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The Formation of Glycerylphosphorylcholine during the Clotting of Plasma

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It has been known since the experiments of Wooldridge (1886) that tissue juices are capable of accelerating the coagulation of blood. The active principle in these extracts is a complex of phospholipid and protein (Howell, 1935), and the phospholipid portion of the complex is essential to the activity of the protein (Cohen & Chargaff, 1940). Since then much work has been carried out on the effect of various added phospholipids on the coagulation of plasma.

The purpose of the work described in this paper was to see whether the plasma phospholipids themselves undergo any chemical change during clotting. In most of the experiments Russell's viper venom has been used to coagulate the plasma. It has been shown (Macfarlane & Barnett, 1934) that when this venom was added to plasma in the presence of calcium ions there is a rapid conversion of prothrombin into thrombin.

taining sodium citrate (final concn. 0.38%). The supernatant plasma was centrifuged at 25 000g for 15 min. to remove most of the platelets. This plasma was first dialysed for 2 hr. against Krebs-Ringer phosphate (Krebs & Henseleit, 1932), which contained sodium citrate (final concn. 0.38%), to reduce the specific activity of the exchangeable phosphate. The plasma was then dialysed for 18 hr. in the cold against two changes of a modified Krebs-Ringer bicarbonate solution. The solution had the following composition (μ moles/l.): NaCl, 119; KCl, 4.8; CaCl_2 , 2.6; MgSO_4 , 1.2; NaHCO_3 , 25; sodium citrate, 1.3; $\text{N}_2 + \text{CO}_2$ (95:5) was bubbled through the solution, which had a pH of 7.5.

Platelets. These were obtained from freshly drawn venous blood. The blood was centrifuged at 500g for 20 min. at 0–2° to sediment erythrocytes and white cells. The plasma was then centrifuged at 25 000g to obtain a pellet of platelets. The platelets were resuspended in the dialysed plasma.

Russell's viper venom. The dried venom was a gift from the Wellcome Physiological Research Laboratories.

Methods

Clotting of the plasma. The dialysed plasma (usually 5 ml.) was mixed with fresh platelets derived from the same volume of non-radioactive plasma, and incubated in roughened glass tubes for 10 min. at 37°. The plasma was then transferred to plastic tubes and incubated at 37° with Ca^{2+} ions (0.025 ml. of m-CaCl_2 /ml. of plasma). The plasma clotted about 135 sec. after the addition of the Ca^{2+} ions. In the experiments in which viper venom was used, platelets were not added to plasma but, except where stated, the plasma was incubated with venom (10 μ g./ml. of plasma) which was added at the same time as the Ca^{2+} ions. The plasma usually clotted 30 sec. after the addition of the venom.

Determination of phosphorus. The acid-soluble phosphate

MATERIALS AND METHODS

Materials

Plasma. Blood was withdrawn from patients with polycythaemia vera who had received a single injection of 5–10 mc of [32 P]phosphate. Preliminary experiments showed that plasma phospholipids reached their maximum specific activity about 50 hr. after the injection of the labelled phosphate. The blood was obtained by venepuncture and collected in plastic centrifuge tubes con-

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was extracted from plasma by the addition of 0.2 vol. of ice-cold 60% (w/v) trichloroacetic acid. The mixture was dispersed in an homogenizer and centrifuged at 25 000g for 10 min. at 0–2°. The residue was re-extracted with cold 10% (w/v) trichloroacetic acid and centrifuged as before. The clear supernatants were filtered to remove any traces of the precipitated protein. Organic phosphate was converted into inorganic phosphate by ashing a dried sample of the extract with 0.1 ml. of 5% (w/v) ammonium molybdate and 0.5 ml. of a concentrated H_2SO_4 –70% $HClO_4$ (2:3, v/v) mixture (Werkheiser & Bartley, 1957). Inorganic phosphorus was determined by the method of Ennor & Stocken (1950). The '10 min. phosphorus' was defined as the inorganic phosphorus released from organic compounds after hydrolysis in $N-HCl$ at 100° for 10 min.

