THE EFFECT OF SOME FATTY ACIDS AND PHOSPHOLIPIDS ON BLOOD COAGULATION

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It has been reported that phosphatidyl ethanolamine (P.E.) (Robinson and Poole, 1956) or fatty acids (Poole, 1955) can accelerate the clotting time of plasma in various coagulation systems. O'Brien (1956*a*) suggested that platelets are active in blood coagulation because they contain P.E. or some similar substance. He also suggested (1956*b*) that the acceleration of blood coagulation after a fatty meal might be due to an increase in " available " P.E. or a similar substance in the plasma.

It is, therefore, important to know if P.E. is the only phospholipid which can replace platelets and cause acceleration of coagulation similar to that observed after a fatty meal. This paper reports experiments which show that a number of other phospholipids are active in some coagulation systems. Thus, any one of a number of phospholipids or a mixture of them might be responsible for the activity of platelets and the increased activity of lipaemic plasma.

It has been suspected for a long time that a lipoid substance in platelets was essential for normal coagulation. Bordet and Delange (1913) showed that cytozyme, a lipoid extractable from platelets etc., was essential to their clotting system. They also showed that a commercial " lecithin " could replace cytozyme. Wadsworth, Maltaner and Maltaner (1936) showed that " cephalin " produced marked acceleration when introduced into the same system. They produced several new concepts applicable to the present problem. They showed that " cephalin "—which promotes rapid coagulation—will combine stoichometrically with calcium chloride and that lecithin, which is inactive, will not. They studied four systems : (1) The precipitation of serum in the presence of calcium chloride and cephalin, (2) the clotting system of Bordet and Delange (1913), (3) the clotting time of oxalated plasma and (4) the complement system. In all of these, they found that the proportion of the three reactants, protein, cephalin and calcium, were all critical. If any reactant was increased or decreased, then the reactions were inhibited. Using the same sample of " cephalin ", now twenty years old and shown to be pure phosphatidyl serine, Rapport (1956) has expanded this picture. He showed that phosphatidyl serine by itself was only moderately active in shortening the clotting time of his system. If it was dissolved in a fat solvent with two volumes of lecithin, itself inactive, and the mixture was dried and suspended in water, then the activity of the phosphatidyl serine was considerably enhanced. A tenfold addition of lecithin was inhibitory ; so he argued that this increased activity indicated a specific lipid-lipid interaction and was not simply the physical effect of lecithin increasing the solubility of the phosphatidyl serine through mixed micelle formation. Chargaff (1944) also emphasized the

complexity of this work. Starting with impure fractions that were highly active, he found that the more they were purified beyond a certain point, the less active they became. Using the acceleration of the clotting time of rooster plasma, he found phosphatidyl serine inactive and P.E. only moderately active, while impure fractions were highly active. Turner, Silver and Tocantins (1956), in their attempt to identify the chemical nature of their lipid antithromboplastin, find that purification beyond a certain point does not increase the activity of the fraction ; phosphatidyl serine, however, seemed to be a major component.

In England, Trevan and Macfarlane (1936) showed that crude commercial " lecithin " greatly accelerated the clotting of human plasma by Russell viper venom (the Stypven time). However, pure lecithin is inactive (Poole, Robinson and Macfarlane, 1955). Using platelet-poor rat plasma, Poole and Robinson, (1956) and Robinson and Poole (1956) found that P.E., which is probably a contaminant of commercial " lecithin ", accelerated the Stypven time and increased the thrombin generated, while the serine and inositol phosphatides were almost inactive at a concentration of 1 mg./ml. O'Brien (1956*a*) confirmed and investigated further the activity of P.E. : the purest P.E. obtained from egg was considerably less active than a slightly less pure sample from brain. Maclagan and Billimoria (1956) have shown that a fraction isolated from butter which contains P.E. and galactose is an accelerator of the Stypven time of human plasma. Wallach, Surgenor and Walters (1956) have shown that platelets contain P.E. and phosphatidyl inositide, but have not isolated and tested these substances in clotting systems.

There is less information about the action of free fatty acids on blood coagulation. Poole (1955) reported that the sodium salts of some fatty acids produced moderate acceleration of the Stypven time of platelet-poor plasma. Laurate, oleate and linoleate were active, while palmitate and stearate and other fatty acid salts were inactive. In a thrombin generation test in which the plasma was already moderately active in producing thrombin, the addition of sodium laurate and sodium stearate produced increased activity. The greatest effect was produced by a concentration of 3·3 m-equiv./l but activity could be shown by as little as 20μe-quiv./l.

