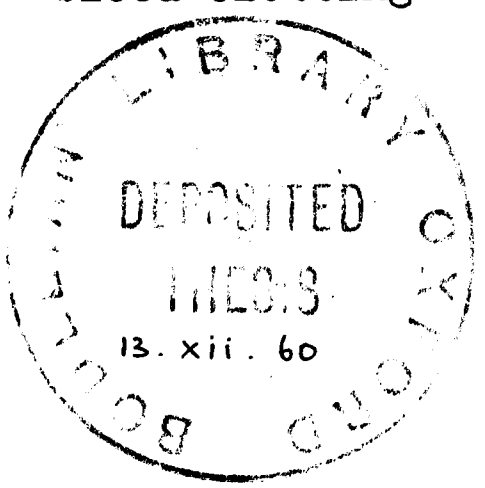
Biochemical changes associated with blood clotting.



A thesis submitted for the Degree of

Doctor of Philosophy

M.P. ESNOUF

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ABSTRACT

It has been recognised for many years that phospholipids are essential for the coagulation of plasma, and the evidence which has been obtained so far suggests that they are involved in the formation in the plasma of a substance which will convert prothrombin. The activity of the various phospholipids has been assessed by their ability either to shorten the clotting of recalcified plasma, or to stimulate the conversion of prothrombin. Using this technique the most active phospholipid has been found to be phosphatidyl ethanolamine, but apart from an investigation of the physical and chemical properties of this phosphatide, no information has been gained as to its mode of action.

Since plasma already contains phospholipids it was thought worthwhile to investigate whether any reaction took place during the coagulation of plasma, in which they were involved. For this investigation the plasma was clotted either in the presence of platelets, or Russell's viper venom. It was found that when the plasma clotted it was accompanied by the release of a small amount of glycerylphosphorylcholine. This substance was formed however the plasma was made to clot. The results

which have been obtained suggest that this compound was formed by the degradation of between 2 - 3% of the plasma lecithin. Glycerylphosphorylcholine is formed from lecithin by the action of two enzymes, namely, a lecithinase and a lysolecithinase. It has been shown that while Russell's viper venom has lecithinase activity, it is unable to hydrolyse lysolecithin. It was found that plasma had a latent lysolecithinase activity, which could be activated by exposing the plasma to a roughened glass surface. In addition the activated lysolecithinase enzyme would in the presence of a tripalmitin emulsion hydrolyse lecithin to give glycerylphosphorylcholine. It is suggested that the probable role of phosphatidyl ethonolamine is to activate the plasma lecithin so that it can be hydrolysed by the plasma enzyme. The results which have been obtained suggest that the degradation of the lecithin was associated with the appearance in the plasma of a substance which will convert prothrombin, and the conclusion has been reached that the developement of both lecithinase and lysolecithinase activity is a necessary prerequisite for the coagulation of the plasma.

Born (1958) showed that during the coagulation of platelet-rich plasma there was a loss of adenosine

triphosphate from the platelets. Experiments have been carried out to determine whether this loss of adenosine triphosphate was concerned in the clotting of the plasma. It has been concluded that the release of adenosine triphosphate from the platelets was only incidental to the clotting of the plasma.

GENERAL INTRODUCTION.

Blood coagulation, in particular the early stages, has received much attention from clinicians, pathologists and physiologists; until recently it was not examined by biochemists. Most of the work carried out so far has resulted in the recognition and, in some instances, in the isolation of active components. The mechanism by which the components react remains for the most part obscure, though the conversion of fibrinogen into fibrin, has been studied more thoroughly and is better understood.

The purpose of the work to be described in this thesis was to investigate those chemical reactions in clotting which may be related to the formation in the plasma of the substance which converts prothrombin to thrombin. This phase of coagulation is at present the subject of many hypotheses as to how the various clotting factors interact. Born (1958) observed that as soon as platelet rich plasma was recalcified there was a rapid loss of adenosine triphosphate from the platelets, and it was suggested that its disappearance might be associated with clotting of the plasma. In the first part of the thesis the fate of the adenosine triphosphate has been investigated

and the significance of its breakdown discussed. The main purpose of the thesis, however, is to describe the work done to follow up a suggestion that the phospholipids in plasma also underwent changes during the early stages of clotting. Much work suggests that phospholipids play an important role during this phase; however, the manner in which they do so is still obscure.

CHAPTER I.

THE MODERN CONCEPT OF BLOOD COAGULATION.

A comprehensive survey of the literature on coagulation would, for the most part, be irrelevant to the main topic of the thesis. Therefore only a brief description of various factors will be given which are thought to be involved in coagulation.

A. A diagramatic representation of the coagulation process is given in Fig. 1.1.; it is an attempt to represent the most probable interactions of the clotting factors. No distinction is made between the prothrombinase activity generated in three different systems, i.e. in platelet-rich plasma, in platelet-free plasma with tissue extract added, and in platelet-free plasma with Russell's viper venom added. This does not mean, however, that the active agent is necessarily the same substance in each of the three systems.

There now follows a summary of the evidence for each of the factors.

Calcium: Since Arthus & Pages (1890) showed that calcium precipitants inhibited coagulation, an effect which was reversed when calcium was added, few have doubted that

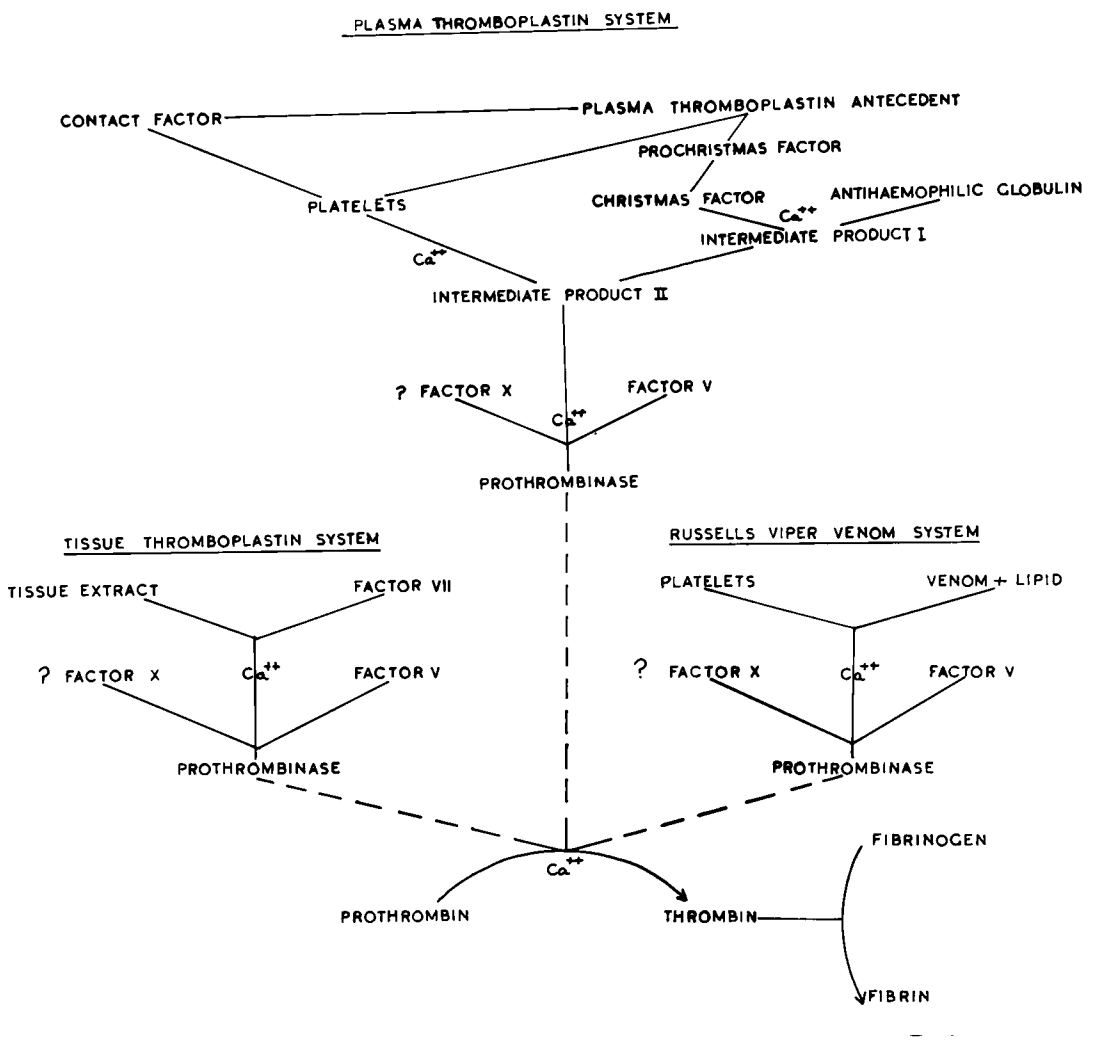


Fig. 1.1.

A diagramatic representation of the interaction of the blood coagulation factors.

calcium is required for coagulation. It has been implicated in all stages of clotting (Fig.1.1) with the exception of the fibrinogen conversion. Lovelock and Porterfield (1952) found that coagulation would only take place within a range of ionic strengths 0.01 - 0.5 of those of the simple electrolytes. Clotting occurred most rapidly when the ratio of calcium ions to other ions was approximately 1 : 16. They suggest that calcium functions by maintaining the surface charge of one or more of the plasma colloids at a value suitable for their interaction with other plasma colloids.

Contact: It has been recognised for many years that blood exposed to a foreign surface coagulates. It was thought that the reaction was initiated by contact with a wettable surface. However, nylon, which is less wettable than glass, is more active in causing clotting, (Rose & Brodia, 1954), and a wettable collodion surface is relatively inert (Hirschboeck, 1940). A second theory suggests that clot inhibiting surfaces are active by virtue of their negative charge. This is supported by the finding of Sawyer & Pate, (1953) and Sawyer (1953) that when the normal negative surface charge of the intima of the dog's aorta is reversed *in vivo* by injury or by electrical means, rapid thrombosis occurs.

It was suggested by Margolis, (1957) that coagulation is initiated as the result of the interaction of the foreign surface and a particular substance in the plasma. Ratnoff & Colopy (1955) have described plasma taken from three patients which did not exhibit this phenomenon (Hageman defect). It is interesting to note that these patients exhibit no haemorrhagic tendencies in vivo.

Plasma Thromboplastin Antecedent.

Rosenthal, Dreskin & Rosenthal (1953, 1955), described a haemorrhagic state which resembled haemophilia, but differed clinically from either haemophilia or christmas disease, and that the prolonged coagulation time which was observed in these patients was only corrected by a hitherto unrecognised factor. Biggs, Sharp, Margolis, Hardisty, Stewart & Davidson (1958) suggested that the product of the reaction between plasma and glass reacts with plasma thromboplastin antecedent, and accelerates the rate of both viscous metamorphosis of platelets and the activation of christmas factor.

Christmas Factor: Biggs, Douglas, Macfarlane, Dacie, Pitney, Merskey & O'Brien (1952) showed that this factor was able to correct christmas disease, a condition formerly confused with haemophilia. Like Antihaemophilic globulin

this factor is associated with the plasma B_2 globulin fraction (Aggeler, Spaet & Emery, 1954). Bergsagel (1955) noticed that while the christmas factor activity of normal and haemophilic plasma was unaffected by decalcification, it was considerably less than the activity found in serum. Further, the depressed christmas factor activity of decalcified serum would return slowly on incubation with calcium alone. This reactivation process was accelerated by the presence of antihaemophilic globulin. Bergsagel (1955) suggested that these two globulins formed a calcium linked complex, and that this complex was formed during the initial stages of coagulation.

Antihaemophilic Globulin: This globulin was recognised for its ability to correct the clotting defect in the blood of human and canine haemophiliacs (Graham, Penick & Brinkhous, 1951). Although this factor is present in normal plasma it is absent in normal serum, which suggests that it is consumed during clotting. The properties of this globulin have been reviewed by Brinkhous & Wagner (1958). It has been suggested (Macfarlane, 1942) that prothrombinase is formed in plasma by the interaction of a thermostable lipoid factor from the platelets and a thermolabile plasma component. Bergsagel & Hougie (1956) proposed that this

plasma component could be identified with the christmas factor-calcium-antihaemophilic globulin complex.

Platelets:

These, like fibrin, are the only visible coagulation factors. Their functions in clotting are complex and have been attributed to a number of separable factors.

Platelet factor 1: This has now been shown to be absorbed plasma factor V (Hjort, Rapaport & Owen, 1955).

Platelet factor 2: Ware, Fahey & Seegers (1948), isolated a heat labile, non dialysable protein from platelets which appeared to accelerate the conversion of fibrinogen to fibrin by thrombin.

Platelet factor 3: This is the platelet lipoid factor mentioned above, and has been shown to be associated with the intact platelet granules (Johnson, Sturrock & Rebuck, 1958). Disintegration of these granules by ultra-sonic vibration resulted in the loss of factor 3 activity.

Platelet factor 4: This factor has an anti-hepanin effect, which was originally ascribed to factor 3, but Deutsch, Johnson & Seegers (1955) have shown this to be a separate substance. Platelets can be seen to undergo morphological changes during coagulation, but the precise relation of this phenomenon to clotting is obscure.

Factor V: Quick (1943) observed that stored oxalated plasma slowly lost a factor which would potentiate prothrombin conversion; this did not happen when citrate was used as an anticoagulant (Fahey, Ware & Seegers, 1948). The factor was isolated from plasma by Owren (1947) who proposed the name. Factor V activity is not found in normal serum.

Factor X: Duckert, Flückiger, Matter & Köller (1955) described a factor that was diminished in patients undergoing dicoumarol therapy. A lack of the factor caused a delay in the formation of prothrombinase but did not reduce the final amount formed. Two similar defects which occur naturally have been described by Telfer, Denson & Wright (1956), and by Hougie, Barrow & Graham (1957); they have been called the Prower and Stuart defects respectively. Initially these defects appeared to be separate, but Denson (1958) has now shown them to be the same. The precise relationship between the Stuart/Prower defect and Köller's factor has not yet been finally established.

Tissue Extracts: This material is referred to by many workers as thromboplastin; it is usually obtained from

brain, lung or placenta. The coagulant activity of these tissues is recovered from the acetone insoluble fraction.

Brain extracts will clot both normal and haemophilic blood, though the ability to clot haemophilic blood is lost on heating to 60° (Quick, Hussey & Stapp , 1952). This would suggest the presence of two components in these extracts. The heat stable component requires anti-haemophilic globulin, and perhaps christmas factor; O'Brien (1956) is of the opinion that this stable component is probably ethanolamine phosphatide. The unstable component presumably has a similar action to antihaemophilic globulin. The nature of the active components found in these preparations will be discussed in greater detail in a later section.

Factor VII: Köller, Loeliger & Duckert (1951) observed that plasma contained a second factor which would accelerate the conversion of prothrombin, and which was diminished in patients receiving dicoumarol treatment. Biggs, Douglas & Macfarlane (1953) have shown that whilst this factor is required in the tissue thromboplastin system, it is not required in the plasma thromboplastin system.

Prothrombinase: This term has here been taken to denote

the 'substance' which is responsible for the conversion of prothrombin to thrombin, and which is formed as the result of any of the three thromboplastin generating systems. (cf. Biggs & Macfarlane, 1957). It is realised that this 'substance' may, in fact, be composed of one or more separable components, and further that the components generated by the three systems may differ. The possible interactions of the various factors which result in the formation of prothrombinase are the subject of an interesting paper by Biggs & Bidwell (1958).

Russell's Viper Venom: Macfarlane & Barnett (1934) showed that when this venom was added to plasma in the presence of calcium an extremely active prothrombinase was produced. The activity could be potentiated by the addition of crude lecithin (Macfarlane, Trevan & Attwood, 1941). Venom added to plasma devoid of platelets and of particulcate fat produced only very little prothrombinase. The prothrombinase activity produced by venom does not depend on the concentration of Factor VII (Biggs & Macfarlane, 1947) but it does require Factor V (Rapaport, Aas & Owren, 1954).

Prothrombin-Thrombin: The classical theory of blood coagulation suggested that thrombin was generated from

an inactive precursor, prothrombin, which was present in the plasma. It was not, however, until comparatively recently that prothrombin was isolated (Seegers, Loomis & Vandenbelt 1945). Analysis of purified preparations of prothrombin have revealed its amino acid content and shown that carbohydrates are also present (Laki, Kominz, Symonds, Lorand & Seegers.1954). It has been known for many years that prothrombin is synthesised in liver, and recently Lasch and Roka (1953) have been able to generate prothrombin <u>in vitro</u> by incubating liver mitochondria in a cell-free system with factor VII. Seegers (1958) has confirmed these observations and has found that the mitochondria could be replaced by cathepsin. When prothrombin is dissolved in 25% citrate solution, thrombin activity gradually appears; this transformation takes place without the addition of any other biological material (Seegers & Alkjaersig.1956). This 'citrate thrombin' is composed of three fragments, two of which have thrombin activity. In the biological conversion of prothrombin to thrombin (biothrombin) three such fragments could not be found. The incubation of biothrombin in 25% citrate gives citrate thrombin. It is worth noting that while the formation of citrate thrombin takes several hours, the

formation of biothrombin requires only a few seconds. Alexander (1958) has been able to obtain a prothrombin preparation of a higher specific activity than preparations made by Seegers. This prothrombin was not converted to thrombin by 20% sodium citrate, and it was suggested that Seeger's preparations were contaminated with Factor VII.

Seegers & Alkjaersig (1956), have reported a third derivative of prothrombin called autoprothrombin, the function of which is obscure. Thrombin formed by biological conversion of prothrombin can be obtained from serum by ammonium sulphate precipitation, (Astrup & Darling 1941), and it has been classified as an albumin.

Thrombin: Fibrinogen: Fibrin: It has been recognised for many years that thrombin reacts with fibrinogen to give fibrin. Bailey, Bettelheim, Lorand & Middlebrook (1951) showed that when thrombin was incubated with fibrinogen there was an appearance of molecules with N-terminal glycine; furthermore, the reaction was apparently specific for fibrinogen, since no reaction was observed when the fibrinogen was replaced by myosin or by ovalbumin. Lorand (1952) showed that a peptide was released

at the same time as the terminal glycine groups appeared. This peptide, called fibrinopeptide, possessed a glutamic acid group on the acidic end. This group which was present in the unchanged fibrinogen, was not found in fibrin, and it was suggested that thrombin acted on fibrinogen as a proteolytic enzyme, splitting off the fibrinopeptide. Sherry & Troll (1954) confirmed this by showing that thrombin could hydrolyse an artificial substrate, namely tosyl arginyl methyl ester. Bailey & Bettelheim (1955), and Bettelheim (1956) found that a second peptide was released from fibrinogen, but at a slower rate than fibrino-peptide. Whilst the appearance of fibrinopeptide was proportional to the appearance of fibrin, Blombäck & Verstermark (1958) have demonstrated that the second peptide only accumulates rapidly after considerable amounts of fibrin have been formed. Probably, therefore, fibrin is an intermediate product of the degradation of fibrinogen <u>in vitro</u>. However, there are distinct differences between the types of fibrin formed <u>in vivo</u> and <u>in vitro</u>. Fibrin formed <u>in vitro</u> is soluble in dilute acids and alkalis (Robbins, 1944) and also in 5 <u>M</u> urea (Lorand 1950). On the other hand, the fibrin clot formed in plasma does not dissolve in these solvents. The stability of the natural product is most probably due to

the presence in the plasma of a factor which stabilises fibrin (Lorand, Chen, Fuchs, Jacobsen & Lorand, 1958).