Measurement of radioactivity. This was carried out in a liquid-counter tube (M.6 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 phosphorus compounds were chromatographed on Whatman no. 1 paper which had been washed in 2*N*-acetic acid (Dawson, 1954). The solvents used were phenol–ammonia (Dawson, 1954) and ethanol–ammonia (Dawson, 1955). Spots which contained [^{32}P]phosphate were detected by scanning the papers with an end-window counter (EHM 2/S of General Electric Co.). A sample of glycerylphosphorylcholine was kindly given by Dr R. M. C. Dawson.

Determination of glycerylphosphorylcholine. The glycerylphosphorylcholine was hydrolysed in a sealed tube with 2*N*-HCl for 18 hr. at 126°. After the hydrolysis a sample was removed for the estimation of inorganic phosphorus. 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 1.5*N*. A third sample of the hydrolysate was dried *in vacuo* over P_2O_5 and KOH pellets. Free choline was estimated in this sample by the iodine-precipitation method of Smits (1957).

Determination of lecithin and lysolecithin in plasma. The lecithin and lysolecithin were separated on silicic acid-impregnated papers by the method of Marinetti, Ford & Stotz (1959). The amount of each phosphatide present was determined from the phosphorus content of the spots after they had been eluted from the paper.

Action of venom on purified plasma lecithin. The lecithinase activity of the viper venom was followed in ethereal solutions as described by Long & Penny (1957), with human plasma lecithin as substrate.

Human plasma lecithin. The phospholipids were obtained from fresh human citrated plasma by extraction into 15 vol. of a chloroform–methanol (1:1, v/v) mixture. This extract was shaken with an equal volume of distilled water and the chloroform layer was concentrated in a rotary evaporator at 20°. The lecithin was recovered from the extract by chromatography on a silicic acid column (Hanahan, Dittmer & Warashina, 1957). The non-phosphorus-containing lipids were removed by prolonged elution of the columns with chloroform before the phospholipids were fractionated. The fractions which contained the lecithin were combined, and evaporated in a rotary evaporator at 20°. The residue was redissolved in a small volume of peroxide-free ether and stored overnight at –5°. The precipitate which formed was removed by filtration and the ethereal solution was adjusted to pH 7.2 with ammonia vapour. The phosphate:acyl ester ratios for several preparations varied from 1:1.99 to 1:1.95.

RESULTS

When plasma containing [^{32}P]phosphorus compounds was clotted in the presence of platelets, there was an increase in the radioactivity of the total acid-soluble phosphorus. The results of four such experiments are summarized in Table 1. This increase in radioactivity appeared in the stable organic phosphorus fraction, i.e. the total phosphorus less both '10 min. phosphorus' and inorganic phosphorus. It was not possible to correlate the increase in radioactivity with an increase in stable organic phosphorus.

If the liberation of the radioactivity were the result of a reaction taking place during coagulation, it would be reasonable to suppose that the same material would be released when plasma was clotted under the influence of viper venom. Table 2 shows that, under these circumstances, there was an increase in both the radioactivity and the net

Table 1. *Appearance of radioactivity and organic phosphorus in the acid-soluble phosphorus fraction during the coagulation of platelet-rich plasma*

In Expts. 1–4*a*, results are for total phosphorus less inorganic phosphorus. In Expt. 4*b*, results are for total phosphorus less '10 min. phosphorus' and inorganic phosphorus. The results of Expts. 4*a* and 4*b* are taken from the same experiment. The specific activity is expressed in counts/min./μg. of phosphorus, and the phosphorus concentration in μg./ml. of plasma.

Time (min.)	Expt. 1		Expt. 2		Expt. 3		Expt. 4 <i>a</i>		Expt. 4 <i>b</i>	
	Sp. activity	Concn. of P	Sp. activity	Concn. of P	Sp. activity	Concn. of P	Sp. activity	Concn. of P	Sp. activity	Concn. of 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

Table 2. *Appearance of radioactivity and organic phosphorus during the incubation of plasma with Russell's viper venom*

Results are for total phosphorus less '10 min. phosphorus' and inorganic phosphorus. The specific activity is expressed in counts/min./ $\mu\text{g.}$ of phosphorus, and the phosphorus concentration in $\mu\text{g./ml.}$ of plasma.