MATERIALS AND METHODS

Each substance to be tested was added in a range of concentrations to a substrate of non-lipaemic platelet-poor human plasma and the effect was compared with a negative control containing saline in place of the test substance, and a positive control containing an optimal number of platelets. Previous experiments had shown that Asolectin, a crude commercial product, simulated the action of platelets in all respects and, in practice, Asolectin (1 mg./ml. normal saline) was usually substituted for platelets.

The substrate for the tests was citrated platelet-poor non-lipaemic human plasma. This was prepared by collecting human blood after a fat-poor breakfast. A silicone technique was used throughout. Sodium citrate was added to the blood in the proportion of 1 part 0·025 M citrate to 9 parts blood and the mixture centrifuged at once for 15 min. at 3500 r.p.m. All experiments were carried out at 37° and the results are expressed as the average of at least two separate tests performed on the same day. All test substances were made up at a concentration of 1 mg./ml. (or stronger, if supplies allowed) and serial tenfold dilutions in distilled water were prepared therefrom. In each test, sufficient dilutions were used to ensure that the optimum concentration had been passed in both directions, or that a plateau of maximal activity had been reached. In all tests the positive and negative controls were carried out at the same time ; the positive control consisted of substituting platelets or Asolectin for the test substance ; in the negative control saline was substituted.

The clotting time.—Plasma, 0·1 ml., was pipetted into each of four small glass test tubes together with 0·1 ml. of the substance to be tested. One minute later, 0·1 ml. of 0·025 M-$CaCl_2$ was added. The clotting time is recorded as the average of the four determinations.

Prothrombin consumption.—Following the determination of the clotting time the serum from the four tubes was pooled and tested by the method of Biggs and Macfarlane (1953).

Thrombin generation.—The substrate plasma was recalcified after adding the test substance and at suitable intervals thereafter subsamples were transferred to tubes containing a fibrinogen solution, and the clotting times of the fibrinogen subsample tubes were recorded. The speed of clotting of the fibrinogen is determined by the amount of thrombin present in the transferred subsample. The result reported is the shortest clotting time in sec. of any of the subsample tubes. This is a measure of the max. concn. of thrombin that occurred during the coagulation of the original plasma : the shorter the reported time, the higher is the peak concentration of thrombin in the orginal plasma (for further details see O'Brien, 1955).

Thromboplastin generation test.—The method of Biggs and Douglas (1953) was used, and the figure reported is the shortest clotting time in sec. in any subsample tube.

Stypven test.—To 0·1 ml. of plasma was added 0·1 ml. of a suspension of the material under test and 0·1 ml. of 1/10,000 dilution of Russell viper venom (Stypven Burroughs Wellcome and Co.) in distilled water ; 1 min. later, 0·1 ml. of 0·025 M-$CaCl_2$ was added and the time then taken for clotting to occur was recorded in sec.

Materials.—Egg lecithin (Sylvana), prepared by the method of Pangborn (1951), may be assumed to be almost pure and free of acetal. It was shown to contain less than 1 per cent P.E. and less than 2 per cent inositides. Egg lecithin, prepared on silica columns by G. B. Robinson, was similarly pure. Beef heart lecithin (Sylvana), prepared by the Pangborn method (1951), contains, as well as lecithin, the acetal or plasmalogen substance called phosphatidal choline (Hack, 1953) which may represent 60 per cent of the total (Rapport and Alonzo, 1955). Cardiolipin (Sylvana), probably has the formula suggested by Pangborn (1947) and was prepared by her method (1942). It was investigated since it has a close chemical relationship to phosphatidic acid. The egg lecithin-cardiolipin mixture was prepared by mixing equal parts of these two substances in alcohol. The alcohol was removed by a stream of warm air and the dry residue was suspended in distilled water. The phosphatidyl ethanolamine (P.E.) used was pure, but was at the time of use of a middle brown colour. The acetal of P.E., phosphatidal ethanolamine was contaminated with 33 per cent P.E. It was a brown powder. The pure P.E. was separated from this mixture. Phosphatidyl serine (Klenk), was a brown powder, containing 20 to 40 per cent P.E. L-α-phosphatidyl serine (Hawthorne), prepared from brain, may contain small quantities of P.E. and inositol phosphatide. For the phosphatidyl serine (Wadsworth *et al.*, 1936) used Rapport, who kindly gave me a part of Wadsworth's original preparation, produced evidence that this sample was pure (Rapport, 1956). The brain diphosphoinositide with the probable formula suggested by Hawthorne (1955) was certainly impure. It may have contained 40 per cent of phosphatidyl serine but did not contain P.E. or lecithin. Liver phosphatidyl inositide probably has the formula of Faure and Morelec-Coulon (1953). The sample was contaminated by phosphatidyl serine but not by P.E. Sphingomyelin was over 98 per cent pure, the only possible contaminant being a trace of cerebroside. Since the Sphingosine and the sphingosine hydrochloride preparations proved to be inactive, the question of their purity, and therefore, a possible content of an active impurity did not arise. Asolectin is a crude commercial product prepared from soya " lecithin "containing about 95 phosphatides, including 30 per cent lecithin, 30 per cent cephalin and 35 per cent " lipositol "—mostly inositides.