From this brief summary of blood coagulation it will be seen that the mechanism of the later stages is gradually becoming clear. This, however, is not true for the earlier stages, i.e. those preceeding the conversion of prothrombin. The purpose of the work described in the following chapters was to investigate chemical changes which might be associated with these early stages of the coagulation process.

CHAPTER II.

THE FATE OF ADENOSINE TRIPHOSPHATE DURING BLOOD COAGULATION.

Born (1956) found that platelets contained a remarkably high concentration of adenosine triphosphate. The problem arose as to whether any particular functions could be assigned to this nucleotide, which might be relevant to the physiological functions of the platelets. It was suggested (Born, Ingram & Stacey, 1956) that adenosine triphosphate was concerned in the accumulation and retention of 5-hydroxytryptamine. Born (1958) showed that as soon as calcium was added to platelet-rich plasma, there was rapid loss of adenosine triphosphate from the platelets. The experiments indicated that for each mole of adenosine triphosphate lost there appeared between one and two moles of phosphate which became temporarily attached to a protein residue. This residue was left after thorough extraction of the plasma with trichloracetic acid and fat solvents. These observations suggest that adenosine mono-and diphosphates might accumulate in the plasma.

METHODS:

Plasma: Human blood was obtained by venepuncture and

collected in plastic centrifuge tubes. The blood was mixed with sodium citrate (final concentration 0.38%) to prevent clotting. The blood was centrifuged at 1500 g for 15-20 minutes at 0-2°. The supernatant plasma was centrifuged at 25,000 g for 15 minutes to remove the platelets. The plasma was then dialysed for 20 hours at 0-4° against 3 litres of modified Krebs-Ringer bicarbonate solution. The solution had the following composition expressed as millimoles per litre: NaCl, 119; KCl, 4.8; $CaCl_2$, 2.6; $MgSO_4$, 1.2; $NaHCO_3$, 25; sodium citrate, 1.3. N_2+CO_2 (95:5) was bubbled through the solution, which had a pH of 7.5. The dialysing fluid was changed twice during dialysis.

Platelets: These were obtained from freshly drawn venous blood. The blood was centrifuged at 500 g for 20 minutes at 0-2°. The platelet-rich plasma was then centrifuged at 25,000 g for 15 minutes at 0-2° to obtain a pellet of platelets. The platelets were resuspended in the dialysed plasma.

Adenosine triphosphate: This was determined by the firefly luminescence reaction (Strehler & Totter, 1954). Internal standards were used because the high concentration of chloride in the plasma inhibits the production of luminescence.

Determination of Phosphate: Acid-soluble phosphate was extracted from plasma by the addition of 1/5 vol. 60% trichloracetic acid which was ice-cold. The mixture was dispersed in a homogenizer and centrifuged at 25,000g for 10 mins. at 0-2°. The residue was re-extracted with cold 10% trichloracetic acid and centrifuged again. The centrifugation was carried out in plastic tubes in order to reduce the risk of breakage. High speeds of centrifugation were employed because they assisted in the breaking up of the precipitated fibrin. The clear supernatants were filtered to remove any traces of protein precipitate. Samples taken for the estimation of the adenosine triphosphate were extracted four times with equal volumes of ether to remove the trichloracetic acid. The ether was removed by bubbling nitrogen through the solutions, which were kept cold. Organic phosphate was determined in a sample which had been digested with 0.5 ml. of a mixture containing 2 parts of conc. H_2SO_4 to 3 parts of 70% $HClO_4$, and 0.1 ml. 5% ammonium molybdate (Werkheiser & Bartley (1957). Inorganic phosphate was determined by the method of Ennor & Stocken (1950). 10 min. phosphate was defined as the phosphate released from organic compounds by hydrolysis in N HCl at 100° for 10 min.

Prothrombinase activity: This was assayed by a method adapted from Hicks & Pitney (1957). Samples of experimental plasma (0.1 ml.) were added to 0.9 ml. 0.15 M NaCl containing 0.1 M imidazole buffer pH 7.0, and rapidly mixed. A sample (0.1 ml.) was immediately added to 0.1 ml. platelet-free plasma at the same moment as 0.1 ml. 0.025 M $CaCl_2$. All reagents were maintained at 37°. The clotting time of the platelet-free plasma was taken as a measure of prothrombinase activity in the experimental plasma.

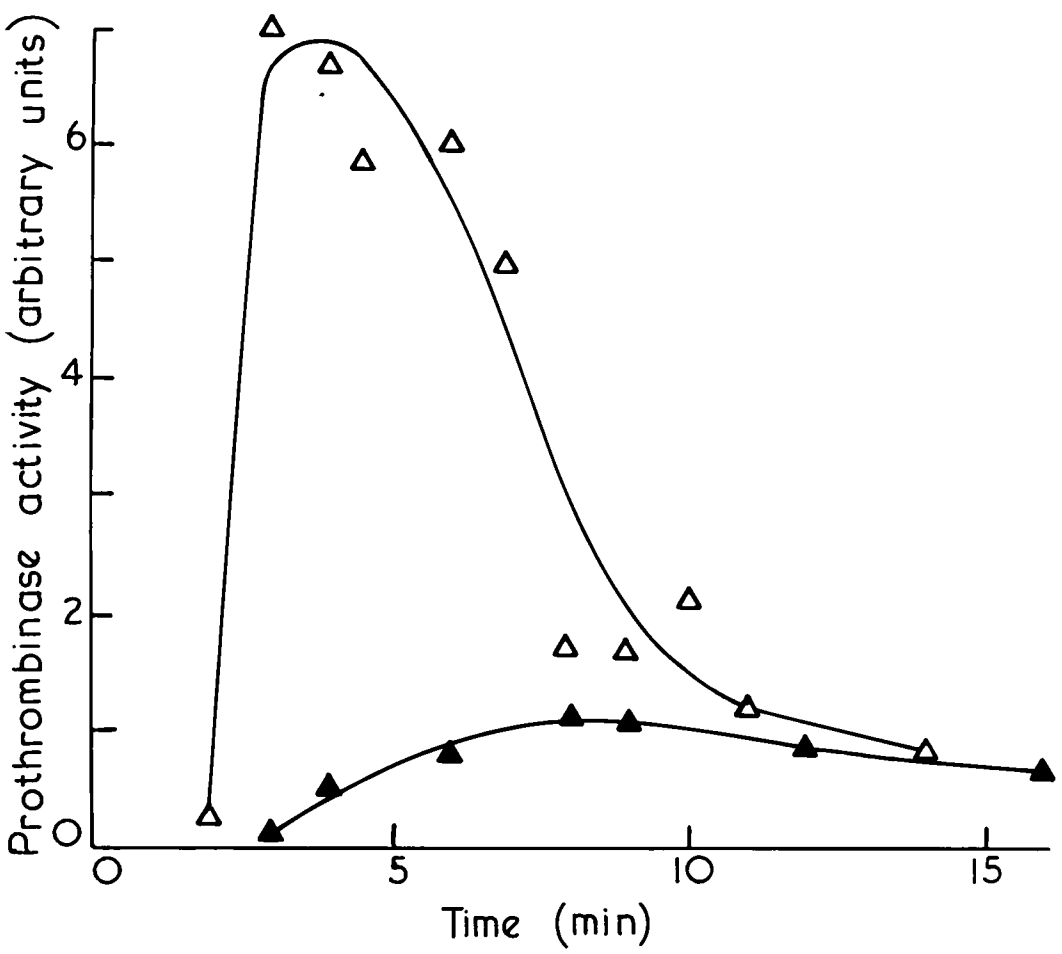


Fig. 2.1.

The appearance of prothrombinase activity in plasma using the technique of Born (1958) ▲—▲ , and the procedure described in the text △—△ .

EXPERIMENTAL AND RESULTS

In early experiments dialysed plasma to which fresh platelets had been added was incubated at 37° in plastic tubes. Pieces of glass tubing were placed in the plasma to provide glass contact and after the plasma had reached 37° calcium was added (0.025 ml. M $CaCl_2$/ml. plasma). In different plasmas fibrin clots formed over a wide range of times, usually after 4-15 minutes, but in some instances not until 20 minutes after the addition of calcium. This was far from satisfactory because no experiment could ever be repeated exactly. Moreover, the level of prothrombinase activity was never high (Fig. 2.1.).

In view of this, experiments were carried out to establish the conditions for obtaining reproducible results. It was found that if the plasma was first incubated with the platelets in roughened glass tubes for 10 min. at 37°, before it was transferred to the plastic tubes, the clot formed in the plasma much more quickly. The average clotting time of seven different plasmas was 2 min. 16 sec. (with a range from 1 min. 45 sec. - 2 min. 45 sec.). Furthermore, it will be seen in (Fig. 2.1.) that a much greater prothrombinase activity was developed (using this

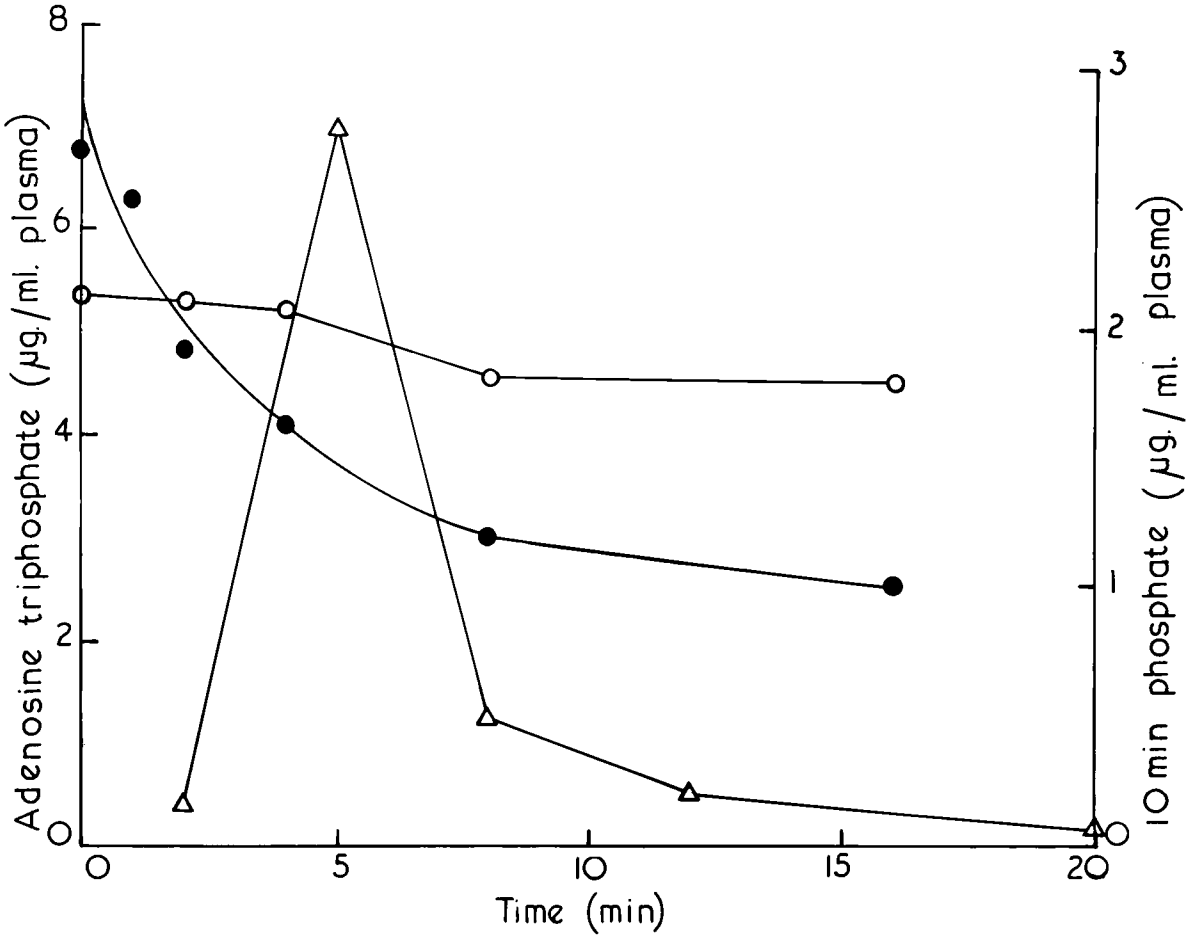


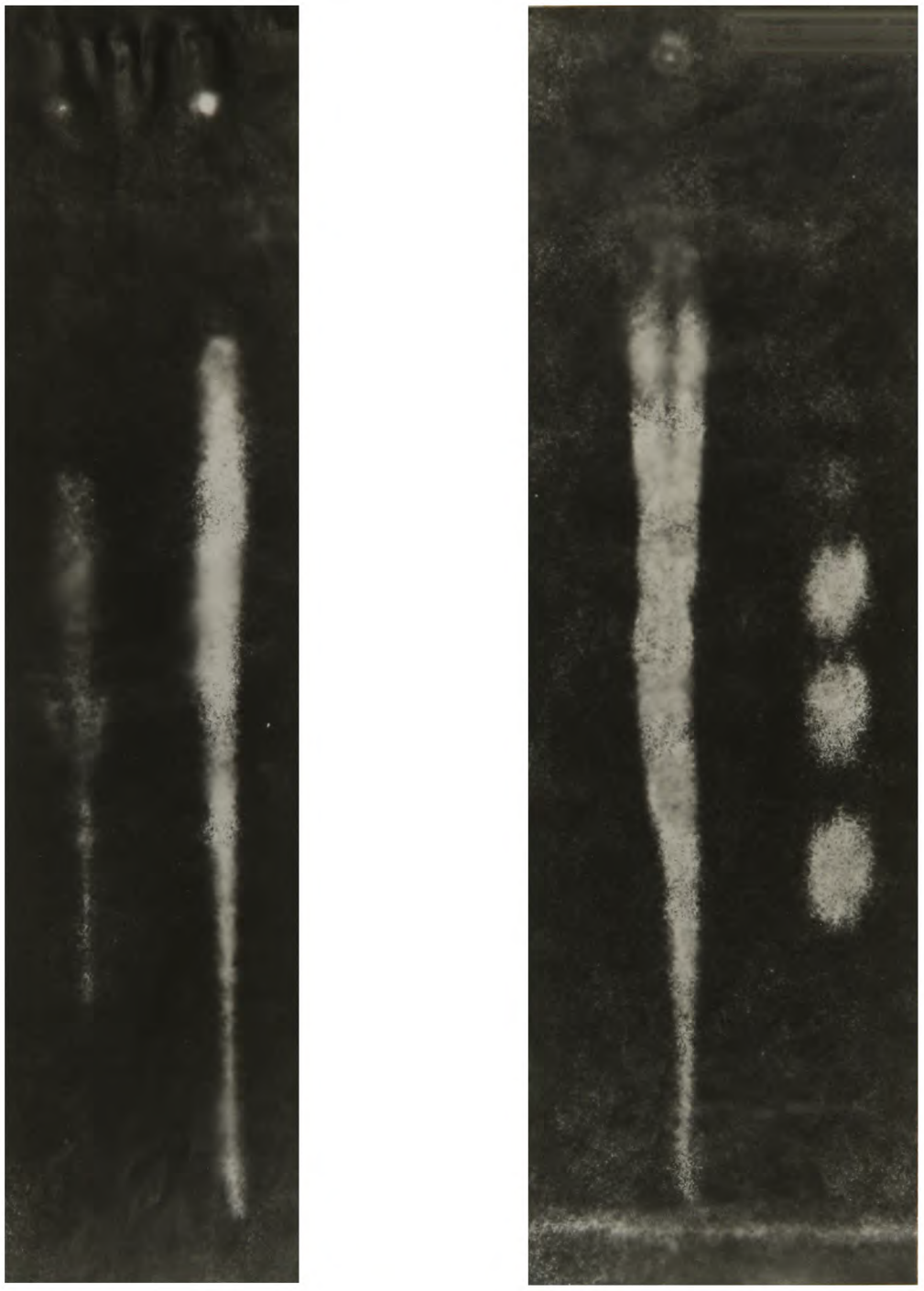
Fig. 2.2.

the loss of adenosine tri-phosphate ●—●, 10 min. phosphate ○—○, and the appearance of prothrombinase activity △—△, during the incubation of platelet-rich plasma with calcium. The plasma clotted after 2 mins. 15 secs.

technique). This procedure was used routinely in all further experiments.

Fig. 2.2. shows that the adenosine triphosphate was broken down immediately after the addition of calcium; the reaction thus began before the appearance of prothrombinase activity. This rapid breakdown of adenosine triphosphate was accompanied by the disappearance of more 10 min. phosphate than could be accounted for by the disappearance of the adenosine triphosphate. Fantl & Ward (1956) showed that platelets contained other nucleotides; the degradation of these may have accounted for the additional loss of 10 min. phosphate.

In the next series of experiments an attempt was made to find out whether the adenosine triphosphate which disappeared during clotting could be recovered as adenosine diphosphate or adenosine monophosphate. Paper chromatography of the acid extracts obtained from the plasma was thought to be the simplest method. However, initial runs were frustrated by the appearance of streaks on the papers, which were due to the large amounts of sodium chloride present in the extracts. (Plate 2.1.). Because of this an attempt was made to separate the chloride from the nucleotides. Out of a variety of solvents tested, the



a b c d

Plate 2.1.

a. Chromatography of the acid-soluble extract obtained from 3 mls. plasma.

b. As (a) except that a nucleotide mixture had been added.

c. The chromatography of the adenine nucleotides in the presence of NaCl ≡ 3 mls. of plasma.

d. Adenine nucleotide markers.



Plate 2.2.

The separation of chloride from a mixture of nucleotides on paper by two dimensional chromatography. The paper was first run with the isopropanol:NH_3 solvent in the direction indicated. The dried paper was then run with the isobutyric acid :NH_3 solvent.

most suitable was a mixture containing 80% isopropanol and 20% 0.5 N ammonia (adapted from Lederer & Lederer, 1957). On the starting spot of a chromatogram (Plate 2.2.) was placed the acid extract obtained from 3 ml. plasma to which had been added a mixture of nucleotides. The chromatogram was developed first with the isopropanol solvent for 4 hours. After this the paper was dried and then developed along the second dimension with a mixture of isobutyric acid and ammonia (Krebs & Hems, 1953). Unfortunately, when acid extracts derived from larger volumes of plasma (10 ml.) were used, some streaking of the nucleotides occurred. To overcome this, experiments were carried out in which columns of cellulose powder (Whatman Standard Grade) were used. An amount of sodium chloride corresponding to that found in 10 ml. plasma was mixed with inorganic phosphate containing ^{32}phosphate and with adenine nucleotides. This was made into a stiff paste with cellulose powder and a little of the isopropanol solvent. The paste was applied to the top of the cellulose column (1 x 10 cm.) which was then eluted with the isopropanol: ammonia solvent. 5 ml. samples of the effluent were collected. These were counted for

TABLE 2.1.

The separation of chloride and phosphate from a mixture nucleotides on a cellulose column.