Time (min.)	Expt. 1		Expt. 2	
	Sp. activity	Concn. of P	Sp. activity	Concn. of P
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

Fig. 1. Effect of added Ca^{2+} ions on the release of acid-soluble organic phosphorus from platelet-free plasma during the incubation with Russell's viper venom. Platelet-free plasma (5 ml.) was incubated at 37° with viper venom (10 $\mu\text{g./ml.}$) for the times stated. The organic acid-soluble phosphorus was determined in the supernatant after precipitation with 0.2 vol. of 60% trichloroacetic acid. $\circ$, Ca^{2+} ions added; $\bullet$, Ca^{2+} ions absent.

concentration of the stable organic phosphorus. These results suggested that, in the corresponding platelet experiments, other reactions were taking place which made it difficult to detect the appearance of the organic phosphorus. When Ca^{2+} ions were omitted from the incubation mixture, the increase in specific activity of the organic-phosphorus fraction was reduced (Fig. 1). In the absence of venom no release of radioactivity could be detected.

Identification of the acid-soluble phosphorus compound

Samples of the acid-soluble extracts obtained from plasma clotted with venom and platelets were chromatographed on paper with various solvent

systems. Initial runs showed that it was necessary to remove chloride from these extracts to obtain satisfactory chromatograms of this fraction. Attempts were made to remove the salt from the extracts by a chromatographic procedure. From a variety of solvents tested the most suitable was a propan-2-ol-0.5N-ammonia (4:1, v/v) mixture.

An extract (from 3–5 ml. of plasma), from which the trichloroacetic acid had been removed by shaking three times with ether and which had been dried by standing over phosphorus pentoxide *in vacuo*, was chromatographed first with the propan-2-ol-ammonia solvent for 4 hr. During this time a ^{32}P -containing compound separated from the other phosphorus compounds and moved (R_f 0.19) half the distance of the chloride (R_f 0.34). This phosphorus compound was present in extracts made from plasma clotted with either venom or platelets. No such spot was detected in extracts obtained from unclotted plasma. When the chromatograms were then 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.

To obtain larger amounts of the radioactive material, 120 ml. samples of plasma were clotted with viper venom. After the removal of the trichloroacetic acid from the acid extract by three ether washes, the extract was divided into two equal portions. Each portion was concentrated in a rotary evaporator, and finally dried *in vacuo* over phosphorus pentoxide. One portion of the dried extract was mixed with a little cellulose powder (Whatman, Standard Grade) and propan-2-ol-ammonia, and applied to a packed column of cellulose powder (1.5 cm. $\times$ 20 cm.) which had been previously washed with propan-2-ol-ammonia. The column was then eluted with the solvent at a flow rate of 1–2 ml./min. Fractions (10 ml.) were collected and tested for the presence of chloride, with silver nitrate, and for radioactivity. The separation of chloride from the radioactive fractions was not complete. However, rechromatography of the pooled radioactive fractions almost completely removed the chloride. Paper chromatography of the concentrated radioactive material with phenol-ammonia as the solvent revealed two spots. The major spot had the same mobility (R_f 0.86) as a sample of glycerylphosphorylcholine (R_f 0.87); the second spot, which could just be detected with the Geiger counter, had the same mobility (R_f 0.65) as a sample of glycerylphosphorylethanolamine (R_f 0.64). With ethanol-ammonia, which does not separate the choline and ethanolamine compounds (Freinkel, 1958), only one spot was detected.

To obtain sufficient of these compounds for chemical analysis, samples of the chloride-free radioactive material were pooled and further purified by ion-exchange chromatography. The radioactive material was taken up in 5 mM-sodium tetraborate. This was applied to a column (1 cm. $\times$ 13 cm.) of Dowex 1X2 resin (200–400 mesh), which was in the formate form (Hawthorne & Hübscher, 1959); 90–95 % of the radioactive material passed through the resin. The remaining 5 % was recovered when the solvent was changed to one containing 5 mM-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. Phosphorus, 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. Interference from free formaldehyde occurred in the glycerol determinations. This was kept to a minimum by the prolonged washing of the resin column with water before use (Hanahan & Olley, 1958). The molar proportions obtained for phosphorus, choline and glycerol were 1.02:1:1.04. This confirmed that the radioactive material was glycerylphosphorylcholine.