RESULTS

The optimal results of from one to six tests for each substance on different plasmas were averaged irrespective of the dilution of the substance used in each test. This average time and that of the positive and negative control for all the clotting tests are presented in Table I. The times for the inactive substances are omitted. Inactivity (—) means that the plasma containing the test substance behaves in the same way as the negative control. Maximal activity, indicated by +++, is defined as an effect by the test substance equivalent to that pro-

TABLE I.—*Activity of Some Phospholipids Compared with Platelets and Saline*

Test substance	Clotting time in min.			Thrombin generation in sec.			Serum prothrombin time in sec.			Thromboplastin generation in sec.			Average activity in the foregoing tests	Stypven time in sec.			
	Saline	Platelets	Test substance	Saline	Platelets	Test substance	Saline	Platelets	Test substance	Saline	Platelets	Test substance		Saline	Platelets	Test substance	Activity
Beef lecithin	5¼	1¼	5	120	21	120	35	>90	65	34	9	60	−	20	5	8	++
Cardiolipin	6	1¼	6	140	21	146	34	>90	40	34	9	37	−	19	5½	11½	+
Mixture of lecithin and cardiolipin	5½	1½	4¾	85	24	86	27	>90	32	33	8	44	−	17	6	6	+++
Phosphatidyl ethanolamine (P.E.)	4	1	1¾	>120	27	26	30	>90	>90	28	10	9	+++	32	5½	5	+++
Phosphatidal ethanolamine	4	1	1¾	>120	16	14	30	>90	>90	28	10	11	+++	32	5½	5	+++
Phosphatidyl serine (Klenk)	4	1	1	>120	16	13	30	>90	>90	28	10	10	+++	32	5½	5½	+++
Phosphatidyl serine (Hawthorne)	5	1¾	4	>120	23	49	38	>90	>90	37	9	20	+	23	4¾	10	++
Phosphatidyl serine (Wadsworth)	5½	1¼	3½	80	25	58	26	>90	>90	30	7¼	18	+	18	5½	6	++
Liver phosphatidyl inositide	5¾	1	2½	95	31	34	27	>90	>90	33	8	19	++	23	4¾	9	++
Brain diphosphoinositide	6	1	1½	90	31	27	26	>90	>90	33	8	9½	+++	19	5	5	+++

Sodium glycerophosphate
Egg lecithin (two sources)
Sphingomyelin
Sphingosine hydrochloride
Sphingosine
} Completely inactive in all tests.

duced in the positive control by the suspension of platelets or Asolectin. Intermediate degrees of activity are indicated by : ++, +, and ±.

The following substances were found to be completely inactive, or slightly inhibitory : egg lecithin (two sources), sphingomyelin, sphingosine hydrochloride, sphingosine, and sodium glycerophosphate. Although egg lecithin was completely inactive, the beef lecithin, containing a considerable proportion of acetals, was moderately active in the Stypven clotting system, but inactive in all the other test systems. Cardiolipin behaved similarly to the lecithin acetal, having a moderate effect only on the Stypven time ; the mixture of cardiolipin and the inactive egg lecithin had maximal activity in the Stypven test, *i.e.*, enhanced activity, but was inactive in the other tests. Two samples of phosphatidyl serine were both slightly active in all the tests not involving Stypven and were moderately active in the Stypven test. The third sample of phosphatidyl serine was fully active in all tests. The liver phosphatidyl inositide was moderately active in all tests while the brain diphosphoinositide was fully active.