SOLVENT	Cl	^{32}P counts/min	ϵ_{250}	ϵ_{260}	ϵ_{280}
isopropanol: ammonia	+++	2100	0.4	0.32	0.58
	+++	546	0.04	0.005	0.01
	+++	3450	0.04	0.05	0.01
	+++	1447	0.03	0.02	0.01
	+	812	0.02	0.005	0
	-	784	0.03	0.003	0
isopropyl ether: formic acid	-	65645			
	-	307			
	-	220			
	-	114			



a b

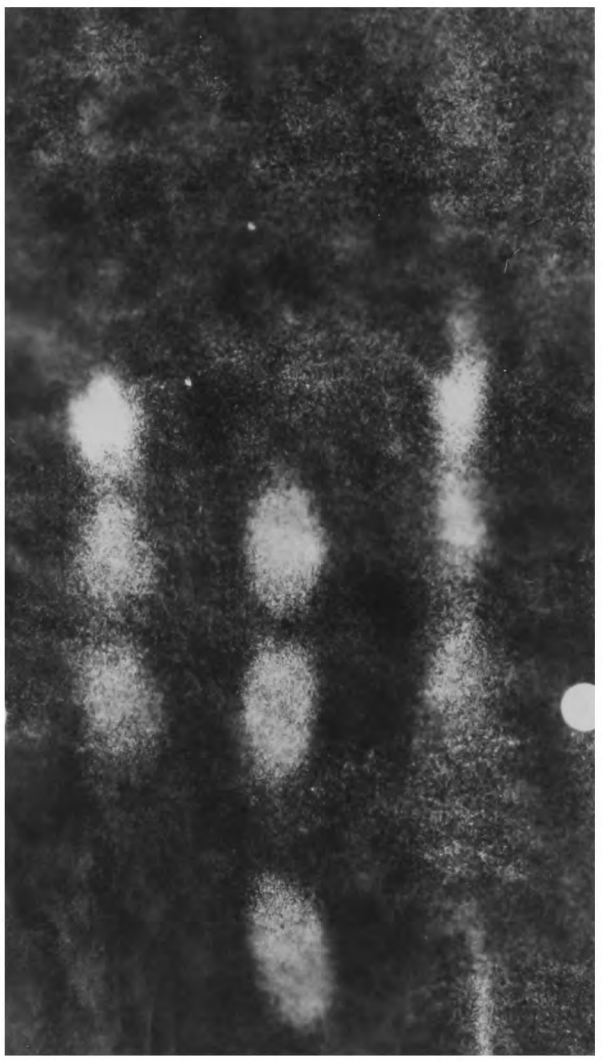
Plate 2.3.

a. Nucleotide markers.

b. The separation of the nucleotides recovered from a cellulose column.

Chromatograms developed with isobutyric acid: ammonia.

radioactivity and tested for chloride with silver nitrate dissolved in conc HNO_3. (Table 2.1.) shows how the substances came off the column. After the chloride had been eluted the column was washed with diethyl ether and then blown dry. Next, the inorganic phosphate was eluted using the isopropyl ether/formic acid solvent (Krebs & Hems, 1953). When no further phosphate could be detected, the column was again washed with diethyl ether and blown dry. The nucleotides were then eluted with 10 ml. water. Plate 2.3. shows the separation of the nucleotides on a paper chromatogram after they had been recovered from the column. The recovery of the individual nucleotides by this procedure was about 90%. Since it was possible to separate the nucleotides from both the chloride and the inorganic phosphate by this technique, undialysed platelet-rich plasma was used in the following experiments. Samples of plasma (12 ml.) were incubated with added calcium (clotted sample) and without added calcium (control sample). After 12 min. they were mixed with trichloracetic acid. When the chloride and inorganic phosphate had been removed from the acid extracts on cellulose columns, the nucleotides were separated on paper using the isobutyric acid: ammonia solvent



a b c

Plate 2.4.

The chromatography of the acid-soluble extracts obtained from unclotted (a), and clotted (c), platelet-rich plasma. The extracts had been freed of chloride and inorganic phosphate on a cellulose column.

(b) Nucleotide markers.

TABLE 2.2.

Changes in the adenine nucleotides during clotting.

		μg phosphate	
		CLOTTED	CONTROL
ATP	1	5.9	0.46
	2	4.75	2.5
ADP	1	2.0	2.3
	2	2.0	1.85
AMP	1	0.0	0.0
	2	0.06	0.08

(Krebs & Hems, 1953). The nucleotides were located on the paper by photography by ultraviolet light (Plate 2.4.). The areas of paper which contained the nucleotides were cut out and ashed for the determination of phosphate.

It will be seen (Table 2.2) that no significant amounts of adenosine monophosphate could be found in either the clotted or the control samples, and further that the breakdown of adenosine triphosphate was not accompanied by a marked change in the amounts of adenosine diphosphate or adenosine monophosphate in the plasma.

DISCUSSION:

Unfortunately it was not possible to demonstrate whether deaminated adenine nucleotides appeared. This was because some coloured material was present in both the clotted and the control samples, and this material ran on the chromatograms to the same positions as would be occupied by inosine nucleotides. It was not possible to detect adenosine or adenine because these substances would have been eluted together with the chloride and phosphate.

Although other attempts were made to detect possible degradation products using ion exchange chromatography,

they proved unsatisfactory. This was mainly because the plasma extracts contained high concentrations of salt. It is, however, clear from the experiments described that adenosine di- and monophosphates do not accumulate as a result of the degradation of the adenosine triphosphate in platelets.

The observations could be explained by the findings of Jørgensen (1956), who showed that in heparinized human plasma adenosine triphosphate was broken down to hypoxanthine. The first stage was the dephosphorylation of the adenine nucleotides to adenosine which was then deaminated to inosine. Hypoxanthine was formed by the removal of the ribose molecule from inosine, and it was this step which was rate-limiting.

Comparing the amount of prothrombinase formed by the technique described by Born (1958) and by the technique described in this chapter, it was observed (Fig. 2.1.) that the modified procedure resulted in the formation of much more prothrombinase. However, the rate of breakdown of the adenosine triphosphate found by Born (1958) is almost identical with the rate observed in these experiments. This would suggest that the disappearance of

adenosine triphosphate occurs independently of prothrombinase formation.

It seems, then, that adenosine triphosphate is not involved in the coagulation process. The function of adenosine triphosphate in platelets may be associated with the uptake and retention of 5-hydroxytryptamine (Born et al, 1956). When the amine is released from platelets it is apparently accompanied by the adenine nucleotide which is then degraded in the plasma.

CHAPTER III.

THE ROLE OF PHOSPHOLIPIDS IN BLOOD COAGULATION.

It has been recognised for many years that tissue juices were capable of coagulating blood. Wooldridge (1886) produced intravascular clotting in animals by injecting extracts of various tissue. It seemed that the active factor in these extracts was a complex of protein with phospholipid and the phospholipid was apparently a cephalin (Howell, 1935). The lipid portion of the complex was found to be essential to the activity of the protein (Cohen & Chargraff, 1940). The phospholipid-protein complex was isolated from ox-lung in a relatively pure state by Chargraff, Moore & Bendich (1942). Subsequent analysis of the lipid fraction (Chargraff, Bendich & Cohen, 1944) which was 40-45% of the whole molecule, showed that it contained free cholesterol 19%, fat 18%, and phospholipid 63%. The phospholipid fraction contained lecithin, 40%, cephalin 39%, sphingomyelin 19% and 2% as acetal phosphatides. This work suggested that phospholipid was essential for producing clotting in vitro but it did not show which kind of phospholipid had this activity.

When it was recognised that cephalin was a complex mixture of phosphatides, workers began to investigate the relative ability of the various components of cephalin to accelerate clotting. Chargraff (1944) found that phosphatidyl serine was unable to accelerate the clotting time of fowl plasma, whereas fractions which were rich in phosphatidyl ethanolamine showed some activity, though not as much as that of the more ethanol-soluble fractions. It was an unresolved problem whether the activity of crude lecithin preparations was due to the lecithin or to an impurity until Poole, Robinson & Macfarlane (1955) showed that pure lecithin did not accelerate the clotting time of plasma to which Russell's viper venom had been added. Crude preparations, on the other hand, had a marked accelerating effect. The nature of the contaminant remained obscure. Poole (1955) noticed that plasma rich in chylomicra, i.e. minute particles of fat, clotted more rapidly on the addition of calcium than plasma freed of these particles. The shortening of the clotting time of the plasma was a function of the concentration of the chylomicra, and was not related to the concentration of platelets.

Extending these observations, Poole & Robinson (1956a) and Robinson & Poole (1956) obtained evidence which suggested that the chylomicra might contain small amounts of ethanolamine phosphatide, and that the activity of the chylomicra was due to the presence of this compound rather than the fat. Poole & Robinson (1956b) investigated the effects of preparations of phosphatidyl ethanolamine obtained from various sources on the clotting time of rats' plasma in the presence of Russell's viper venom. They found that the clotting time decreased as the dilution of the phosphatide increased from 1 mg./ml. to 0.01 mg./ml. plasma. Ethanolamine phosphatide prepared from egg yolk was just as active as synthetic preparations containing fully saturated fatty acids. Acting on a suggestion that the synthetic phosphatides probably contained traces of the lyso compounds, Poole & Robinson (1956b) tested the activity of the α-stearoyl lyso phosphatidyl ethanolamine and found it to be inactive. Poole & Robinson also correlated the activity of brain phospholipid preparations with their content of ethanolamine. Robinson, Harris & Poole (1957) showed that when plasma was incubated with an enzyme obtained from Bacillus cereus and the coagulation

time of the plasma was prolonged. This increase in the clotting time of the plasma which had been incubated with the enzyme could be reversed by the addition of ethanolamine phosphatide. This enzyme had been described by Chu (1949), who showed that it attacked both lecithins and cephalins, and split off the phosphate esters of their corresponding bases. If the plasma was incubated with the enzyme for longer than 3 hours, the reversal brought about by the addition of ethanolamine phosphatide was incomplete. Some plasma was freed of platelets and chylomicra, and incubated with the enzyme until 75% of the phospholipid had been destroyed. The plasma was then almost incoagulable, even after the addition of ethanolamine phosphatide.

These results suggest that ethanolamine phosphatide is necessary for the coagulation of plasma; they do not exclude the possibility that other phospholipids also take part. Evidence that the active phospholipid present in platelets is ethanolamine phosphatide came from the observation that platelets and ethanolamine phosphatide behaved similarly after treatment with various agents (O'Brien, 1956). For example, platelet extracts lost their coagulant effect after they had been passed through

an alumina column which adsorbs all phosphatides except those containing choline. Incubation of both platelet extracts and phosphatidyl ethanolamine with bee venom, cobra venom, or Cl. welchii α toxin, resulted in the loss of activity. These results were confirmed by Barkhan, Newlands & Wilds (1956); they separated a phospholipid fraction from platelets which was rich in ethanolamine phosphatide, and which accelerated the coagulation of plasma. A second fraction was also obtained from platelets which had an inhibitory effect; this fraction was found to be rich in phosphatidyl serine. The inhibitory action of preparations rich in phosphatidyl serine has been confirmed by Turner, Silver & Tocantins (1958), who isolated a fraction containing this phosphatide from brain. Phosphatidyl serine was found to be inhibitory at concentrations of 1 mg./ml. The phosphatide was inhibitory only when it contained unsaturated fatty acids, since catalytic hydrogenation resulted in the loss of activity. Other alterations in its structure produced by deamination with pyridoxal, by treatment with diazomethane, or by the removal of one of the fatty acids, also abolished the inhibitory effect. In all these experiments the serine

phosphatide was dispersed with the aid of sodium desoxycholate.

The work described so far suggests that while ethanolamine phosphatide is an accelerator of plasma clotting and serine phosphatide is an inhibitor, lecithin appears to be without effect. However, such a generalization must be regarded with caution. Thus, Biggs & Bidwell (1957) investigated the effect of various phospholipid fractions obtained from brain (Folch, 1942) and found that fractions rich in phosphatidyl ethanolamine had no greater activity than the crude cephalin from which they were obtained. In fact, these workers were unable to concentrate the coagulant activity into any fraction.

These fractions were not pure, and the results could be explained on the supposition that the ethanolamine phosphatide was not separated from an inhibitor such as serine phosphatide, or that the presence of a second phospholipid modified the activity. That this can happen has been demonstrated by Rapport (1956), who observed that a preparation of brain phospholipid rich in phosphatidyl serine accelerated the clotting time of plasma, and that the activity of this preparation was increased by the addition of lecithin. This activating effect of lecithin

was only obtained if the two phosphatides were first mixed in toluene. The toluene was then removed and the phospholipid mixture was dispersed in plasma for the assay of its activity. The potentiating effect of lecithin was not observed if the phospholipids were first mixed in water. The activation was maximal when lecithin and phosphatidyl serine were present in a ratio of 2:1; when the activity was increased ten-fold. These observations have been confirmed by Therriault, Nicholls & Jensen (1958). They purified Folch fraction III of brain by the technique of countercurrent distribution. In this way they obtained pure serine phosphatide, the activity of which was almost the same as that of the crude fraction. The coagulant activity of both the crude and purified phosphatide was greatly enhanced by the addition of lecithin in the ratio of 1:2. This increase in activity appeared only when the phosphatides were mixed in an organic solvent, namely chloroform. Infra-red measurements carried out on the mixtures indicated that the interaction of the phospholipids was not covalent. One possible explanation for the phenomenon would be that the phosphatides mix completely in the organic solvent and that, when the mixture is transferred

to an aqueous phase, the result is the formation of micelles which possess certain critical physical properties (Wallach, Maurice & Surgenor, 1959: see below). This hypothesis is strengthened by the observation of Rapport (1956) that the activity of the mixture depended on its composition.

Marcus & Spaet (1958) investigated the coagulant properties of platelet phosphatides, and isolated phosphatidyl serine chromatographically. On comparing the activity of this pure phosphatide with that of brain preparations, it was found that mole for mole the serine phosphatide was approximately 200 times more active than standard brain preparation. (The optimal concentration was 0.3 mg. phospholipid phosphate/ml. plasma.) The activity of the serine phosphatide decreased when larger amounts were added, and at high concentrations the activity disappeared altogether. Marcus & Spaet did not compare the coagulant activity of phosphatidyl ethanolamine with that of serine phosphatide but they found that a third component, probably a phosphatidic acid, also had coagulant activity. Troup & Reed (1958) confirmed that phosphatidyl serine obtained from platelets and from red cells had coagulant activity but in their

experiments it was optimally active at a concentration of 2 mg./ml. Samples of ethanolamine phosphatide obtained from the same source were also active. The optimum concentration was considerably greater for the ethanolamine phosphatide than for the serine phosphatide. Troup & Reed concluded that the phosphatides obtained from red cells and from platelets were similar and were equally active in thromboplastin generating systems.

Rouser, White & Schloredt (1958) and Rouser & Schloredt (1958) have investigated the coagulant activity of neutral lipids, fatty acids and phospholipids and have found that only the phospholipids had marked coagulant properties. They next tested the coagulant effect of the individual phospholipids which were obtained from both natural and synthetic sources and found that the only compounds which possessed marked activity were phosphatidyl ethanolamine obtained from both natural and synthetic sources and phosphatidic acid. The other phospholipids were inactive as were the compounds formed after hydrolysis of the phospholipids.

Rouser et al. (1958) found that ethanolamine phosphatide extracted from various tissues had different potencies.

The sources arranged in increasing orders of activity were: egg, soya bean, rabbit appendix, human blood platelets. A synthetic ethanolamine phosphatide which contained only saturated fatty acids was less active than the phosphatides extracted from the tissues. An analysis of the fatty acids contained in the ethanolamine phosphatides which had been isolated from the tissues revealed a progressive change in structure. The egg ethanolamine phosphatide contained fatty acids of which approximately half were unsaturated, the remainder being saturated. The more active soya bean phosphatide contained fatty acids which were predominantly unsaturated and was primarily the dilinoleyl phosphatide. The rabbit appendix phosphatide differed from the soya bean product only in the degree of unsaturation of the fatty acids, which were arachidonic and oleic.

The platelet phosphatide which was the most active contained 72% of arachidonic acid. The remaining fatty acids were highly unsaturated containing five and six double bonds. Since Rouser et al. (1958) were unable to correlate the variation in the activity of the ethanolamine phosphatide obtained from the various sources with the

extent to which the preparations were contaminated with other phosphatides or plasmologens, it was concluded that the variations in activity were primarily due to the degree of unsaturation of the fatty acids. Rouser & Schloredt (1958) suggested that the presence of highly unsaturated acids controlled the size of the colloidal particles obtained when the phosphatide was dispersed in aqueous media. Wallach, Maurice & Surgenor (1959) have investigated the relationship between size of the colloidal particles formed by ethanolamine phosphatide and its coagulant properties. They used purified plasma reagents to assay the coagulant activity of a pure egg ethanolamine phosphatide, which contained fatty acids of which 92% were unsaturated. Slight oxidation of the phosphatide resulted in an increase in its coagulant activity, but this decreased rapidly as the oxidation was continued. The phosphatide exhibited its maximal coagulant activity at 20 µg./ml; at higher concentrations the activity decreased. A suspension of the phosphatide was centrifuged for ten minutes at 100,000 g., and Wallach et al. (1959) found that the coagulant activity of this suspension was concentrated into the sediment, which suggested that the coagulant activity exhibited by ethanolamine phosphatide

in their system was due to the formation of small particles or micelles. When the temperature, the hydrogen-ion concentration and the ionic strength of the suspending medium favoured the formation of micelles the phosphatide exhibited its greatest coagulant activity. Wallach et al. (1959) therefore suggested that ethanolamine phosphatide takes part in the formation of micelles which catalyse the conversion of prothrombin to thrombin.

The observations reported by the groups of Rouser and Surgenor have shown that the coagulant activity of phosphatidyl ethanolamine is due to the formation of micelles which have a certain critical size, and that this physical property of the molecule is controlled to a great extent by the degree of unsaturation of the fatty acids.

In the light of these observations it is possible to suggest an interpretation of the apparently contradictory results obtained by other workers who have investigated the coagulant properties of phosphatidyl serine. Troup & Reed (1958) found that serine phosphatide had maximal coagulant activity at lower concentrations than ethanolamine phosphatide, which suggests that in their experiments the

serine phosphatide formed active micelles at a lower concentration than did the ethanolamine analogue. At higher concentrations (1 mg./ml.) serine phosphatide was found to be an inhibitor (Turner et al., 1958); presumably at this concentration the micelles had become aggregated into larger particles which were inactive. Since the phosphatidyl serine was dispersed in these experiments with sodium desoxycholate which is itself a weak inhibitor of coagulation (Silver, Turner & Tocantins, 1957), the apparent increase in inhibitory activity may be due to the association of the phosphatide with the detergent, which results in the reduction of active micelles already present in the plasma.

In the presence of lecithin (Rapport, 1956 and Therriault et al., 1958) the aggregation of the micelles might take place at higher concentrations, with the result that a greater number of active surfaces can be obtained.

All the evidence summarised in this chapter suggests that phospholipids play an essential part in the coagulation of blood. However, most of the information about this has been obtained by experiments in which highly artificial systems were used. It is very probable, for example, that

the addition of extraneous phospholipids to plasma disturbs the equilibrium which determines the association of phospholipids already present with the plasma proteins (Oncley, 1954).

The work done in the remainder of this thesis, therefore, had as its basis the idea that it might be fruitful to find out whether during clotting the plasma phospholipids themselves underwent any chemical change.

CHAPTER IV.

THE ISOLATION AND IDENTIFICATION OF A PHOSPHATE-CONTAINING COMPOUND WHICH APPEARS DURING THE COAGULATION OF PLASMA.

Although phospholipids have been shown to be essential for the coagulation of blood, no chemical reactions have been demonstrated in which they take part. Born (1958) observed that during the clotting of recalcified platelet-rich plasma, a fraction of the phospholipid phosphorus became unextractable in chloroform: methanol. Later it was found that a slight modification in the extraction procedure resulted in the disappearance of this effect. Born & Esnouf (1958) pointed out that the redistribution of the phospholipid might be an artefact introduced by the use of trichloracetic acid as a precipitant. While these experiments were in progress, it was noticed that a phosphate compound appeared in acid extracts of plasma during clotting. This chapter will describe the experiments in which this compound was isolated and identified.

METHODS

Platelets and Plasma: Platelets and dialysed plasma were

obtained as described in a previous chapter.