To test whether the organic phosphorus released during clotting could be accounted for solely by the formation of glycerylphosphorylcholine, the phosphorus:choline ratio was determined in the acid-soluble fraction obtained at various times during the incubation of plasma (10 ml.) with venom and calcium ions. The small amounts of glycerylphosphorylethanolamine which might also be formed would not seriously affect the result. The acid-soluble extracts were concentrated, made 1N with respect to hydrochloric acid, and, after the removal of a sample for the determination of total phosphate, the remainder was heated to 100° for 20 min. to release the choline from the glycerylphosphorylcholine (Dawson, 1956). Samples were then removed for the estimation of choline. Table 3 shows that the release of the organic phosphorus was accompanied by the appearance of choline, and

that molar ratios were 1.0:0.99 (mean). Thus the increase in acid-soluble organic phosphorus can be accounted for by the release of glycerylphosphorylcholine, and the amount of glycerylphosphorylethanolamine formed is too small to be detected by this technique. Further confirmation of this was obtained from an experiment in which the specific activity of the organic acid-soluble phosphorus released from a sample of plasma by the action of viper venom was compared with that of the phosphate in the glycerylphosphorylcholine (phosphate:choline ratio 1:1.02) isolated from the same extract; the specific activities were 20.6 and 20.2 counts/min./ μ g. of phosphorus respectively. In one experiment the plasma was clotted with the acetone-insoluble fraction obtained from brain, and, as with the platelets, the specific activity of the acid-soluble organic-phosphorus fraction increased, although the presence of other organic phosphorus compounds introduced with the brain preparation made it difficult to detect the release of organic phosphorus.

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, namely phenol-ammonia, ethanol-ammonia, and propan-2-ol-ammonia, it is reasonable to assume that this compound was released from plasma in the presence of platelets.

Hydrolysis of plasma lecithin by viper venom

Glycerylphosphorylcholine can be formed by the hydrolysis of lecithin or lysolecithin. Both of these are present in plasma and represent about 70 and 9 % of the plasma phospholipids, respectively (Phillips, 1958; Marinetti *et al.* 1959). The amount of glycerylphosphorylcholine released during incubation of plasma with viper venom for 16 min. is equivalent to the hydrolysis of only 2–3 % of the plasma lecithin.

To test whether the slow rate of formation of glycerylphosphorylcholine was governed by the rate at which plasma lecithin was hydrolysed, the amounts of lecithin, lysolecithin and glycerylphosphorylcholine were estimated at different time intervals during the incubation of plasma with

Table 3. *Appearance of organic phosphorus and choline in the acid-soluble extracts obtained during the incubation of plasma with Russell's viper venom and Ca²⁺ ions*

Time (min.)	Phosphorus (μ moles)	Choline (μ moles)	Phosphorus:choline ratio	Phosphorus (μ moles)	Choline (μ moles)	Phosphorus: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

viper venom. Fig. 2 shows that there was a rapid conversion of lecithin into lysolecithin which was complete at about 16 min. If the glycerylphosphorylcholine were formed by the degradation of the lysolecithin formed from the lecithin, one might expect the rate at which glycerylphosphorylcholine was released to increase with the concentration of lysolecithin. However, glycerylphosphorylcholine was liberated most rapidly during the first 2 min. after the addition of the venom and Ca^{2+} ions, when the concentration of lysolecithin in the plasma was only half that found after 16 min.

When platelet-rich plasma was incubated with Ca^{2+} ions, no changes in the amounts of lecithin and lysolecithin could be detected, although glycerylphosphorylcholine was liberated (measured by the increased specific activity of the acid-soluble organic-phosphorus fraction). If the glycerylphosphorylcholine were derived from plasma lysolecithin, then one would expect to detect a fall in the concentration of lysolecithin in the plasma, since the amount of glycerylphosphorylcholine released would correspond to the hydrolysis of 30–40 % of the lysolecithin.

It appears from these experiments that viper venom has an active lecithinase enzyme. This has been demonstrated by MacLagan, Billimoria & Curtis (1958). Therefore a considerably enhanced

release of glycerylphosphorylcholine would be expected in plasma incubated with the venom; however, this is not found, and it may be that the lecithinase activity of the venom is here coincidental with the formation of glycerylphosphorylcholine.