The fully active and the inactive substances were consistent in their behaviour, but this was not so with the intermediate substances. Table II gives two examples.

TABLE II.—*The Variability of the Effect of Phospholipids in Coagulation Tests*

	Thrombin generation (sec.)				Stypven time (sec.)		
Test substance	Saline	Platelets	Test substance	Test substance	Saline	Platelets	Test substance
Phosphatidyl serine (Hawthorne)	70	25	80	Cardiolipin	16	6	16
	105	21	21		22	5	7
	>120	22	60		18	4½	12
	78	13	24		—	—	—
	>120	26	34		—	—	—
	>120	25	60		—	—	—
	>120	26	63		—	—	—
Average	120	23	49	Average	19	5½	11½

Details of some individual experiments averaged in Fig. 1.

Had stronger concentrations of all the substances been available, they would probably all have had an inhibitory effect at high concentration. Brain inositide showed no evidence of inhibition at a concentration of 1 mg./ml. but all the other substances showed some evidence of inhibition at this strength, either in the dilute system used in the thromboplastin generation test or in the Stypven test, and often in both.

Slightly different types of inhibition and acceleration were encountered. In the Figure the Stypven times of fasting platelet-poor plasma containing logarithmically increasing amounts of phospholipids are plotted against the concentration of the phospholipid added. It will be seen that Asolectin produced maximal acceleration (*i.e.*, as good as platelets) over a considerable range. Beef heart lecithin on one occasion produced maximal acceleration but at one concentration only, and any increase in concentration thereafter produced inhibition. On another occasion, using different plasma, the beef heart lecithin behaved quite differently. A plateau of optimal activity was reached at 11 sec. and further

increments only produced inhibition. Two samples of phosphatidyl serine regularly produced inhibition in high concentration and, as will be seen, there is a critical concentration which gives the optimal acceleration of the Stypven test. The variability of this optimal effect has already been stressed in Table II. Egg lecithin is inactive at all concentrations used.

The minimum concentration necessary to produce demonstrable acceleration of the Stypven time was about 0·1 to 1·0 μg. per ml. This concentration was about

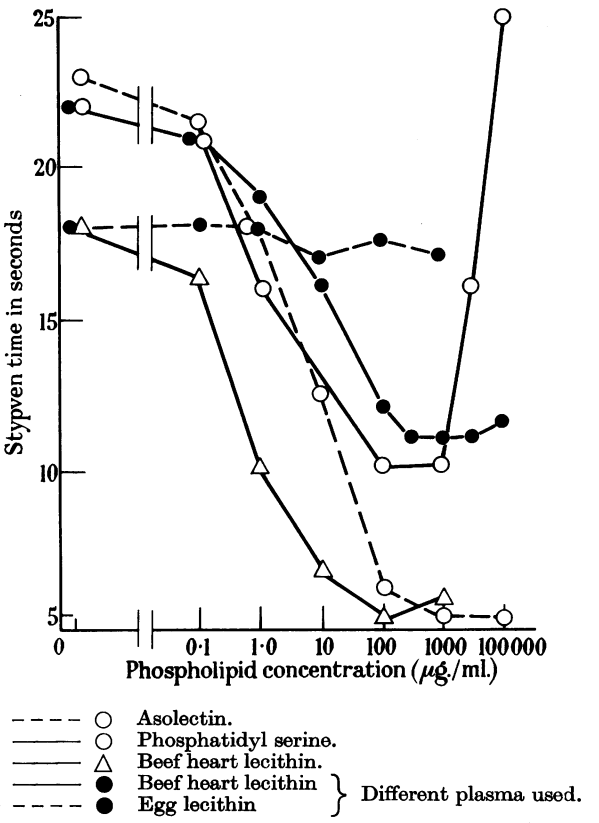

FIGURE.—The effect on the clotting time of the concentration of phospholipids added to platelet-poor plasma and Russell's viper venom.

To 0·1 ml. of platelet-poor plasma and 0·1 ml. Russell's viper venom, 1/10,000, was added 0·1 ml. of the phospholipid at the concentration indicated. One min. later 0·1 ml. 0·025 M-$CaCl_2$ was added and the clotting time recorded: each point is the average of at least two determinations.

the same for all substances showing any activity, and was independent of the degree of activity shown at optimal concentration.