Radioactive Plasma: Blood was withdrawn from patients who were suffering from polycythaemia vera and who were receiving radioactive phosphate, i.e. ^{32}P. Preliminary experiments showed that the plasma phospholipids reached their maximum specific activity about 50 hours after an injection of ^{32}P. Blood was therefore withdrawn about 48 hours after the injection of the isotope. Platelet-free plasma was obtained as described earlier. The plasma was dialysed for two hours against Krebs-Ringer phosphate solution which contained sodium citrate (final conc. 0.38%), so as to reduce the specific activity of exchangeable phosphate. The plasma was then dialysed against Krebs-Ringer bicarbonate solution as described previously; in this way the concentration of dialysable phosphate was greatly diminished.

Clotting of the Plasma: Either radioactive or non-radioactive plasma, usually 5 ml., was mixed with fresh platelets derived from the same volume of non-radioactive plasma. After an initial period of activation in glass, the plasma was recalcified as described in a previous chapter. In experiments in which Russell's viper venom was used, platelets were not added to the plasma. Except

where stated, the plasma was incubated with 10 µg. venom/ml. plasma; after preliminary glass activation the venom was added at the same time as the calcium. The acid-soluble extracts were obtained as already described.

Russell's viper venom: This was a gift from the Wellcome laboratories.

Prothrombinase Activity: Assayed by the method described on p. 19.

Determination of phosphate: Organic, 10 min. and inorganic phosphate was estimated by the methods described on p. 18.

Measurement of radioactivity: This was measured in a liquid counter (M6 of 20th Century Electronics Ltd.). Specific activity measurements were made by determining the radioactivity in the isobutanol layer which contained the phosphomolybdate complex.

Chromatography: Acid soluble phosphate compounds were chromatographed on Whatman No. 1 paper which had been washed in 2N acetic acid (Dawson, 1954). The solvents used were phenol:ammonia (Dawson, 1954) and ethanol:ammonia (Dawson, 1955). Spots were located by scanning the papers with an end-window counter (EHM2/S of General Electric Co.). A sample of glycerylphosphorylcholine was kindly given by Dr. R.M.C. Dawson; this was used as a marker on the chromatograms.

TABLE 4.1.

The appearance of radioactivity in acid soluble phosphate fraction during the coagulation of platelet-rich plasma.

TIME (mins.)	Expt. 1.		Expt. 2.		Expt. 3.		Expt. 4a.		Expt. 4b.	
	S.A.	µg.P.	S.A.	µg.P.	S.A.	µg.P.	S.A.	µg.P.	S.A.	µg.P.
0	0.68	7.0	5.4	10.9	1.86	7.1	0.38	4.80	0.96	1.80
1	1.35	9.3	6.0	9.6	3.19	7.5	0.92	6.80	1.71	3.70
2	3.16	6.0	6.6	8.6	4.0	6.2	1.59	5.70	3.05	2.90
4	-	-	6.8	7.9	-	-	2.14	5.20	4.40	2.50
8	3.9	4.1	6.6	8.2	5.0	6.5	3.34	4.90	6.03	2.70
16	3.5	4.4	7.8	7.1	5.2	7.4	3.34	4.50	6.03	2.50

phosphate expressed as µg/ml. plasma.

Experiments 1-4a phosphate is the total phosphate less inorganic phosphate. 4a and 4b are the same experiments. The phosphate figure in 4b is the total phosphate less 10 min. and inorganic phosphate.

S.A. is specific activity in counts/min/µg phosphorus.

Determination of Glycerylphosphorylcholine: The glyceryl-phosphorylcholine was hydrolysed in a sealed tube with 2N HCl. for 18 hrs. at 126°. After the hydrolysis a sample was removed for the estimation of inorganic phosphate. A second sample was taken for the determination of glycerol by the method of Hanahan & Olley (1958). Care was taken to ensure that the periodate oxidation took place at an acid concentration not exceeding 1.5N. The HCl. was removed from a third sample of the hydrolysate by standing it *in vacuo* over phosphorous pentoxide and potassium hydroxide pellets. Free choline was then estimated in this sample by the iodine precipitation method of Smits (1957).

EXPERIMENTAL & RESULTS

When plasma containing ^{32}phosphate was clotted in the presence of platelets, it was noticed that there was an increase in the radioactivity of the total acid-soluble phosphate. This increase in radioactivity was not due to the appearance of labelled inorganic phosphate. The results of four such experiments are summarized in (Table 4.1.). It will be noticed that this increase in radioactivity appeared in what was called the organic phosphate fraction, i.e. the total phosphate less 10 min. plus inorganic phosphate.

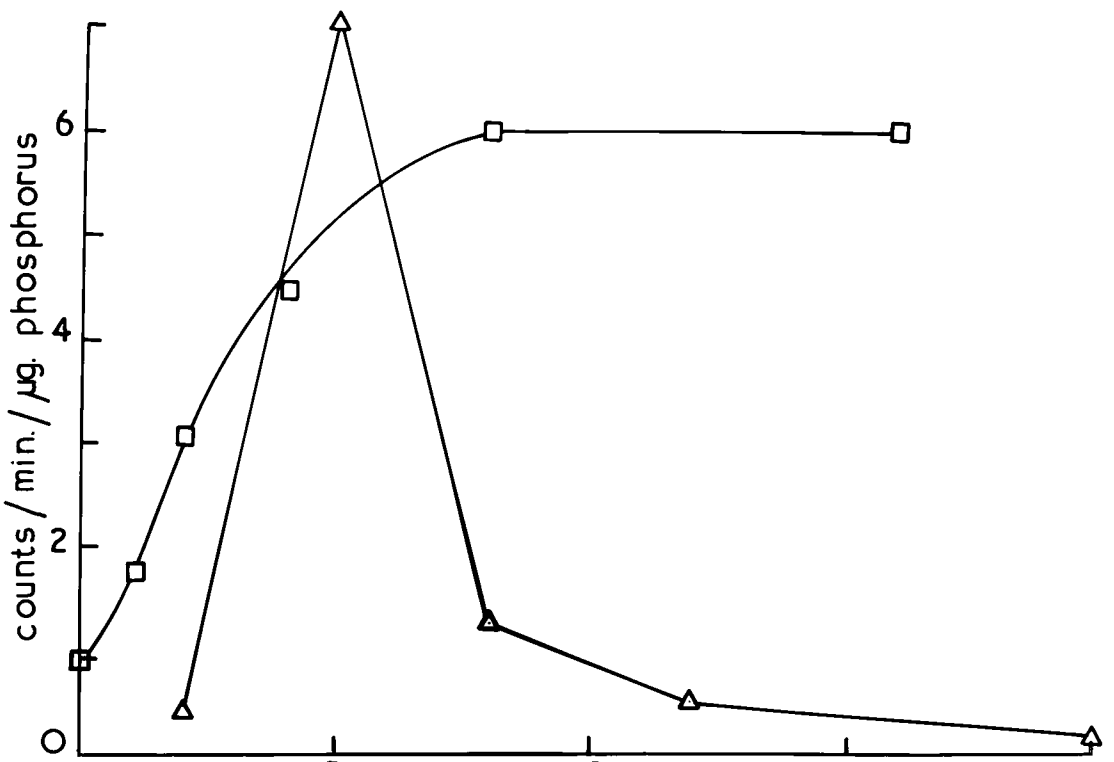


Fig. 4.1.

The release of radioactivity □—□ into the acid soluble phosphate fraction, and the appearance of the prothrombinase activity Δ—Δ, during the incubation of platelet-rich plasma with calcium. The plasma clotted after 2 mins. 15 secs.

TABLE 4.2.

The appearance of radioactivity and organic phosphate during the incubation of plasma with Russell's viper venom.

TIME (mins.)	Expt. 1.		Expt. 2.	
	S.A.	µgP/ml. plasma	S.A.	µgP/ml. plasma
0	3.7	0.12	1.35	1.48
1	19.0	0.76	-	-
2	16.5	0.96	-	-
4	14.5	1.35	6.50	1.80
8	16.6	1.90	7.45	1.90
12	-	-	7.56	1.96
16	16.0	1.80	7.80	2.34
20	-	-	7.40	2.30

S.A. specific activity counts /min/µg phosphorus.

Unfortunately, it was not possible to correlate the increase in radioactivity with an increase in organic phosphate. The increase in radioactivity is compared with the appearance of prothrombinase activity in (Fig. 4.1.), and it will be seen that the appearance of the radioactivity ceased shortly after maximum prothrombinase activity had developed.

If the liberation of the radioactivity was the result of a reaction taking place during coagulation, it was reasonable to suppose that the same material would be released when plasma was made to clot under the influence of Russell's viper venom. To test this supposition, samples of radioactive dialysed plasma were clotted with venom as described in the methods section. (Table 4.2.) shows that, using venom, the increase in radioactivity was accompanied by the appearance of organic phosphate. These results suggested that, in the corresponding platelet experiments, other reactions were taking place which made it difficult to detect the appearance of this phosphate compound.

Samples of the acid-soluble extracts obtained from plasma clotted with venom and platelets were chromatographed on paper, using various solvent systems. It was evident from initial runs that it was necessary to remove the salt from the extracts. Using the isopropanol:ammonia

solvent described in a previous chapter, it was found that a radioactive spot separated from the other phosphate compounds. This radioactive material (rf 0.19) moved about half the distance of the chloride (rf 0.34), and was present in extracts obtained from plasma clotted with either venom or platelets. No such spot was detected in extracts obtained from unclotted plasma. When chromatograms which had been run previously in the isopropanol:ammonia solvent were run along the second dimension with phenol:ammonia or ethanol:ammonia, the radioactive material appeared in the same position as an authentic sample of glycerylphosphorylcholine. In addition, a second very weak radioactive spot appeared in the phenol:ammonia solvent which ran behind the main spot. In order to obtain large amounts of the radioactive material, 120 ml. samples of plasma were clotted with viper venom. After the removal of the trichloracetic acid from the acid extract with ether, the extract was then divided into two equal portions (≡ 60 ml. plasma). Each portion was concentrated in a rotary evaporator, and finally dried <u>in vacuo</u> over phosphorus pentoxide. One portion of the dried extract was mixed with a little cellulose powder (Whatman, Standard Grade) and isopropanol:ammonia

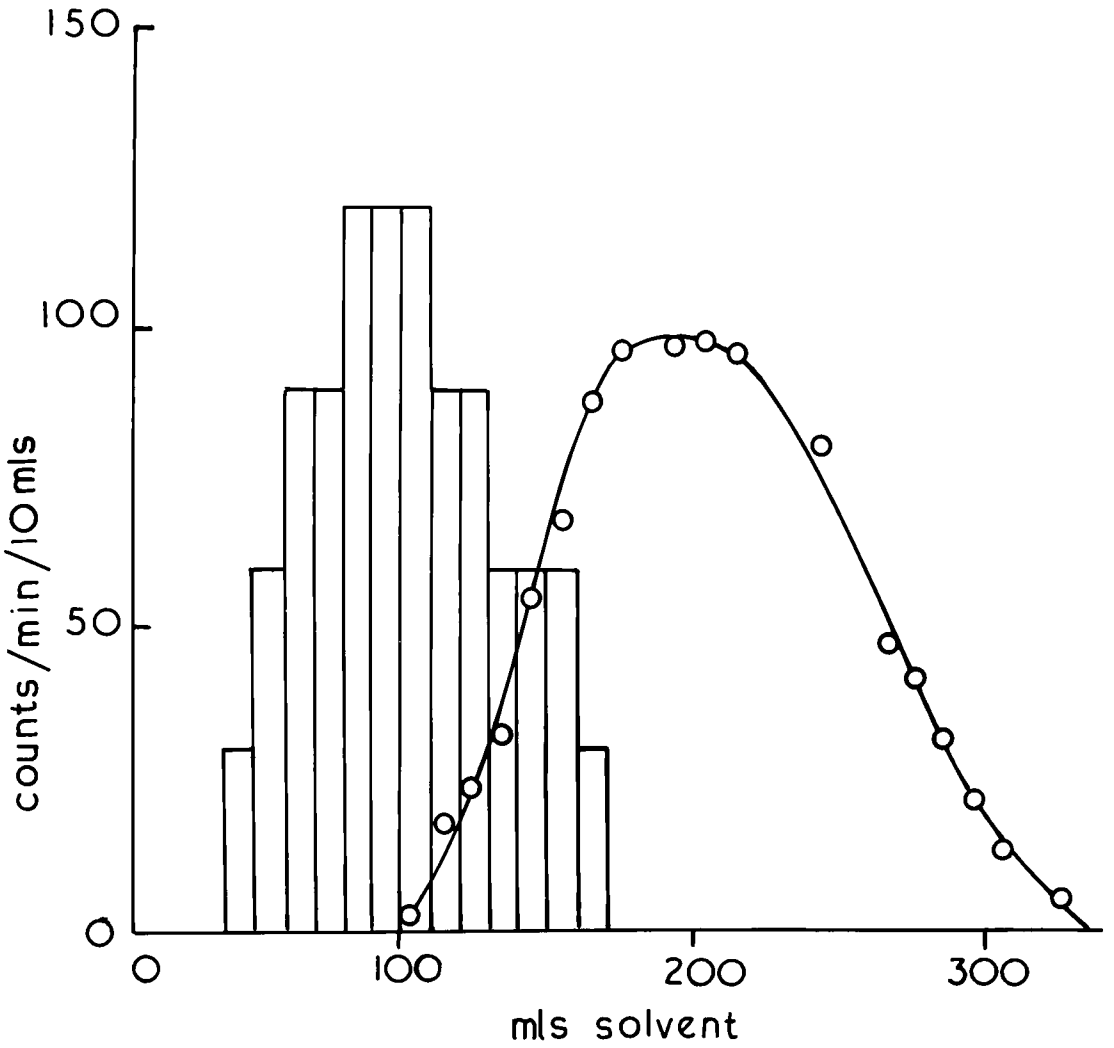


Fig. 4.2.

The elution of chloride (histogram) and radioactivity O——O from a cellulose column with isopropanol:NH_3.

TABLE 4.3.

Fractionation of the acid soluble extracts obtained from clotted and unclotted plasma, on a cellulose column.

SOLVENT	TOTAL COUNTS/MIN.		
	Venom + Ca^{++}	Venom - Ca^{++}	CONTROL
Isopropanol:NH_3	743.1	358.7	90.6
N HCl.	1245.4	1162.0	1160.0

plasma (30 mls.) incubated with venom 10 µg/ml. for 8 mins.

and applied to the top of a packed column of cellulose powder (1.5 x 20 cm.), which had been previously washed with isopropanol:ammonia. The column was then eluted with the solvent at a flow rate of 1-2 ml./min. 10 ml. fractions were collected which were assayed for chloride (as in an earlier chapter), and for radioactivity. The first three samples contained a heavy white gelatinous precipitate, which was presumably due to the formation of metallic hydroxides. It will be seen (Fig. 4.2.) that the separation of the chloride ion from the radioactivity was not complete. However, rechromatography of the pooled radioactive fractions resulted in almost complete removal of the chloride. Chromatography of acid extracts obtained from unclotted samples of plasma gave only very small amounts of the radioactive material (Table 4.3.). Paper chromatography of the concentrated radioactive material using phenol:ammonia as the solvent, revealed two spots. The major spot had the same mobility (rf 0.86) as a sample of glycerylphosphorylcholine (rf 0.87); the second spot, which could just be detected with the geiger counter, had the same mobility (rf 0.65) as a sample of glycerylphosphorylethanolamine (rf 0.64). Using ethanol:ammonia, which does not separate the choline and

ethanolamine compounds (Freinkel, 1958), only one spot was detected.

In order to obtain sufficient of these compounds for chemical analysis, samples of the radioactive material which had been freed of chloride were pooled and further purified by ion exchange chromatography. The radioactive material was taken up in 0.005M sodium tetraborate. This was applied to a column (1 x 13 cm.) of dowex 1 x 2 resin (200-400 mesh), which was in the formate form (Hawthorne & Hübscher, 1959). Between 90-95% of the radioactive material was not retained by the resin. The remaining 5% was recovered when the solvent was changed to a mixture containing 0.0005 M sodium tetraborate and 0.04M ammonium formate. This was presumably glycerylphosphorylethanolamine; however, it was not possible to obtain this compound in sufficient amounts for a chemical analysis. The radioactive material which was not retained by the resin was concentrated and hydrolysed in a sealed tube. Phosphate, choline and glycerol analyses were carried out on the hydrolysate, and this was compared with the result obtained by hydrolysing a pure sample of glycerylphosphorylcholine. In the case of the glycerol estimations, interference from free formaldehyde was found. This was kept to a minimum

TABLE 4.4.

Phosphate, glycerol and choline analyses on the hydrolysates of glycerylphosphorylcholine and the radioactive compound.

	Pure GPC	Radioactive compound
	μ moles	μ moles
PHOSPHATE	2.20	0.118
GLYCEROL	2.20	0.120
CHOLINE	2.28	0.115
MOLAR RATIOS	1.0:1.0:1.04	1.02:1.04:1.00.

TABLE 4.5.

Appearance of phosphate and choline in the acid soluble extracts obtained during the incubation of plasma with venom and calcium.

	Expt. 1.			Expt. 2.		
TIME (mins)	PHOSPHATE µ moles	CHOLINE µ moles	PHOSPHATE: CHOLINE ratio	PHOSPHATE µ moles	CHOLINE µ moles	PHOSPHATE: CHOLINE ratio
4	0.36	0.37	1:1.03	0.49	0.43	1:0.89
8	0.54	0.46	1:0.86	0.32	0.39	1:1.20
12	-	-	-	0.42	0.42	1:1.0
16	0.61	0.59	1:0.97	-	-	-
20	0.59	0.58	1:0.99	0.50	0.49	1:0.99

Mean Phosphate:Choline ratio 1:0.99.

by the prolonged washing of the resin column with distilled water before use (Hanahan & Olley, 1958). The molar ratios obtained for phosphate, choline and glycerol (Table 4.4.) confirmed that the radioactive material was glyceryl-phosphorylcholine.

To test whether the organic phosphate which was released during clotting could be accounted for solely by the formation of glycerylphosphorylcholine, the phosphate to choline ratio was determined in the acid-soluble fraction obtained at various times during the incubation of plasma (10ml.) with venom and calcium. The small amounts of glycerylphosphorylethanolamine which might also be formed would not be detected. The acid soluble extracts were concentrated, made N with respect to HCl, and after the removal of a sample for the determination of total phosphate, the remainder was heated to 100° for 20 minutes to release the choline from the glycerylphosphorylcholine (Dawson, 1956). Samples were then removed for the estimation of choline. It will be seen (Table 4.5.) that the release of the organic phosphate was accompanied by the appearance of choline, and that molar ratios were very near to 1:1. This result indicated that glycerylphosphorylcholine was the only detectable organic phosphate containing compound

which was released during the incubation of plasma with viper venom. Further confirmation of this was obtained from an experiment in which the specific activity of the organic acid-soluble phosphate which was released from a sample plasma by the action of viper venom was compared with specific activity of the phosphate in the glycerylphosphorylcholine isolated from the same extract. The organic phosphate had a specific activity of 20.6 compared with 20.2 found for the glyceryl-phosphorylcholine (phosphate:choline ratio 1:1.02). In one experiment the plasma was clotted with the acetone insoluble fraction obtained from brain, and as in the case of the platelets, the specific activity of the acid-soluble organic phosphate fraction increased, although the presence of other organic phosphate compounds introduced with the brain preparation made it difficult to detect the release of organic phosphate.

Since the radioactive material obtained from experiments in which platelets were used to clot the plasma had the same mobility as glycerylphosphorylcholine in the three chromatographic solvents, phenol:ammonia, ethanol:ammonia, and isopropanol:ammonia, it is reasonable to assume that this compound was released from plasma in the presence of platelets.