The possibility that the venom might hydrolyse lecithin to glycerylphosphorylcholine was investigated by incubating samples of purified human plasma lecithin ($2\text{ }\mu\text{moles/ml.}$) dissolved in ether with venom ($10\text{ }\mu\text{g.}$) for periods up to 18 hr. The phosphate:acyl ester ratio of the reaction mixture never fell below 1:1.01. Thus under these conditions the venom readily hydrolysed lecithin to lysolecithin but was not able to hydrolyse lysolecithin to glycerylphosphorylcholine.

In addition, the lecithinase activity of the viper venom was not affected by Ca^{2+} ions over a concentration range of 25–50 mM; also, in the presence of EDTA (1 mM), there was some activation of the enzyme. These findings are in contrast with the activation by Ca^{2+} ions and inhibition by EDTA of the lecithinase activity found in other snake venoms (Long & Penny, 1957).

DISCUSSION

Although Dawson (1956) showed that lysolecithin is hydrolysed in whole rat blood and to a slight extent in sheep blood, Marinetti *et al.* (1959) were unable to demonstrate any significant hydrolysis of lysolecithin in human oxalated plasma, even after incubation for 2 hr. However, oxalate is an anticoagulant and, since glycerylphosphorylcholine is released during coagulation, it suggests that the lysolecithinase activity is generated in plasma during coagulation. However, in platelet-rich plasma it was not possible to demonstrate any hydrolysis of lysolecithin already present in the plasma which would account for the formation of glycerylphosphorylcholine. Possibly the precursor of this substance is a specific plasma lecithin, which is first hydrolysed to lysolecithin and subsequently degraded to glycerylphosphorylcholine.

Dawson (1958) has shown that an enzyme obtained from *Penicillium notatum* which will hydrolyse lysolecithin will, in the presence of certain activating lipids, cleave both the fatty acids from lecithin. Marcus & Spaet (1958) and Rousser, White & Schloredt (1958) have demonstrated that platelets possess phospholipids which accelerate the coagulation of plasma. It is possible, therefore, that during coagulation the plasma lecithin is hydrolysed to glycerylphosphorylcholine by a reaction which is analogous to that described by Dawson (1958). Such a phenomenon might serve as a basis for the mechanism by which phospholipids shorten the clotting time of plasma in the

Fig. 2. Hydrolysis of plasma lecithin to give lysolecithin and glycerylphosphorylcholine during the incubation of recalcified platelet-free plasma (8 ml.) with Russell's viper venom ($10\text{ }\mu\text{g./ml.}$) at 37° for the times stated. The phospholipids were estimated by the chromatographic technique of Marinetti *et al.* (1959) on 3 ml. samples of the plasma. The glycerylphosphorylcholine phosphorus was determined in the acid-soluble fraction obtained after mixing the remaining plasma (5 ml.) with 0.2 vol. of 60% trichloroacetic acid. ○, Lecithin; ●, lysolecithin; △, glycerylphosphorylcholine.

presence of Russell's viper venom (Macfarlane, Trevan & Attwood, 1941) or of purified plasma reagents (Wallach, Maurice, Steel & Surgenor, 1959).

An alternative explanation for the release of glycerylphosphorylcholine is that choline plasmalogen which is present in small amounts in human blood (Christl, 1953) undergoes enzymic deacylation and that the resultant lysoplasmalogen is chemically hydrolysed to glycerylphosphorylcholine by the acid precipitation with trichloroacetic acid. However, since the lecithinase activity of the venom is unaffected by Ca^{2+} ions whereas the formation of glycerylphosphorylcholine is stimulated by Ca^{2+} ions, it seems unlikely that this interpretation is correct.

The suggestion that the lecithinase activity of Russell's viper venom is only coincidental with its coagulant activity has been confirmed by experiments in which the various components of the venom have been resolved by column chromatography (Williams & Esnouf, 1961). It was found that the lecithinase activity of the venom was not associated with the coagulant activity of the venom. The only enzymic activity which was associated with the coagulant protein was a slight proteinase activity and an esterase activity in the presence of toluene-*p*-sulphonylarginine methyl ester.

SUMMARY

1. During the incubation of plasma with viper venom and Ca^{2+} ions, glycerylphosphorylcholine is released.

2. Glycerylphosphorylcholine is formed during the incubation of platelet-rich plasma with Ca^{2+} ions.

3. It is likely that the source of the glycerylphosphorylcholine is plasma lecithin.

4. The mechanism by which glycerylphosphorylcholine is released is discussed.

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