Table III summarizes the results of introducing optimal concentrations of the sodium salts of three fatty acids into various clotting systems. It will be seen that sodium laurate was moderately active in the Stypven test but inactive in all the others. Sodium oleate was moderately active in the Stypven test and sodium stearate slightly so. Sodium oleate and stearate repeatedly produced

some shortening of the clotting time of plasma, but were virtually inactive in the other tests.

TABLE III.—*Activity of Fatty Acids in Various Clotting Tests*

Test substance	Clotting time (min.)			Thrombin generation (sec.)			Serum prothrombin time (sec.)			Average activity in foregoing tests	Stypven time (sec.)			
	Saline	Platelets	Test substance	Saline	Platelets	Test substance	Saline	Platelets	Test substance		Saline	Platelets	Test substance	Activity
Sodium laurate	4¼	1¾	4½	120	24	110	22	>120	21	—	33	5	10	++
Sodium oleate	4¼	1¾	2½	160	30	73	—	—	—	±	18	4½	9	++
Sodium stearate	4¼	1¾	2¾	120	24	120	22	>120	23	±	33	5	21	+

DISCUSSION

The variability of the results using substances of moderate activity is emphasized in the Figure and in Table II. This variability may be due in part to the difficulty of getting the substances, all insoluble in water, into a suitable state of dispersion. It was found, however, that even when the same suspension was tested day after day with different plasmas, some, but less, variation persisted. When the clotting time of plasma with saline added (the negative control) was long, the test substance usually gave a long clotting time, although the maximal acceleration with platelets (the positive control) was constant. This suggests that the Russell viper venom was probably behaving consistently; but the presence of lipids or other substances in the plasmas, which were all prepared identically, may have influenced the effect of the added phospholipid.

Rapport (1956) has shown that the activity of phosphatidyl serine in a different clotting test is increased by dissolving it in a fat solvent with lecithin. It has now been shown that the mixture of cardiolipin and lecithin is more active than cardiolipin by itself. These two observations support the general proposition that the action of a particular phospholipid may be modified by the presence of other phospholipids, as for example, in the Wassermann Reaction. Wadsworth *et al.* (1936) have shown that behaviour of phospholipids may depend on the relative concentration of these substances to protein and to calcium ions.

The variability of the results reported here does not represent technical ineptitude, but emphasizes an important aspect of the behaviour of phospholipids in biological systems. Without more knowledge of the relevant factors it is impossible to define accurately the degree of activity of each phospholipid, since the activity may depend on unknown factors in the plasma.

Since P.E. is known to be active, it might be argued that all these substances were active by virtue of their contamination by P.E., but it can be shown that this is not true. Wadsworth's sample of phosphatidyl serine, which was pure according to Rapport (1956), was found to be moderately active in the present investigation. The greater activity of Professor Klenk's sample might be due to degradation products, since it was middle-brown in colour while the other two samples were cream coloured. The phosphatidyl serine preparation of Hawthorne certainly

contained some P.E., but these inositide preparations contained none (although they contained phosphatidyl serine).

The interpretation of the results with P.E. and the acetal of P.E. are more difficult. Both were brown and so might have contained active degradation products. However, the preparation containing 66 per cent acetal and 33 per cent P.E. was weight for weight at all dilutions as active as the pure P.E. It is therefore probable that the acetal was itself active or enhanced the activity of the P.E.

The active phospholipids including the pure phosphatidyl serine all showed some activity in a concentration of about 1 μg./ml. It seems impossible that at this high dilution they were all active by virtue of an impurity. The observation that the minimum concentration showing detectable activity was approximately the same for all the active phospholipids irrespective of the degree of activity shown at optimal concentration is curious, but at present unexplained.

Poole (1955) reported that sodium stearate increased the amount of thrombin generated in a test system where a considerable quantity of thrombin was being generated by the plasma. In the present work, when the plasma alone generated almost negligible quantities of thrombin, the addition of sodium laurate and stearate in all concentrations repeatedly failed to increase the quantity of thrombin generated ; the results with the Stypven test are in complete agreement with those of Poole.

The present results do not support the suggestion that the clotting changes after a fatty meal are due to an increase in free fatty acid in the plasma (Poole, 1955). O'Brien (1955) claimed that after a fatty meal there was a slight increase in the thrombin generated by platelet-poor plasma. If the present observation that fatty acids do not affect thrombin generation in plasma can be confirmed, then it is most unlikely that the increase in unesterified fatty acids is solely responsible for all the coagulation changes observed after a fatty meal.