These results suggest that the formation of prothrombinase activity in the plasma is accompanied by the formation of glycerylphosphorylcholine.

CHAPTER V.

THE ACTION OF RUSSELL'S VIPER VENOM AND COBRA VENOM ON PLASMA LECITHIN.

In the preceeding chapter it was shown that glycerylphosphorylcholine was released during the coagulation of plasma in the presence of Russell's viper venom. Glycerylphosphorylcholine can be formed by the degradation of lecithin or lysolecithin. Both of these compounds are present in the plasma and represent 70% and 9% respectively of the plasma phospholipids (Phillips, 1958 and Marinetti, Ford & Stotz, 1959). The amount of glycerylphosphoryl-choline released during coagulation corresponds to the hydrolysis of between 2 - 3% of the plasma lecithin. Glycerylphosphorylcholine is formed from lecithin by the action of two enzymes, namely a lecithinase (Hanahan, 1954) and a lysolecithinase (Contardi & Ercoli, 1933). Russell's viper venom has been shown to contain lecithinase activity (Maclagan, Billimoria & Curtis, 1958). In this chapter the action of Russell's viper venom on native and purified plasma lecithin has been investigated. In addition the lecithinase activity of the viper venom has been compared with that of the venom of the Indian cobra (Naja Naja).

METHODS

The reaction of the venom on native plasma lecithin was studied as follows. Plasma (3 ml.) was incubated with either oobra venom or Russell's viper venom (10 µg/ml.) by the procedure described in the previous chapter. At the end of the incubation period the reaction was stopped by the addition of methanol (22.5 ml.). After mixing thoroughly, chloroform (22.5 ml.) was added and the sample was left to stand at room temperature for about 2 hours, by which time the fibrin clot had disintegrated. The sample was centrifuged, the clear supernatant removed and the precipitate was washed with 10 ml. of a 1:1 chloroform/methanol mixture and centrifuged again. The combined supernatants were evaporated to dryness on a rotary evaporator at 20°. The lecithin and lysolecithin were separated by a chromatographic procedure exactly as described by Marinetti et al, (1959), except that the proportions of diisobutyl ketone : acetic acid : water in the solvent were changed to 40 ; 30 : 2 (v/v). After the phospholipids had been eluted from the paper they were digested and their phosphate content determined by the methods described in the previous chapter.

Cobra venom (Naja Naja) was purchased from the Zoological Society.

Isolation of plasma lecithin.

150 ml. platelet-free plasma containing ^{32}phosphate was mixed with 15 volumes of a 1:1 chloroform/methanol mixture at room temperature. After 30 mins. the precipitated plasma proteins were removed by filtration in a Buchner funnel. The filtrate was mixed with an equal volume of distilled water, and after centrifuging the aqueous phase was removed together with the interfacial precipitate. The chloroform layer was concentrated to about 15 ml. in a rotary evaporator at 20°. The phospholipids were then separated on a silicic acid column (Hanahan, Dittmer & Warashina, 1957). 60 gm. of silicic acid (A.R. 100 mesh. Mallinckrodt) was heated overnight at 110°, after this it was washed three times with chloroform and poured into a column 2.5 cm. in diameter. The phospholipid extract was placed onto the column after the silicic acid had settled. The column was eluted with about 2 l. of chloroform at a flow-rate of 1-2 ml./min. for 20 hr., during this time the bile-pigments, steroids, mono-, di-, and tri- glycerides were eluted from the column. The flow-rate was maintained by connecting the solvent reservoir to a supply of nitrogen at a pressure between 1-2 lb./square inch. The eluate was tested at

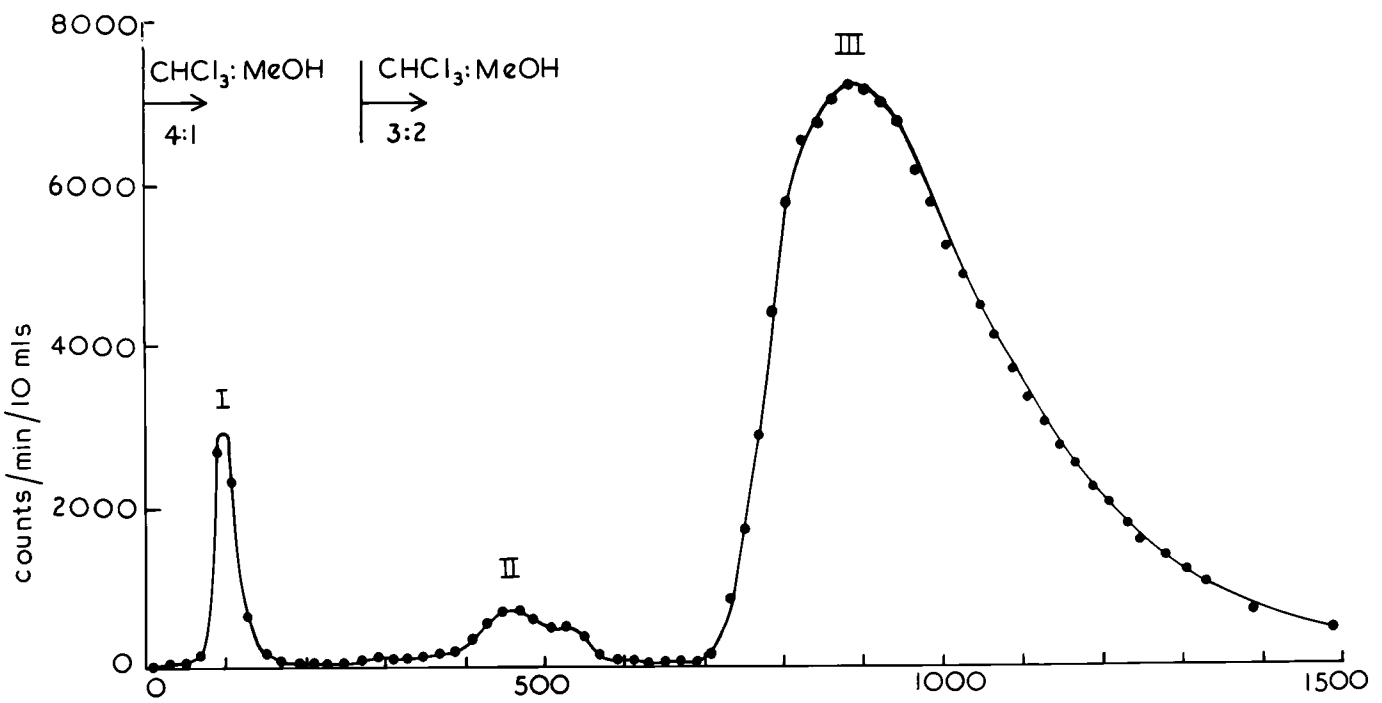


Fig. 5.1.

The elution of the plasma phospholipids from a silicic acid column using mixtures of chloroform and methanol.

intervals for acyl-ester linkage (Rapport & Alonzo, 1956). When no further ester reacting material was eluted from the column by the chloroform (the last 100-200 ml. free of ester reacting material), the solvent was changed. If the non-phosphorus-containing lipids were not removed at this stage they appeared later with each of the phospholipid fractions.

The column was next eluted with a 4:1 chloroform/methanol mixture, and 10 ml. fractions were collected. In the earlier runs the radioactivity was assayed in an M.6. liquid counter; in later experiments the column effluent was continuously monitored with a scintillation counter, the output from which was recorded. After the appearance of the first peak, the solvent was changed to a 3:2 chloroform/methanol mixture and the elution continued until the lecithin had been removed from the column. The solvent in the collecting tubes which contained the lecithin was pooled and the solvent removed in a rotary evaporator. The lecithin was redissolved in peroxide-free ether.

The elution diagram of the phospholipids from the column is shown in (Fig. 5.1.), and little variation was observed in the volume of solvent required to elute each

particular phosphatide during the course of six separate fractionations. Peak I was presumably ethanolamine phosphatide, and peak II was thought to be mono-phosphoinositide (Marinetti et al, 1959). Peak III was identified by analysis as lecithin. An analysis of the material in this peak obtained from each fractionation, showed that it had a phosphate:ester ratio of 1: 1.97 - 1.99, and that glycerol, phosphate and choline were present in almost equimolar proportions.

The action of the venoms on purified plasma lecithin:

The action of the venoms on purified plasma lecithin was followed in ethereal solutions exactly as described by (Long & Penny, 1957).

In the experiments in which the fatty acids which were released by the viper venom were investigated, the following procedure was adopted. To a sample of purified plasma lecithin (40 mg.) dissolved in 20 ml. ether at pH 7.2, was added 300 µg of the venom dissolved in 0.1 ml. water. The reaction was stopped by the addition of 20 ml. ethanol. The sample was then taken to dryness on a rotary evaporator and the residue was taken up in ether. Traces of lysolecithin were removed by filtering the ether extract. The ether was then evaporated off and the

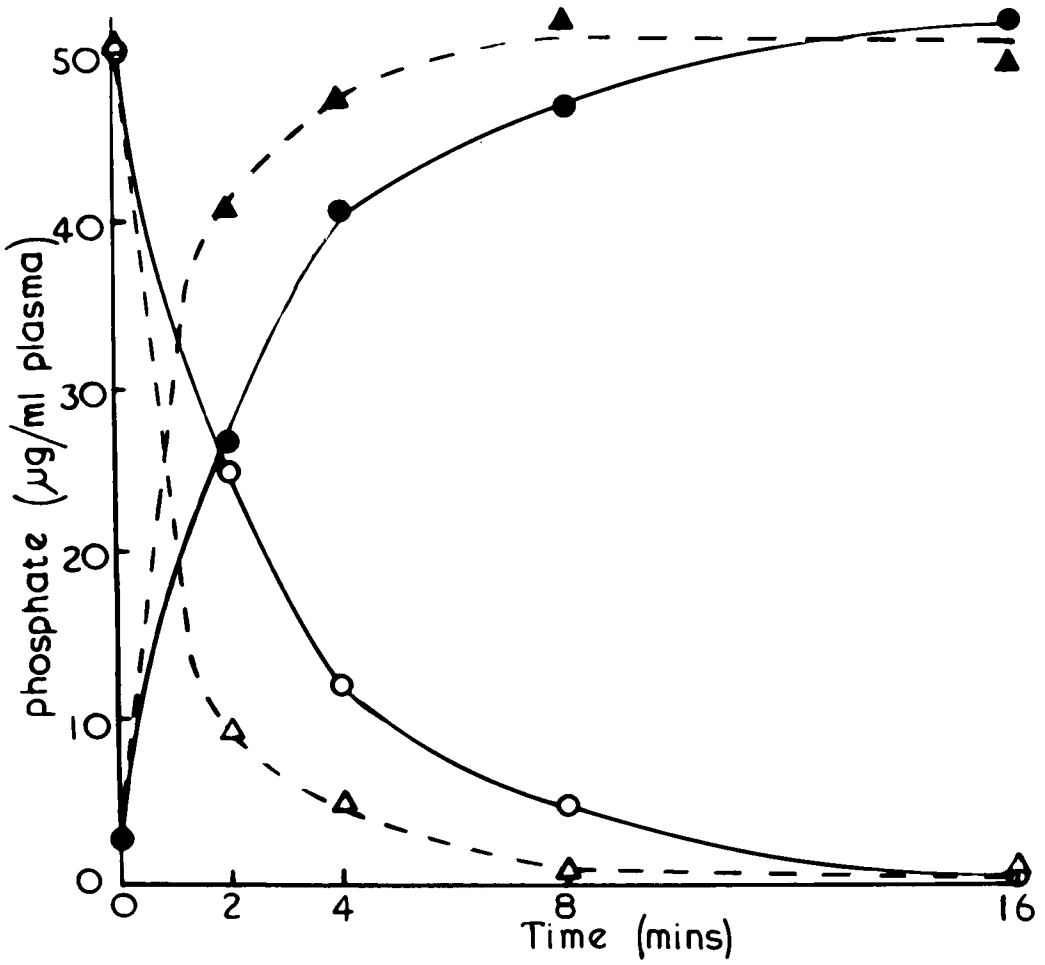


Fig. 5.2.

The hydrolysis of native plasma lecithin by Russell's viper venom O—O, and by cobra venom Δ---Δ, to give lysolecithin ●—● (viper), ▲---▲ (cobra).

residue was redissolved in petrol ether. The fatty acids were separated from the unchanged lecithin by shaking the petrol ether extract with an equal volume of 0.5% potassium carbonate in 50% methanol. When the two phases had separated the methanol layer was run off, this was acidified with HCl. and the fatty acids were recovered by reextraction into petrol ether. The fatty acids present in this extract were identified by gas-liquid chromatography. This was kindly undertaken by Dr. G.S. Getz.

RESULTS

It will be seen in (Fig. 5.2.), that both the cobra and the Russell's viper venom rapidly hydrolysed the native plasma lecithin to lysolecithin, and that this reaction was complete after 16 min. It was immediately apparent from this result that the slow rate at which glycerylphosphorylcholine was formed in plasma during its incubation with Russell's viper venom, was not limited by the rate at which the lecithin was hydrolysed. One explanation for the low yield of glycerylphosphorylcholine may be that only a small proportion of the lysolecithin formed could be hydrolysed further to glycerylphosphoryl-choline. Since viper venom was such an active coagulant

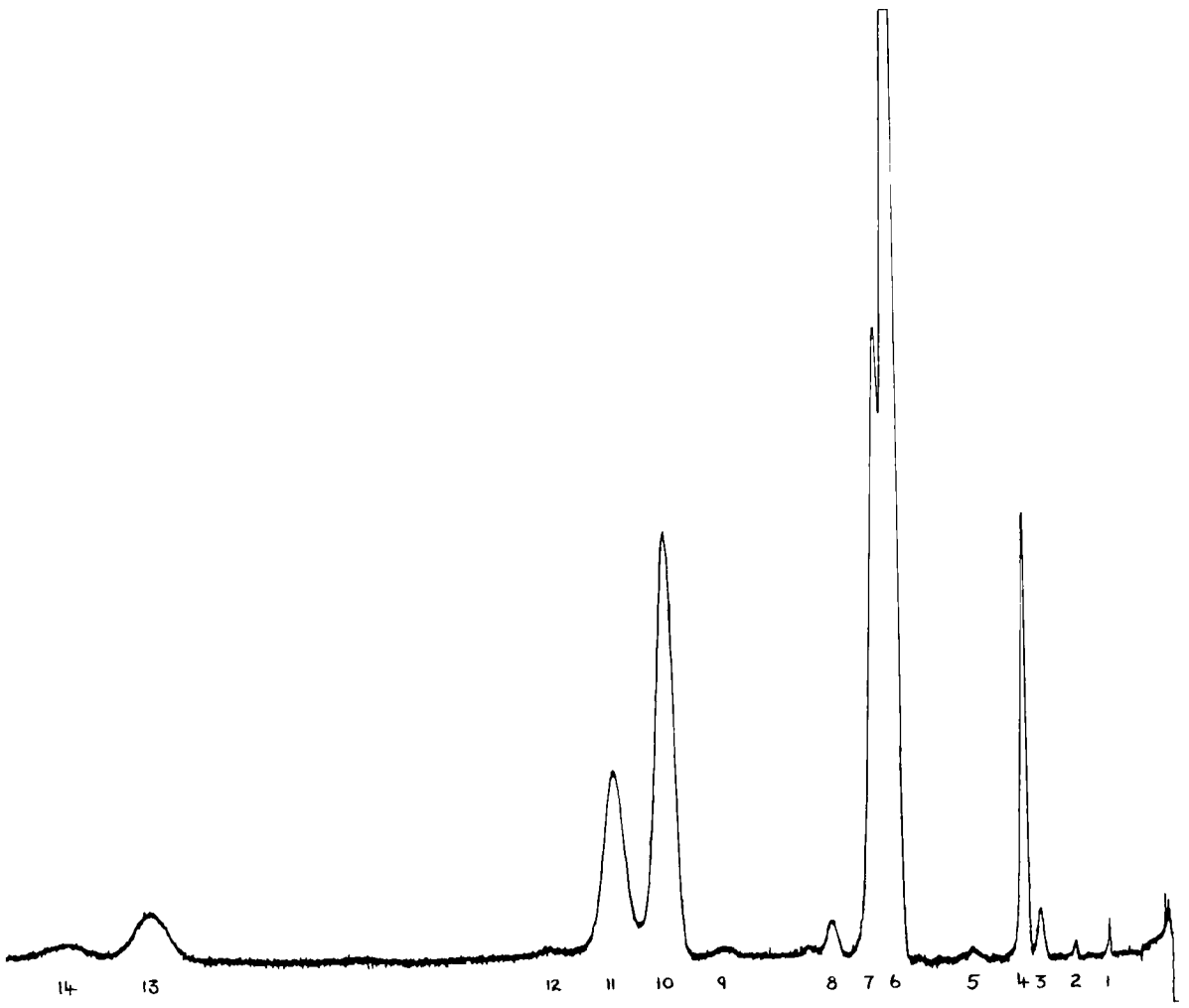


Fig. 5.3.

The separation of the fatty acids released from purified plasma lecithin by Russell's viper venom, by gas-liquid chromatography. The fatty acids are listed in (Table 5.1.).

TABLE 5.1.

The fatty acids released from purified lecithin during its incubation with Russell's viper venom.

PEAK No.	FATTY ACID		% COMPOSITION 5 mins.	10 mins.	30 mins.
1	C_{14}-0	myristic	0.15	0.14	0.13
2	C_{15}-0		-	0.13	0.16
3	C_{16}-1	palmitoleic	0.75	0.76	0.72
4	C_{16}-0	palmitic	6.3	5.6	7.15
5	C_{17}-0		0.32	0.27	0.40
6	C_{18}-2	linoleic	36.6	35.0	35.4
7	C_{18}-1	oleic	24.0	24.9	23.4
8	C_{18}-0	stearic	1.07	1.04	1.80
9	? C_{19}	(branched)	1.18	0.52	0.50
10	C_{20}-4	arachidonic	15.5	17.8	15.2
11	C_{20}-3		10.5	9.4	7.8
12	C_{20}-2		-	0.69	0.78
13	C_{22}-6		2.42	3.44	4.90
14	C_{22}-5		1.43	0.69	1.80

Subscript refers to the number of carbon atoms. Figures denote the number of unsaturated bonds in the molecule.

Table 5.2.

The lecithinase activity of Russell's viper venom.

Incubation time (hrs.)	Phosphate : Ester ratio
0	1:1.99
0.16	1:1.71
0.33	1:1.45
0.5	1:1.23
1.0	1:1.12
1.5	1:1.01
2.0	1:1.03
6.0	1:1.02
18.0	1:1.01

10 µg venom incubated with 2 µ moles purified lecithin.

it was possible that a specific lecithin was hydrolysed as soon as the venom was added to the plasma.

To test this hypothesis the fatty acids released from purified plasma lecithin were investigated at different times during the incubation with viper venom. The times chosen were 5, 10, and 30 min. The elution diagram for the fatty acids present in the petrol ether extracts is given (Fig. 5.3.). It will be seen (Table 5.1.) that plasma lecithin is composed of a mixture of lecithins. There was, however, no indication of a specific hydrolysis of any one of the lecithins. It may be concluded therefore, that if Russell's viper venom has a specific action on the lecithin in the plasma, then the specificity must be influenced by other factors not determined by the structure of the lecithin itself.

