcium in the normal coagulation process is likely to be difficult. Nevertheless, from a knowledge of the nature of the coagulation process, namely the serial interaction of a number of electrically charged macromolecules, the following conclusions can be drawn: calcium ions as such are unlikely to enter directly into the coagulation reaction, but their presence is necessary in order to maintain the requisite amount of calcium adsorbed upon the various plasma colloids. According to the evidence presented earlier, calcium in firm non-ionized combination with either the molecules, or macromolecules of plasma does not appear to be necessary for coagulation.

It follows therefore that the most probable condition of the element calcium necessary for coagulation is the adsorbed state. It seems possible that the function of the adsorbed calcium is to maintain the surface charge of one or more of the plasma colloids at a value suitable for its proper interaction with the other plasma colloids. There is as yet no

direct evidence to suggest which of the plasma colloids require adsorbed calcium for their proper function, although the sequence and degree of activity of calcium, magnesium and barium ions suggests that a phosphate colloid may be involved. Overman (1949) has suggested from other evidence that calcium may function by neutralizing a phosphatide inhibitor of coagulation.

SUMMARY

1. Experiments are described which indicate that the activity of oxalate, citrate and fluoride ions in preventing coagulation is complex, and is due not only to their ability to depress the calcium ion concentration of blood, but also to their ionic charge.

2. The concentrations of calcium, magnesium and barium ions needed for the coagulation of plasma containing various concentrations of salts has been measured. These measurements indicate that human plasma will not coagulate if the ionic strength is less than 0.01 or more than 0.5. The concentration of alkaline earth elements required for coagulation was found to vary with ionic strength, calcium concentration as low as 0.08 mM causing coagulation in plasma of ionic strength 0.03.

3. The function of calcium in coagulation is discussed. It is suggested that it is concerned with the maintenance of an electrolyte balance suitable for the interaction of the plasma colloids.

REFERENCES

- Arthus, M. & Pagés, C. (1890). *Arch. Physiol. norm. path.* **2**, 739.
- Astrup, T. (1944). *Acta physiol. Scand.* **7**, 11.
- Bungenberg de Jong, H. G. (1949). *Colloid Science*. London: Elsevier.
- Bungenberg de Jong, H. G., Teunissen-Van Zijp, L. & Teunissen, P. H. (1940). *Kolloidzshr.* **91**, 311.
- Fuld, E. & Spiro, K. (1904). *Beitr. chem. Physiol. Path.* **5**, 171.
- Glazko, A. J. & Greenburg, D. M. (1939). *Amer. J. Physiol.* **125**, 108.
- International Critical Tables* (1929). **4**, 229–31.
- Jacques, L. B., Fidler, E., Feldstedt, E. T. & Macdonald, A. G. (1946). *Can. med. Ass. J.* **55**, 26.
- Lovelock, J. E. & Burch, B. M. (1951). *Biochem. J.* **48**, xxxiv.
- Mommaerts, W. F. H. M. (1945). *J. gen. Physiol.* **29**, 103.
- Morawitz, P. (1904). *Beitr. chem. Physiol. Path.* **5**, 133.
- Norbo, R. (1936). *Skand. Arch. Physiol.* **75**, Suppl. 11.
- Overman, R. S. (1949). *Blood Clotting and Allied Problems*. New York: J. Macey.
- Pekelharing, C. A. (1892). *Dtsch. med. Wschr.* **18**, 1133.
- Quick, A. J. & Stephanini, M. (1948). *J. gen. Physiol.* **32**, 191.
- Sabbatani, L. (1908). *Mem. R. Accad. Torino*, **51**, 257.
- Schmidt, A. (1895). *Weitere Beitrage zur Blutlehre*. Wiesbaden: Bergmann.
- Schubert, J. & Lindenbaum, A. (1950). *Nature, Lond.*, **166**, 913.
- Steinberg, A. (1944). *Proc. Soc. exp. Biol., N.Y.*, **56**, 124.
- Stephanini, M. (1948). *Proc. Soc. exp. Biol., N.Y.*, **67**, 22.
- Stewart, C. P. & Percival, G. (1928). *Biochem. J.* **22**, 559.
- Vines, H. W. C. (1921). *J. Physiol.* **55**, 86.