Gordon and Cherkes (1956) reported that the unesterified fatty acids increase in the plasma after a fatty meal, and that they also increase after a twelve hour period of starvation, but there is no increase after a carbohydrate meal. No coagulation changes have been described in starvation or after a carbohydrate meal.

O'Brien (1956*a*) has shown that P.E. can simulate the action of platelets in blood coagulation, and therefore might be the " active " substance in platelets. It is now seen that phosphatidyl serine, phosphoinositide and diphosphoinositide which also may be present in platelets, may play a part in normal blood coagulation ; and there is no evidence that the list is complete. It is even possible that the appearance or increase in a substance like lecithin which is inactive alone, might potentiate other active phospholipids previously present but in small and inactive quantities.

The increase of free fatty acids after a fatty meal may possibly be responsible for the post-prandial changes in blood coagulation. On the other hand, an increase in active phospholipids in the plasma post-prandially seems more likely to play a major part. Further evidence in support of this hypothesis is adduced elsewhere (O'Brien, 1957). The present work suggests that the observed results in lipaemic plasma could be produced by an increase in the plasma of free available diphosphoinositide, phosphatidylinositide, phosphatidyl serine or P.E. Furthermore, it has already been shown (O'Brien, 1956*b*) that in some tests the P.E.-

like substance in platelets is not immediately available. P.E. is essential for blood coagulation *in vitro*, but presumably only becomes available during the process of blood clotting after the platelets have been " activated ". Thus, a small amount of free active phospholipid might have a disproportionate effect on blood coagulation by short-circuiting this initial activating stage involving the platelets.

It has previously been tentatively assumed that in experiments on lipaemic plasma the highly reproducible Stypven time and the plasma clotting time, notoriously susceptible to minute changes in technique, were, in fact, measuring the same phenomenon ; and that is why the Stypven time has sometimes been preferred in such investigations (O'Brien, 1956*b*, 1957). This assumption was based on the observation that the two tests varied roughly in parallel (O'Brien, 1955). It is now shown that cardiolipin, the acetal of lecithin, and some free fatty acids all accelerate the Stypven time but do not affect the plasma clotting time or thrombin generation. The above assumption is thus not always justified.

The degree of activity of some phospholipids in various clotting systems probably depends on the presence of other lipids in the plasma and other unknown variables. The optimal concentration of such phospholipids is often critical and most of them show inhibition when present in excess.

O'Brien (1956*a*) has previously shown that the lyso-compound of P.E. is inactive. It is now shown that, while lecithin is completely inactive, the acetal of lecithin is active in the Stypven test and the acetal of P.E. is probably active in many systems. Thus, the arrangement of the fatty acids in the α- and β-positions of the glycerol molecule is also important. On the other hand, the presence of double bonds in the fatty acids may be unimportant since O'Brien (1956*a*) did not detect much difference in activity in the natural unsaturated P.E. and a hydrogenated P.E.

The chemical properties of sphingomyelin and lecithin, which are inactive in coagulation systems, differ from those of P.E., phosphatidyl serine and phosphatidyl inositide, which are active. With their quaternary ammonium group, they have a stronger positive charge and are almost neutral at physiological pH. Unlike lecithin and sphingomyelin the more basic cephalins can combine with calcium and also form salts with tissue proteins (Wadsworth *et al.*, 1936; Chargaff, 1946). These properties may be related to their action in blood coagulation.

SUMMARY

Using the plasma clotting time, the thrombin generation test, the prothrombin consumption test, the thromboplastin generation test and the Russell viper venom accelerated clotting time (Stypven time), it has been shown that the diphosphoinositide, phosphatidyl inositide, phosphatidyl serine and the acetal of phosphatidyl ethanolamine (phosphatidal ethanolamine) can to varying extents mimic the action of platelets. It has previously been shown that phosphatidyl ethanolamine can also substitute for platelets. Some fatty acids accelerate the plasma clotting time and stypen time only. Cardiolipin and the acetal of lecithin accelerate the Stypven time only. Egg lecithin, sphingosine and sphingomyelin are inactive. The activity of one phospholipid may be influenced by the presence of another inactive phospholipid.

It is concluded that platelets may be active in *in vitro* clotting systems by virtue of their content of any one of a number of phospholipids. The coagulation

changes observed in platelet-poor plasma after a fatty meal are not likely to be due to an increase of unesterified fatty acids, but could be fully explained if there was an increase in the plasma of any one of several, or a combined increase in several free and available phospholipids.

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