These experiments clearly demonstrate that Russell's viper venom has a potent lecithinase activity, but so far the possibility that viper venom might also convert lysolecithin to glycerylphosphorylcholine had not been excluded. To investigate this, samples of purified lecithin were incubated with the venom for periods up to 18 hrs. It will be seen (Table 5.2.) that the phosphate:ester ratio never fell below 1:1.01, which shows that under these

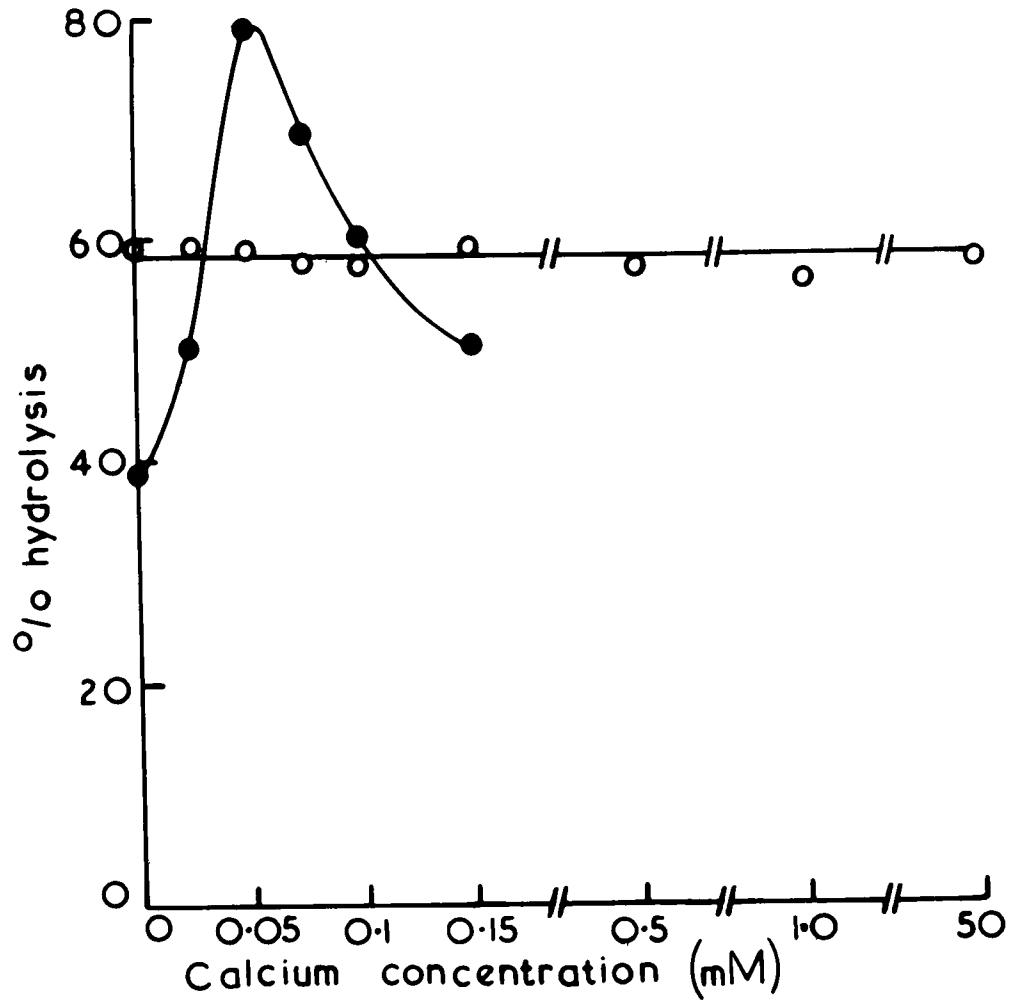


Fig. 5.4.

The effect of the calcium concentration on the hydrolysis of purfied plasma lecithin (2 μ moles) by cobra venom ●——●, and Russell's viper venom O——O.

TABLE 5.3.

The effect of E.D.T.A. and calcium on the lecithinase activity of Russell's viper venom.

E.D.T.A. μmoles $\times 10^2$	Ca^{++} μmoles $\times 10^2$	acyl ester μ moles
-	-	4.0
-	0.5	4.0
-	3.5	4.03
1	-	3.06
2	-	3.14
1	3.5	4.63
2	3.5	4.58

Ethelyene diamine tetraacetic acid (E.D.T.A.) was neutralized to pH 7.2.

10 μ. venom incubated with 2.33 μ moles purified lecithin for 30 mins.

conditions the viper venom was unable to hydrolyse lysolecithin. This would suggest that the production of glycerylphosphorylcholine during the incubation of plasma with venom was due to an enzyme in the plasma.

Long & Penny, (1957) found that the rate at which cobra venom hydrolysed ovolecithin was increased by optimal amounts of calcium. These experiments were repeated using purified plasma lecithin as the substrate and the activity of cobra venom was compared with that of Russell's viper venom. It will be seen (Fig. 5.4.) that whereas the activity of the cobra venom was stimulated by the presence of an optimal amount of calcium, the activity of Russell's viper venom was unaffected by calcium up to a concentration of 50 millimolar.

Long & Penny also showed that ethylenediaminetetraacetic acid (E.D.T.A.) would inactivate cobra venom, and that the venom could be reactivated by the addition of excess calcium ions. However this was not found to be the case with the viper venom. (Table 5.3.) shows that at the concentration of E.D.T.A. chosen the venom was activated, and on the addition of excess calcium ions the venom became inactive. This would suggest that the venom contained diavalent ions other than calcium and that these

can be removed by E.D.T.A. When these ions are replaced by calcium the venom loses its activity.

It was also noticed that aqueous solutions of Russell's viper venom rapidly lost their lecithinase activity when this was assayed in ethereal solutions. This however was not the case when native plasma was used as the substrate, and solutions of the venom were active after storage for several days in the cold.

It can be concluded from the experiments described in this chapter that Russell's viper venom has an unspecific lecithinase activity which is unaffected by calcium ions. Further the appearance of glyceryl-phosphorylcholine in plasma in the presence of viper venom must be due to the activity of an enzyme which is in the plasma.

It is interesting to note that cobra venom was able to hydrolyse native plasma lecithin at the same rate as the viper venom in the presence of a high concentration of calcium ions (25 mM.). This does not compare with the observations made when the lecithinase activity was assayed in an ethereal solution, and it would suggest that the native plasma lecithin is unable to associate with an excess of calcium ions which would inhibit the action of the venom on the lecithin.

CHAPTER VI

THE LYSOLECITHINASE ACTIVITY OF THE PLASMA.

It has been shown that when purified plasma lecithin is incubated with Russell's viper venom lysolecithin is the only product. Therefore the appearance of glycerylphosphorylcholine in the plasma during the incubation of plasma with viper venom must be brought about by the action of a lysolecithinase enzyme in the plasma. Dawson (1956) showed that such an enzyme was present in rat blood and to lesser extent in sheep blood. Marinetti et al (1959), however, have been unable to demonstrate any significant hydrolysis of lysolecithin in human oxalated plasma, even after incubation periods lasting for two hours. Since, during coagulation, there is an apparent hydrolysis of lysolecithin, it may be supposed that this enzyme is activated during coagulation.

METHODS

Preparation of lysolecithin: The lysolecithin used contained ^{32}phosphate and was prepared by the action of cobra venom on plasma lecithin isolated as described in

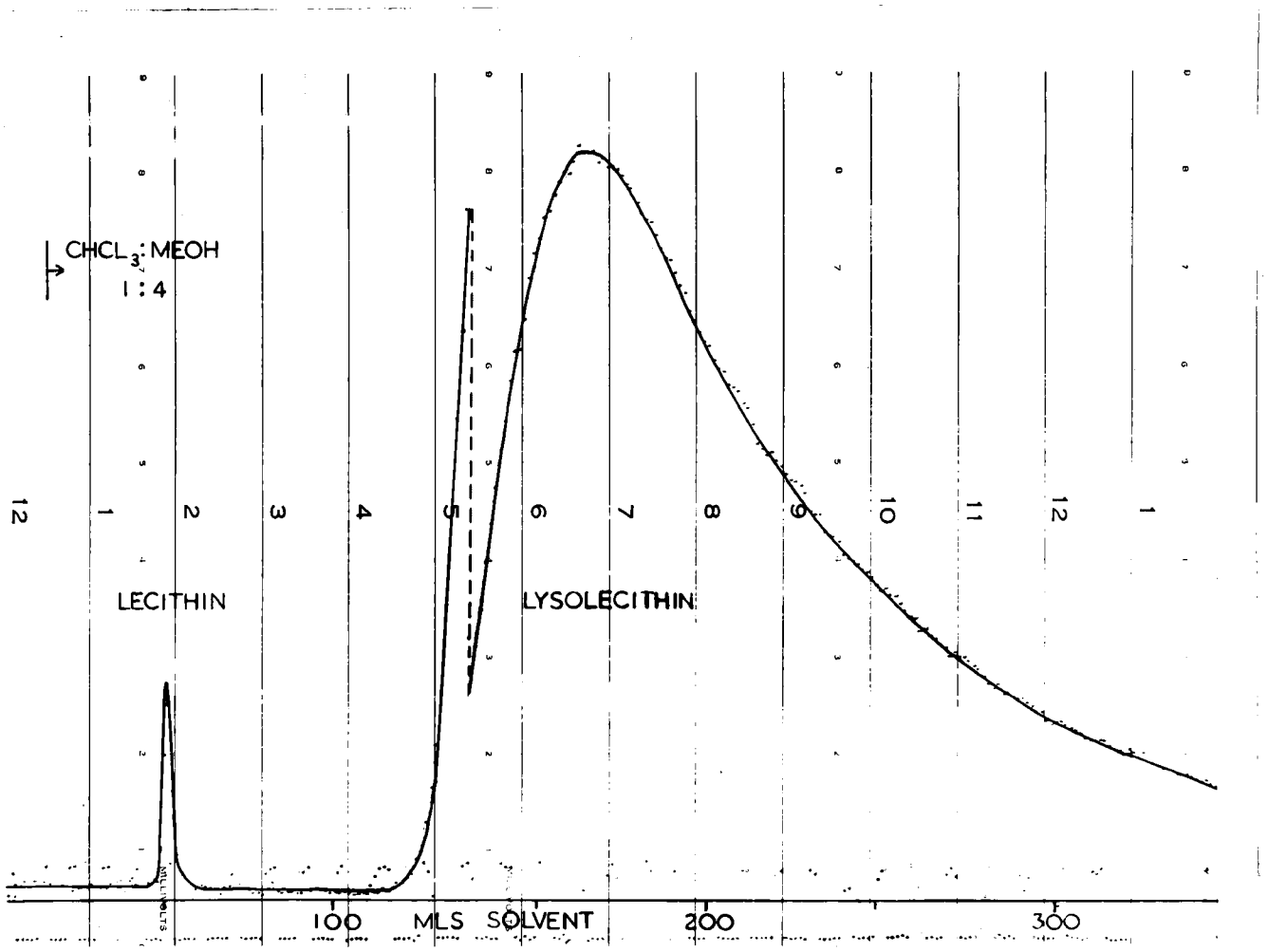


Fig. 6.1.

The separation of lecithin from lysolecithin on a silicic acid column, using a mixture of chloroform and methanol (1:4 v/v). The dotted line indicates a change in the range of the ratemeter.

the previous chapter. Lecithin (260 μ moles) was dissolved in 100 ml. ether, and the pH was adjusted to 7.2 with ammonia. To this was added 2 mg. cobra venom and calcium (0.03 μ moles, Ca^{++}/μ mole lecithin) dissolved in 1 ml. water. The reaction was allowed to proceed over night at room temperature. The ether was then removed and the lysolecithin was dissolved in chloroform, which was filtered to remove a slight turbidity. The lysolecithin was separated from any unchanged lecithin, fatty acids and venom on a silicic acid column (Dittmer & Hanahan, 1959). The silicic acid (1 g. for each 0.5 mg. phospholipid phosphorus) was heated overnight at 110°. After washing with chloroform three times, the silicic acid was poured into a column (2.5 cms. diameter) and allowed to settle. The crude lysolecithin dissolved in a 4:1 chloroform/methanol mixture was applied to the column. The fatty acids were eluted with 4 to 5 column volumes of the solvent. The solvent was then changed to 1:4 chloroform/methanol. The effluent was monitored for radioactivity with a scintillation counter (Fig. 6.1.), and 10 ml. fractions were collected. The fractions which contained lysolecithin were pooled, and concentrated in a rotary evaporator. The lysolecithin was redissolved in

a small volume of chloroform and precipitated by the addition of 6 volumes of ether. The precipitate was redissolved in chloroform and stored at -20°.

Lysolecithinase activity: In preliminary experiments an attempt was made to prepare extracts of plasma which possessed enzymic activity. These extracts were obtained by extracting the precipitate formed by adding acetone to plasma, with a glycerol solution (Dawson, 1956). Unfortunately, no activity was detected in several preparations which were made. Because of this, the lysolecithinase activity was assayed directly in dialysed plasma. The lysolecithin dissolved in chloroform was introduced into the incubation tube, the chloroform was removed in vacuo and the lysolecithin was redissolved in 2 ml. plasma to give a final concentration of 2 μ moles/ml. plasma. The incubation was carried out at 37° in the presence of 0.025M $CaCl_2$. The reaction was stopped by the addition of trichloracetic acid, the acid soluble phosphate was extracted as described in an earlier chapter. Radioactivity and phosphate were determined in these extracts as has been described already. It was found in initial experiments that the acid-soluble extracts were contaminated with small amounts of lysolecithin. This

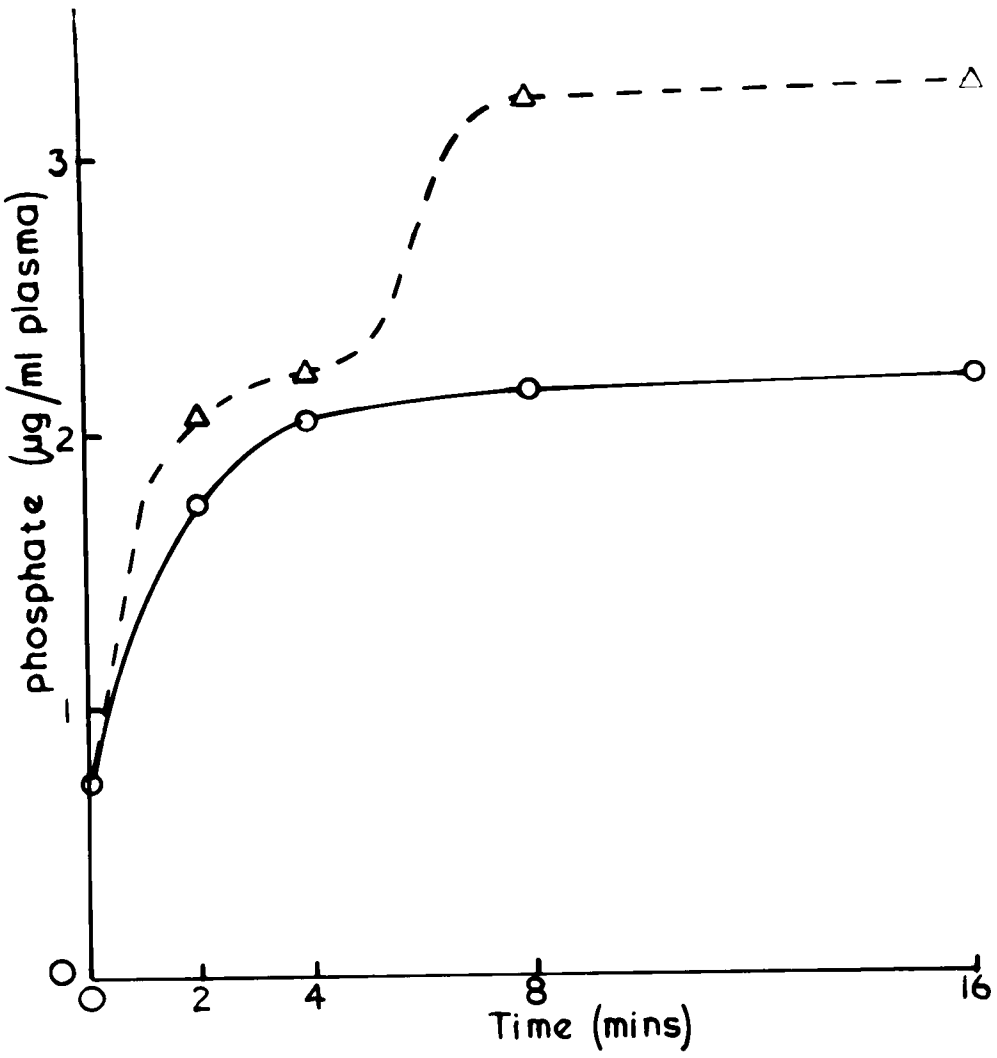


Fig. 6.2.

The release of acid soluble organic phosphate from dialysed platelet-free plasma during the incubation with Russell's viper venom O——O, and cobra venom △--△.

was removed by shaking the extracts with an equal volume of isobutanol, leaving the glycerylphosphorylcholine in the aqueous layer.

The release of glycerylphosphorylcholine: Dialysed plasma was used throughout and was obtained by the procedure already described. The plasma was incubated with Russell's viper venom at a concentration of 10 µg/ml. except where stated. The details of the incubation and of the extraction and estimation of the acid-soluble phosphate are as described previously. Using these techniques the release of glycerylphosphorylcholine could be conveniently followed by measuring the appearance of the acid-soluble organic phosphate.

RESULTS.

It was shown in the previous chapter that when plasma was incubated with Russell's viper venom there was a rapid hydrolysis of plasma lecithin which was complete after 16 mins. In the same experiment the release of glycerylphosphorylcholine was also followed, it will be seen (Fig. 6.2.), that the amount of glycerylphosphorylcholine formed represents the hydrolysis of only 3% of

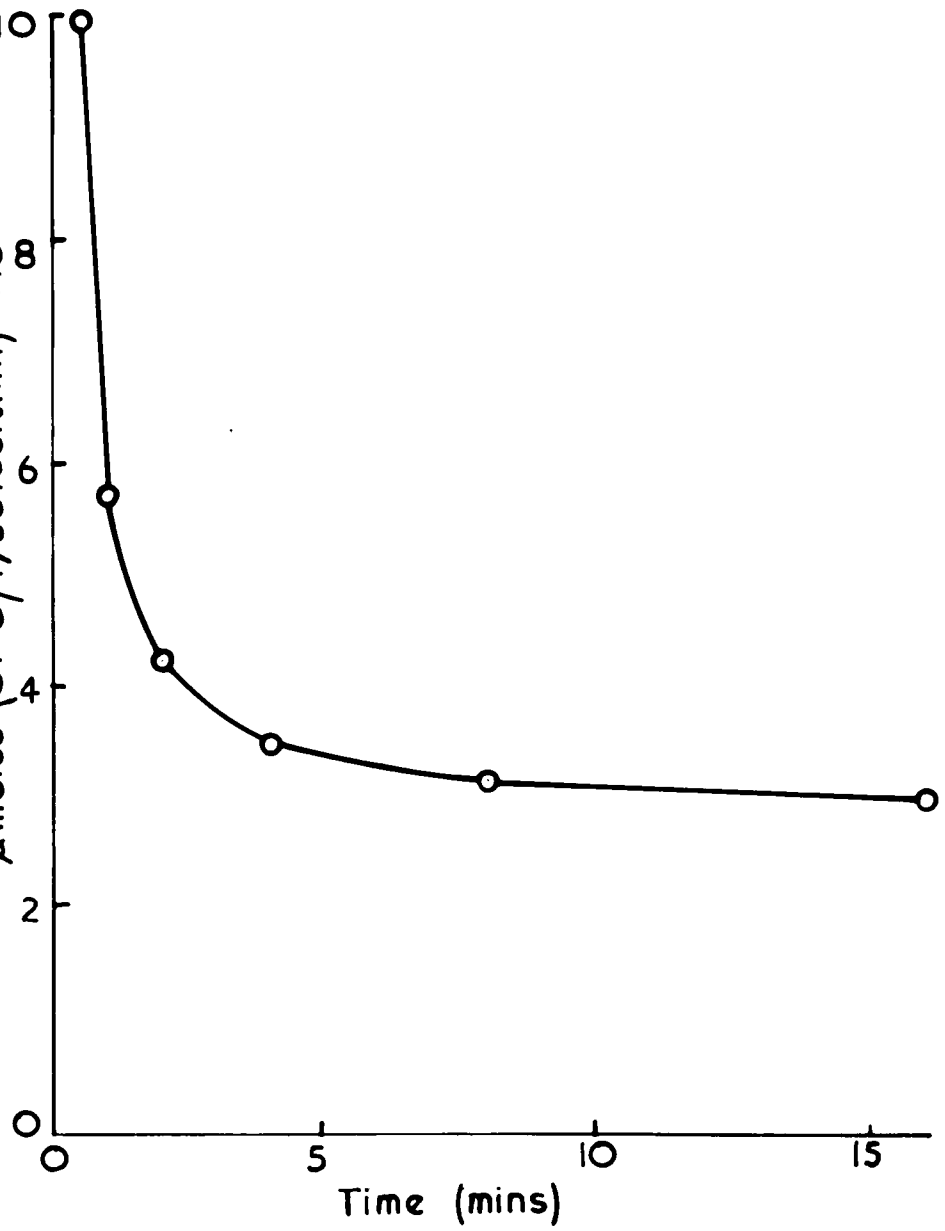


Fig. 6.3.

The ratio of glycerylphosphorylcholine formed to the amount of lysolecithin present in plasma during the incubation with Russell's viper venom.

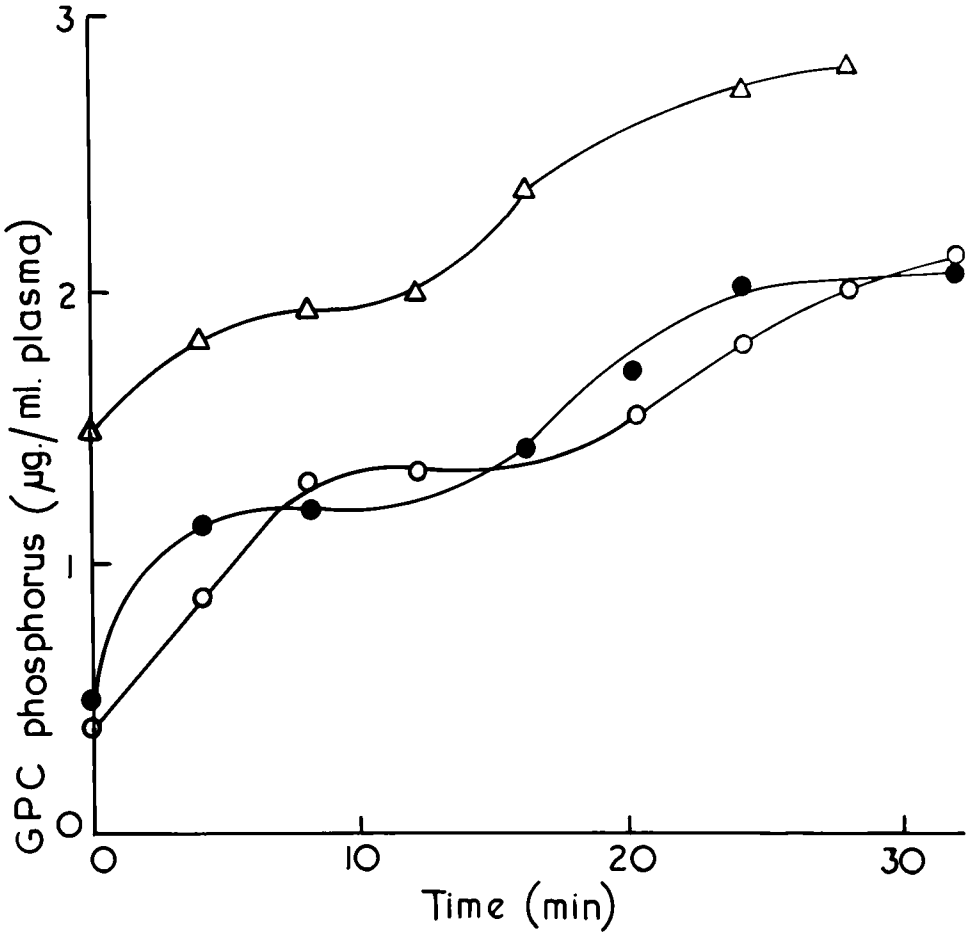


Fig. 6.4.

The release of glycerylphosphorylcholine from platelet-free plasma during the incubation with Russell's viper venom.

the lysolecithin. If the ratio of the amount of glycerylphosphorylcholine formed to the amount of lysolecithin present at any given time is plotted (Fig. 6.3.), it will be seen that there is more glycerylphosphorylcholine formed at low concentrations of lysolecithin than when the concentration of lysolecithin has increased. This may be due to either a rapid loss in activity of the enzyme, or that the reaction is inhibited by an increased concentration of the substrate or the product. It is of interest to observe in this connection that lysolecithin was found to inhibit the coagulation of plasma (Maclagan et al, 1958). This finding has been confirmed; it was observed that when platelet-free plasma was incubated with Russell's viper venom, calcium and lysolecithin (4 μ moles/ml.), the plasma failed to clot within 30 mins. A sample from which the lysolecithin had been omitted clotted in 30 secs.

However, the suggestion that the decrease in the production glycerylphosphorylcholine was due to a loss in the activity of the enzyme, rather than an effect of the lysolecithin concentration, was supported by the observations made when the incubation time of the plasma with venom was extended to 32 min. It was found (Fig. 6.4.) that during the first 10 min. glycerylphosphorylcholine

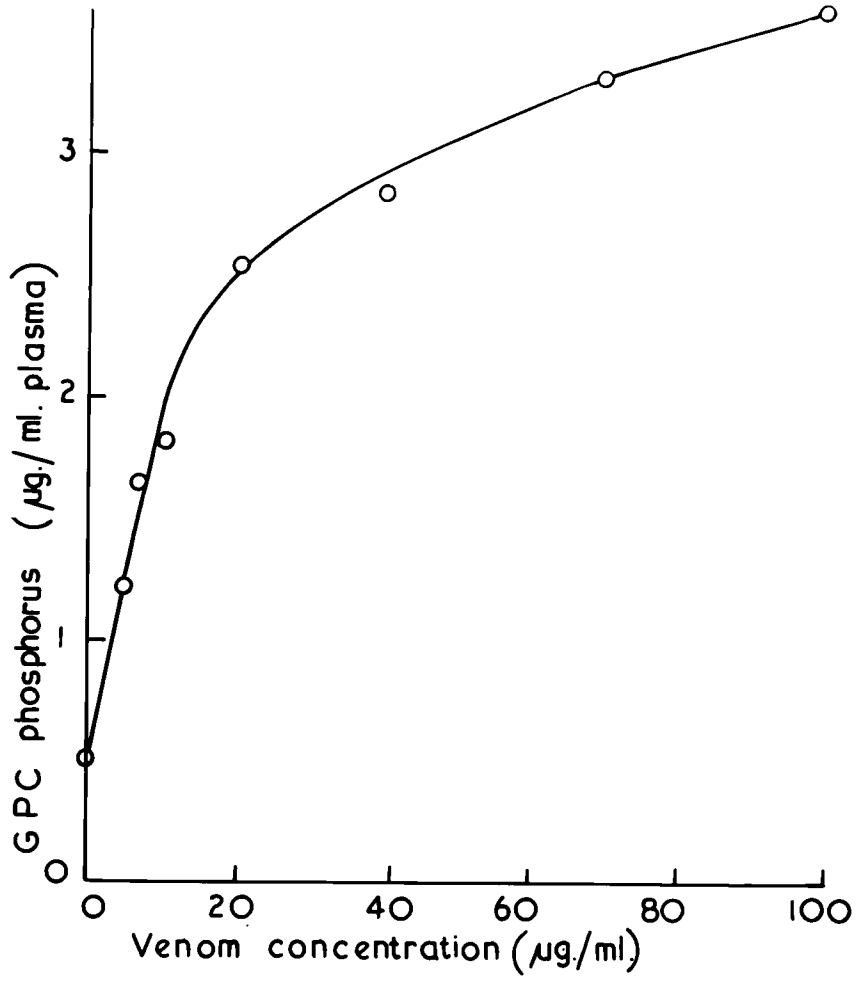


Fig. 6.5.

The effect of increasing the concentration of Russell's viper venom on the amount of glycerylphosphorylcholine released from platelet-free plasma. Incubation time 30 min.

was formed, and that in the next 5 min. no further glycerylphosphorylcholine appeared. This was then followed by the further release of glycerylphosphoryl-choline. It may be concluded that the plasma enzyme loses its activity during the first 10 min., and there follows a period in which the enzyme is reactivated. Since the plasma clots within 1 min. from the addition of the venom and calcium, it is unlikely therefore that the reactivation of the enzyme is directly concerned with the clotting mechanism.

An indication that the viper venom might influence the rate at which glycerylphosphorylcholine was formed was obtained from the following experiment. Plasma was incubated for 30 mins. with calcium and increasing con-centrations of viper venom. It was found (Fig. 6.5.) that up to a venom concentration of 15 µg/ml. the amount of glycerylphosphorylcholine formed increased rapidly with the venom concentration. At higher concentrations of the venom the effect was much less marked. Presumably at the lower venom concentrations the amount of glyceryl-phosphorylcholine which was formed was controlled by the rate at which the lecithin was hydrolysed, while at the higher venom concentrations, the venom stimulated the

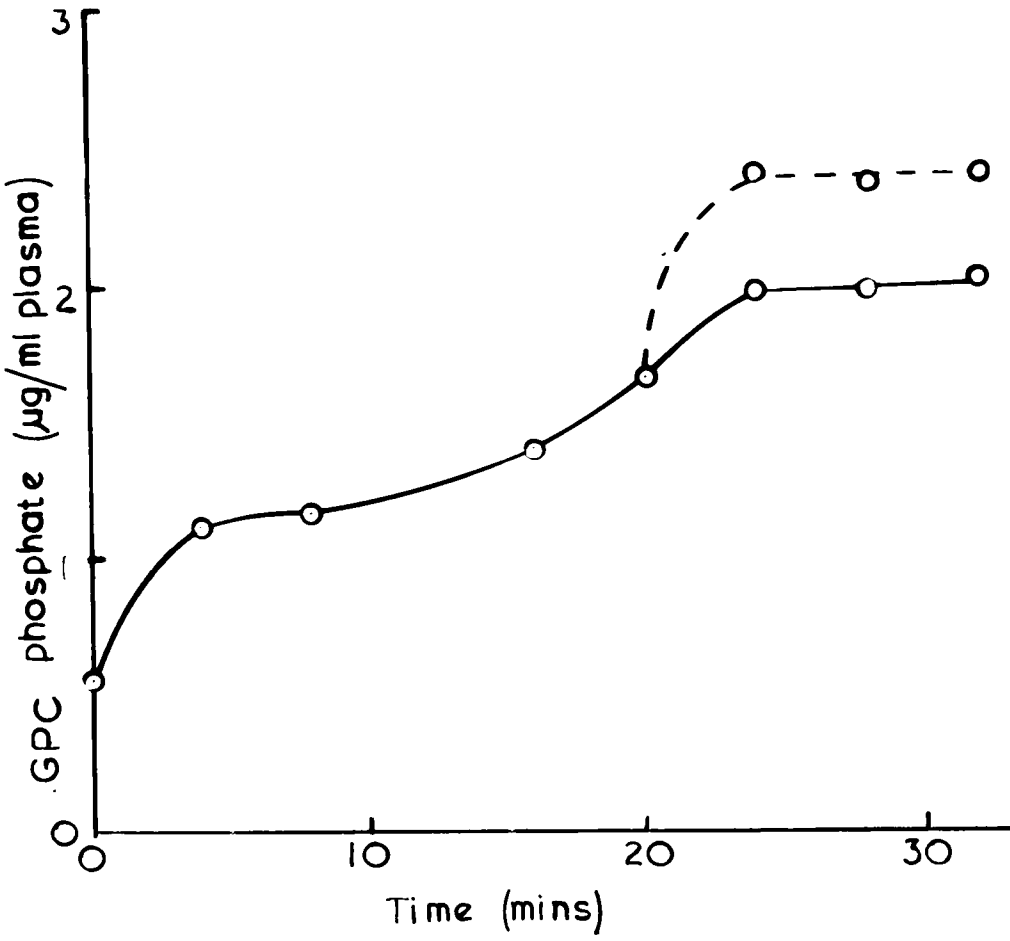


Fig. 6.6.

The release of glycerylphosphorylcholine from platelet-free plasma in the presence of Russell's viper venom (10µ/ml.). The venom concentration was increased to (20µ/ml.) after 20 mins. in the samples indicated by the dotted line.

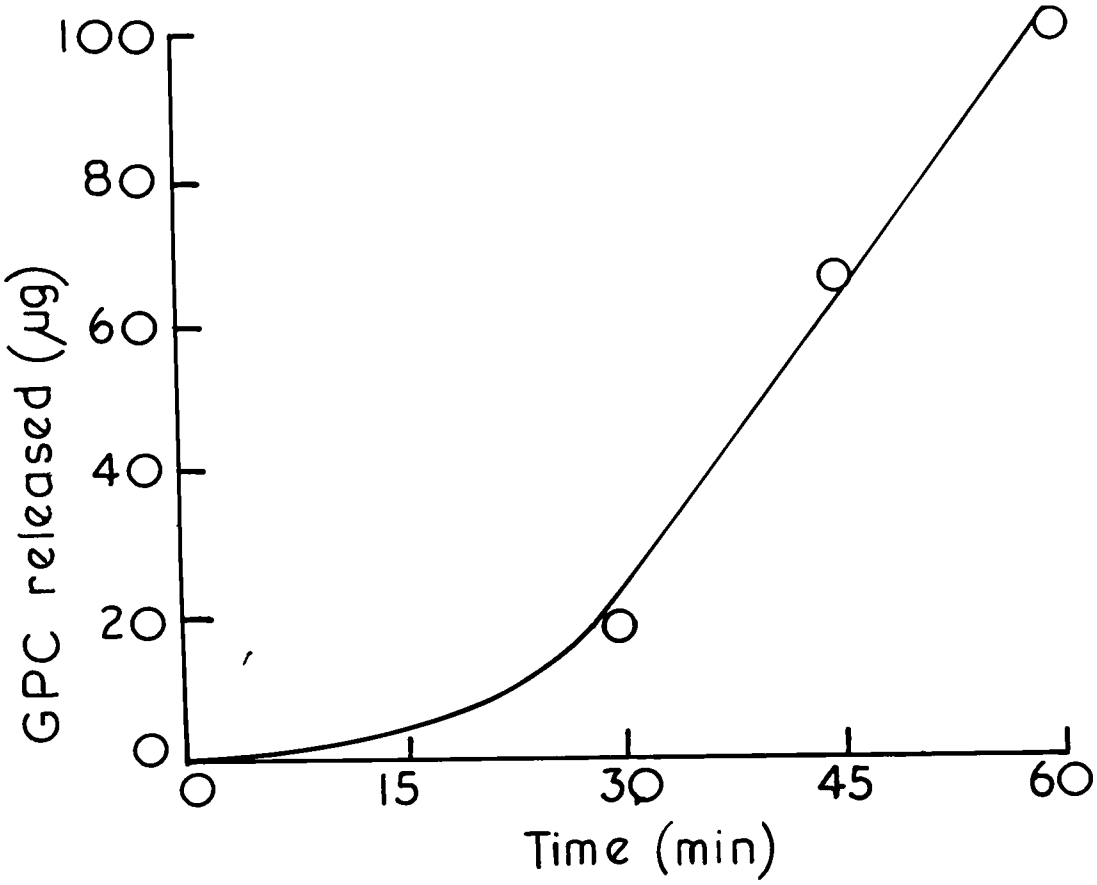


Fig. 6.7.

The appearance of lysolecithinase activity in platelet-free plasma during its contact with a roughened glass surface.

hydrolysis of the lysolecithin by the plasma enzyme. Further support of this idea was obtained in the next experiment, in which plasma was incubated with viper venom (10 µg/ml.) for 20 mins., by which time all the lecithin in the plasma had been hydrolysed. Then the concentration of the venom was increased to 20 µg/ml. and the incubation continued for a further 12 mins. It will be seen (Fig. 6.6.), that the increase in the venom concentration stimulated the production of glyceryl-phosphorylcholine.

An attempt was made next to assay the lysolecithinase activity of the plasma using radioactive lysolecithin as the substrate. When citrated plasma was incubated with the lysolecithin in plastic tubes without added calcium, no enzymic activity was detected, an observation which confirms the findings of Marinetti et al (1959). The release of glycerylphosphorylcholine was not stimulated by the addition of calcium (final strength 0.025M). If, however, the plasma was incubated with the lysolecithin in roughened glass tubes, then lysolecithinase activity gradually developed (Fig. 6.7.) and the maximum activity was obtained after the plasma had been in contact with

the glass for 25 mins. This experiment confirms the hypothesis that plasma has lysolecithinase activity. Although the enzyme is not active under normal conditions, it is activated when the plasma is exposed either to glass, or as it would appear, to Russell's viper venom.

The experiments which have been described so far have demonstrated a mechanism by which glycerylphosphorylcholine can be formed in plasma during its incubation with Russell's viper venom. However, glycerylphosphorylcholine is also formed during the coagulation of platelet-rich plasma, therefore, the plasma must also be capable of hydrolysing lecithin. Dawson (1958) showed that a lysolecithinase enzyme which was obtained from Penicillium notatum, would in the presence of tripalmitin and certain other activating lipids, cleave both the fatty acids from lecithin. A similar enzyme system has been isolated from the intestinal mucosa of the rat by (Epstein & Shapiro, 1957). In order to test whether the plasma enzyme would attack lecithin, plasma which had previously been incubated in roughened glass tubes was incubated at 37° for 30 mins. with calcium and increasing concentrations of a tripalmitin emulsion. This emulsion was prepared by dispersing the lipid in saline containing a trace of "Tween 80" by

TABLE 6.1.

Effect of tripalmitin on the release of organic phosphate and on the clotting time of platelet free plasma.

SAMPLE:	TRIPALMITIN μ moles/ml.	μg org P/ml. plasma	Clotting time (mins.)
Control - Ca^{++}	0	0.24	NO CLOT at 30 mins.
Control + Ca^{++}	0	0.54	NO CLOT at 30 mins.
	4	0.88	26
	16	0.84	24
	32	1.48	22
	64	1.79	18

ultra-sonic vibration. It was found (Table 6.1.) that in the absence of the lipid only a very small amount of acid-soluble phosphate appeared, and that as the concentration of the tripalmitin was increased, not only was there an increase in the amount of acid-soluble phosphate released, which was accompanied by an equivalent release in bound choline, but also the clotting time of the plasma was shortened. These observations suggest that the plasma enzyme in the presence of tripalmitin will release both the fatty acids from plasma lecithin and that the formation of glycerylphosphorylcholine was closely connected with the process of coagulation.

CHAPTER VII.

THE RELEASE OF GLYCERYLPHOSPHORYLCHOLINE AND ITS RELATION TO BLOOD COAGULATION

The experiments which have been described in the previous chapters show that during the clotting of plasma glycerylphosphorylcholine is released. This reaction occurs when the plasma is made to clot with either platelets or Russell's viper venom, and probably with brain extracts. This would suggest that the three thromboplastin generating systems, outlined in the introduction, have at least one stage in common. The experiments which will be described in this chapter are an attempt to identify this stage.

METHODS

The techniques which have been used in this chapter have been described previously.

RESULTS

The possibility that the formation of glyceryl-phosphorylcholine was associated with the conversion of

fibrinogen to fibrin, was excluded by the observation that no acid-soluble organic phosphate or bound choline were released when plasma was made to clot by the addition of thrombin.

Macfarlane (1947), investigating the anticoagulant activity of soya-bean trypsin inhibitor, concluded that the inhibitor depressed the prothrombinase activity, while the prothrombin was not affected. If, therefore, the release of glycerylphosphorylcholine was related to the conversion of prothrombin to thrombin, then the amount of acid-soluble phosphate released would be reduced when the plasma was incubated with the Russell's viper venom in the presence of the inhibitor. It was found that when dialysed platelet-free plasma was incubated with the inhibitor (1mg/ml.) for 2 min., before the addition of calcium (final strength 0.025M) and Russell's viper venom (10 µg/ml.), there was no change in the amount of organic phosphate released during the next 15 min., when this was compared with a control sample of plasma from which the inhibitor had been omitted. The control sample clotted in 15 secs. after the addition of the venom and the calcium, while the plasma which contained the inhibitor did not clot during the 15 min. incubation period. This

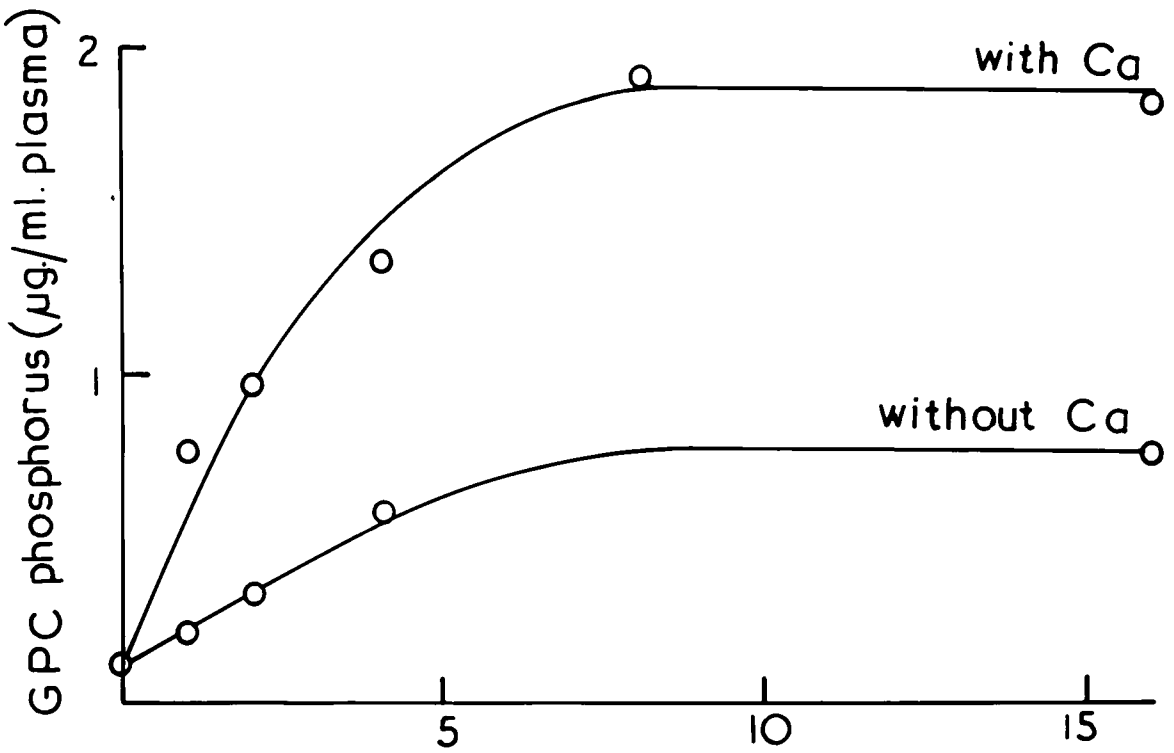


Fig. 7.1.

The effect of calcium on the formation of glyceryl-phosphorylcholine in platelet-free plasma in the presence of Russell's viper venom.

result, taken with the finding that glycerylphosphoryl-choline was liberated before prothrombinase activity could be detected, suggests that the formation of this compound was associated with the early stages of coagulation. Since glycerylphosphorylcholine was released by the three thromboplastin generating systems which have only the formation of thromboplastin in common, it is possible that this chemical reaction is associated with the synthesis of prothrombinase and that glyceryl-phosphorylcholine is liberated as a by-product. Since glycerylphosphorylcholine arises as the result of two consecutive chemical reactions, experiments were carried out to assess the relative importance of each of these reactions to the coagulation of the plasma. It was shown in a previous chapter that when plasma was incubated with Russell's viper venom there was a rapid destruction of the plasma lecithin, it was also found that the lecithinase activity of viper venom was not influenced by calcium and that in the presence of E.D.T.A. is was activated slightly. However, when citrated platelet-free plasma was incubated with viper venom in the absence of added calcium the amount of glycerylphosphorylcholine which appeared was decreased (Fig. 7.1.), its formation was completely

TABLE 7.1.

The effect of incubating plasma with Russell's viper venom in the absence of Ca^{++} on the recalcification time.

Incubation Time (mins.)	Clotting Time (secs.)
2	60
4	66
6	54
8	58
10	59
12	70
14	63
16	69
18	58
20	80
22	83
24	87

Russell's viper venom 10 µg/ml. incubated with platelet-free plasma at 37^{o} for the times shown. 0.2 ml. incubation mixture added to 0.2 mls. 0.025 M $CaCl_2$ and the clotting time recorded.

inhibited by the addition of E.D.T.A. (final strength 0.01M). The requirement for calcium appeared to be specific, since the addition of magnesium (final strength 0.025M) to citrated plasma did not stimulate the production of glycerylphosphorylcholine. It can be concluded from these experiments that the conversion of lecithin to lysolecithin can proceed without the subsequent conversion of the lysolecithin to glycerylphosphorylcholine.

To test whether the coagulation time of plasma would be altered when all the plasma lecithin had been hydrolysed, platelet-free plasma (10 ml.) was incubated with 100 µg of Russell's viper venom at 37° in the absence of calcium. At intervals samples (0.2 ml .) were withdrawn and mixed with an equal volume of a 0.025 M. $CaCl_2$ solution, and the clotting time of the recalcified plasma was recorded. It will be seen (Table 7.1.) that the time at which the plasma clotted did not alter for 18 min., since by this time all the plasma lecithin would have been converted to lysolecithin, the result suggests that one of the fatty acids can be removed from lecithin without affecting the clotting time of the plasma.

This hypothesis is further supported by the finding that plasma will not clot in the presence of added lysolecithin. The lysolecithin presumably acts as an anticoagulant because it would compete with the lysolecithin formed in the plasma for the lysolecithinase enzyme.

It seems likely, therefore, that the hydrolysis of the lecithin is only a preliminary reaction to the more physiologically important hydrolysis of lysolecithin. If this idea is correct then recalcified platelet-free plasma which has been exposed to glass should coagulate after it has been mixed with an enzyme which will hydrolyse lecithin. Cobra venom was chosen as a convenient source of lecithinase activity. Although Mellanby (1909) had found that this venom inhibited the clotting of blood, the concentrations he used were of the order of 500 µg/ml. It was hoped that by using more dilute preparations of the venom its anticoagulant effect would be reduced sufficiently to allow the plasma to clot. It had already been shown (Fig. 5.2.) that when radioactive platelet-free plasma, which had been dialysed, was incubated with cobra venom (10 µg/ml.) there was a rapid hydrolysis of the lecithin. In the same experiment the release of acid-

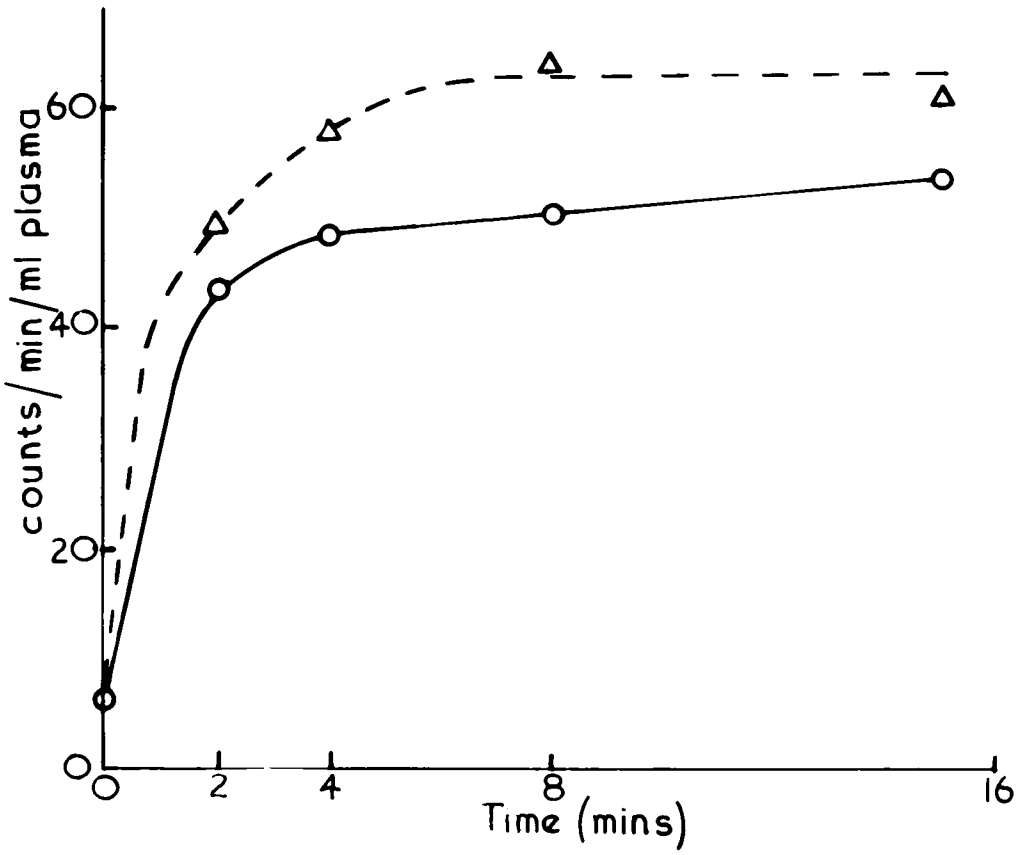


Fig. 7. 2.

The appearance of radioactivity in the acid-soluble organic phosphate fraction of platelet-free plasma, which contained ^{32}P, during the incubation with Russell's viper venom O—O , and cobra venom Δ--Δ .

soluble radioactive and organic phosphate was also determined, this was compared with observations made with Russell's viper venom (10 µg/ml.). It will be seen (Fig. 7.2.), that almost the same amount of radio-activity was released in plasma incubated with the cobra venom as with the viper venom, but when the amount of organic phosphate which was released was determined it was found (Fig. 6.2.) that in the case of the cobra venom some phosphate appeared which was not radioactive. However, the plasma which had been incubated with the cobra venom clotted after 3 mins.; a control sample which contained no venom clotted after $2\frac{3}{4}$ hours. The plasma which contained the viper venom clotted after only 30 secs. The slightly longer clotting time obtained with the cobra venom was most probably due to its inhibitory activity. The fact that cobra venom has other actions on the plasma is clearly shown by the release of the non-radioactive phosphate, which must have arisen from some substance in the plasma which had a low turnover rate.

These observations strengthen the hypothesis that platelet-free plasma which has been exposed to glass will coagulate in the presence of calcium ions and an enzyme which will hydrolyse lecithin.

CHAPTER VIII.

DISCUSSION

It has been shown that whenever plasma is made to clot glycerylphosphorylcholine is released, and that this reaction starts immediately the plasma is recalcified. Rouser et al (1958) showed that the clotting time of plasma was unaffected by the addition of glyceryl-phosphorylcholine, which suggests that it is an inactive by-product formed during the degradation of either lecithin or lysolecithin, both of which are present in the plasma. (Phillips, 1958 and Marinetti et al, 1959).

Experiments have shown that Russell's viper venom can only convert lecithin to lysolecithin, thus the appearance of glycerylphosphorylcholine in the plasma can only be explained by the presence of lysolecithinase activity in the plasma. Although both sheep and rat blood have been shown to possess this activity (Dawson, 1956), attempts to detect this enzyme in human plasma have failed (Marinetti et al, 1959). At first sight it seems peculiar that plasma should contain this enzyme, since the plasma already contains lysolecithin. This anomaly can be explained by supposing that either the native lysolecithin

is in an unreactive state, or that the enzyme only becomes active when the plasma is exposed to a suitable surface. Both these interpretations may be correct, lysolecithinase activity was only detected when the plasma was incubated in roughened glass tubes, and that even when the enzyme was activated there was no significant release of glycerylphosphorylcholine in the absence of added lysolecithin.

Evidence has also been obtained which suggests that Russell's viper venom is also able to activate this enzyme: Because the release of glycerylphosphorylcholine was stimulated by the addition of venom to the plasma at a time when all the lecithin in it had been converted to lysolecithin.

As lysolecithin is the intermediate product formed during the degradation of lecithin to glycerylphosphoryl-choline, it has not yet been possible to determine whether the glycerylphosphorylcholine is formed from the native lecithin or lysolecithin. However, since only small amounts of glycerylphosphorylcholine are formed in platelet-free plasma in the presence of calcium, when the lysolecithinase enzyme has been activated, it would

suggest that the enzyme was unable to hydrolyse the lysolecithin already present in the plasma, and that the glycerylphosphorylcholine was formed from the lysolecithin produced by the action of the venom.

It has been shown that Russell's viper venom acts as a nonspecific lecithinase and that plasma has lysolecithinase activity, thus the mechanism by which glycerylphosphorylcholine is formed during the incubation of plasma with venom is explained. However, as glycerylphosphorylcholine is also formed during the incubation of platelet-rich plasma in the presence of calcium, there must be an alternative mechanism. Dawson (1958) had already shown that an enzyme obtained from P. notatum which hydrolysed lysolecithin, could in the presence of certain activating lipids, of which tripalmitin was an example, cleave both the fatty acids from lecithin. It was observed that when platelet-free plasma was incubated with calcium and increasing amounts of tripalmitin, there was an increase in the amount of glycerylphosphorylcholine formed and at the same time there was a decrease in the coagulation time of the plasma. From this it would appear that under certain conditions the plasma itself can exhibit

both lecithinase and lysolecithinase activities. It may be, therefore, that in platelet-rich plasma the platelets are the source of activating lipid, which is most probably platelet factor 3 (Johnson et al., 1958).

Bangham & Dawson, (1959), and Dawson & Bangham, (1959) have investigated the mechanism by which the various lipids activated the P. notatum enzyme. To do this they studied the reaction of the purified enzyme on an emulsion of lecithin. They found that the same enzyme seemed to be responsible for the hydrolysis of both lecithin and lysolecithin, and that the activating lipids were able to modify the surface charge on the lecithin particles, in such a way as to allow the enzyme to approach them.

In plasma lecithin does not exist in the form of insoluble particles. Instead the phospholipid is associated with protein and water to give a water soluble complex molecule, which is thought to be spherical in shape. On the surface of the molecules are exposed the peptides and the polar part of the phospholipids (Oncley, 1954). Since the surface of the phospholipoprotein complex is not the same as that of the lecithin micelle, it is reasonable to suppose that the activation of the native plasma lecithin would be achieved by different lipids

than were found by Dawson (1958) to activate the lecithin. Moreover the P. notatum enzyme was maximally active at pH 3.0, whereas the plasma system has a pH of about 7.4; this difference in the hydrogen-ion concentration could also affect the nature of the activating lipid.

If this hypothesis is correct, then the activation of the plasma lecithin may be brought about by the phospholipids which have been described by Rouser et al (1958) and Wallach et al (1959), during the initial stages of the coagulation of plasma.

The activating effect of ethanolamine phosphatide on the action of Russell's viper venom on plasma, may also be explained in terms of the activation of the plasma lecithin.

The coagulant activity of tissue extracts (Chargaff et al, 1942) is more difficult to interpret. However since it has been shown that their activity is partly due to the phospholipid which they contain (Chargaff et al, 1944), it is possible that one or other of the phosphatides activates the plasma lecithin. It is also possible that the heat labile protein factor (Quick et al, 1952), may activate the lysolecithinase enzyme in the plasma.

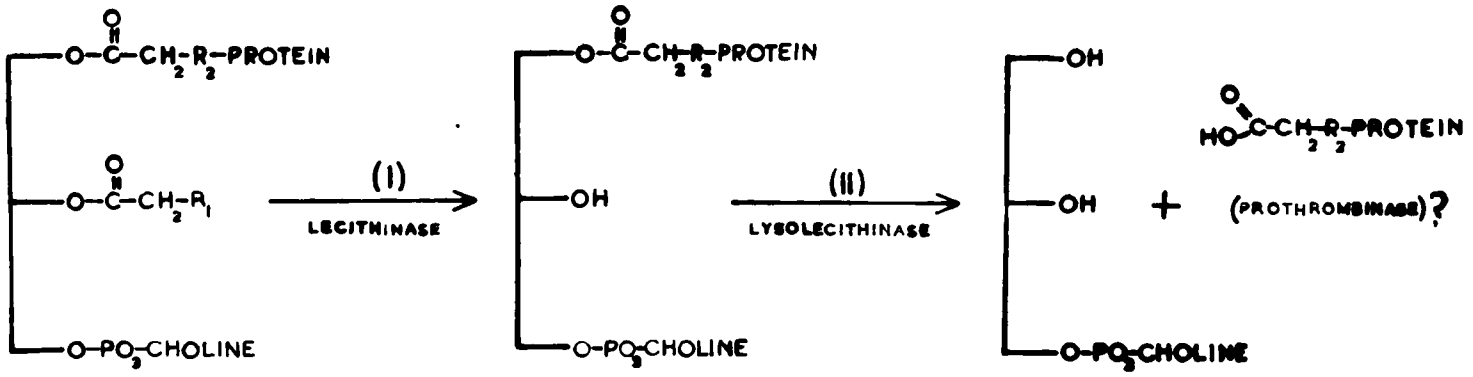


Fig. 8.1.

(R_1 and R_2 are fatty acid residues.)

Any interpretation of the physiological significance of the observations which have been made in this thesis, must take into account the rapidity with which the prothrombin converting substance is formed, especially in the presence of Russell's viper venom. The results which have been obtained show that the processes which lead to the formation of glycerylphosphorylcholine are closely associated in time with the appearance of prothrombinase activity. One possible interpretation may be that a latent prothrombin converting substance exists in the plasma as a protein-lecithin complex. This complex is activated by the removal of lysolecithin to give a protein-lipid complex which will convert prothrombin. This reaction is given in diagramatic form (Fig. 8.1.). It has been shown that reaction (I) can proceed without any loss in the coagulant activity of the plasma, and that whenever the plasma clots it is accompanied by the formation of glycerylphosphorylcholine (II).

Nour-Eldin & Wilkinson (1956) claim to have isolated from plasma during its coagulation a substance which will convert prothrombin to thrombin. They have shown that this contains lipid and protein but no

phosphate, an observation which is in agreement with the reaction proposed above. However, in experiments not described in this thesis, attempts were made to repeat the isolation procedure as described by these authors. Unfortunately only 5% of the original prothrombinase activity was recovered from the plasma. A similar result was also obtained by Bidwell (personal communication). Consequently the properties of the substance obtained by Nour-Eldin & Wilkinson (1956) must be regarded with caution.

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