PHOSPHOLIPID STRUCTURE AND THROMBOPLASTIC ACTIVITY*

II. THE FATTY ACID COMPOSITION OF THE ACTIVE PHOSPHATIDYL ETHANOLAMINES

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Phosphatidyl ethanolamine is the phosphatide that accelerates the rate of clotting of recalcified human plasma[1], the differing activities of preparations from various sources being related to differences in fatty acid composition. In the present communication the fatty acid composition of phosphatidyl ethanolamine preparations from several sources will be described, and it will be shown that as the degree of unsaturation of symmetrically distributed fatty acids of the molecule increases, the thromboplastic activity increases.

MATERIALS AND METHODS

The coagulation tests and source of materials have been described[1]. Phosphatide mixtures were prepared and phosphatidyl ethanolamine isolated by silicic acid column chromatography[1]. The soybean and egg preparations examined did not contain appreciable amounts of phosphatidyl serine and thus the phosphatidyl ethanolamine from these sources was essentially free of other phosphatides as it came off the column. Both rabbit-appendix and human blood-platelet preparations contained phosphatidyl serine. This could not be removed by a preliminary FOLCH[2] fractionation procedure because the additional manipulation was found to increase the extent of oxidative alteration of the highly unsaturated phosphatides. It was first shown by paper-chromatographic isolations that the phosphatidyl serine from both of these sources contained virtually all of the saturated fatty acids found in the hydrolysates of the mixture of the amino phosphatides and was very low in unsaturated fatty acids. The "aged" octanol solvent[1] was used with well-washed Whatman 3 MM paper. Phosphatidyl serine and phosphatidyl ethanolamine were separated completely in the system. After elution and hydrolysis, phosphatidyl serine showed the presence of saturated fatty acids, and the major unsaturated fatty acid detected was oleic acid. Only more highly unsaturated fatty acids were detected in the phosphatidyl ethanolamine. The study of the unsaturated fatty acids in phosphatidyl ethanolamine could thus be carried out on the mixture of amino phosphatides and steps likely to cause oxidative change could be avoided.

The phosphatidyl ethanolamine preparations were hydrolyzed as follows: 4 mg was mixed with 4 ml of 1 *N* hydrochloric acid in a sealed tube, heated to 100° for 10 min, removed briefly and shaken vigorously, and heating continued for an additional 50 min. Fatty acids were extracted into diethyl ether or petroleum ether. This procedure, although producing some slight oxidation of the highly unsaturated fatty acids, was found to be the most useful for chromatographic studies.

The fatty acids of the phosphatide preparations from silicic acid were studied spectrophotometrically by the micro alkali-isomerization technique described by HERB AND RIEMENSCHNEIDER[3]. The method was selected because it gives information pertaining to the number of double bonds in the fatty acids of a mixture and can be used for determining the percentage of di-, tri-, tetra-, penta- and hexaenoic acids. The specific extinction coefficients were those of

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HERB AND RIEMENSCHNEIDER[3] and HAMMOND AND LUNDBERG[4]. These determinations are in error to an undetermined extent since some unavoidable oxidation of the highly unsaturated fatty acids occurred during the preparations of the samples for analysis. The additional uncertainty with platelet phosphatide arises from the fact that chain length and position of the double bonds of penta- and hexaenoic acids found in significant amounts are uncertain. Paper-chromatographic examination of the phosphatide preparations was also conducted. Chromatography on an acid hydrolysate was performed. Catalytic hydrogenation of the same mixture was used to determine the chain length of the major unsaturated fatty acids encountered. Chromatographic examination of the fatty acids released by moccasin venom[1] was used to study the relative distribution of the fatty acids between the α'- and β-positions.

A modification of the chromatographic method of KAUFMANN AND NITSCH[5] was used. Whatman No. 1 paper washed with 2 N acetic acid and 95% ethanol was impregnated with mineral oil (10 g/100 ml petroleum ether). Chromatograms were developed by the descending technique using 90% acetic acid as solvent. The solvent was allowed to migrate to the end of the paper or preferably to drip over the end of a serrated paper strip for a total running time of 18–24 h. The chromatograms were dipped into 1% potassium permanganate solution for visualization of unsaturated fatty acids. Excess permanganate was washed off in running tap water and the chromatograms were then photographed.

Two spots indicating oxidation of fatty acids appear on the chromatograms. One of the spots appears at the origin and the other near the solvent front (see Fig. 1A). These spots have been produced from linolenic acid by the acid hydrolysis technique used for phosphatides.

The saturated fatty acids are difficult to detect specifically when mixed with unsaturated fatty acids since the spots overlap. It was found, however, that after bromination, all of the unsaturated fatty acids, regardless of number of double bonds or chain length, migrate to the same position on the chromatogram and the single spot can be separated from the saturated fatty acids. Bromination was carried out in ethanol or chloroform and excess bromine was removed by evaporation under nitrogen. The saturated fatty acids were located on chromatograms after spraying with the bromothymol blue reagent of ASHLEY AND WESTPHAL[6]. The spots were outlined, but since rapid fading occurs, no attempt was made to photograph the original spots.

Catalytic hydrogenation was carried out with fatty acids dissolved in 95% ethanol (1 mg/ml). The catalyst was prepared by adding palladium chloride (1–2 mg/4 ml) to ethanol followed by exposure to hydrogen until the material turned black.

RESULTS

Table I shows the relative clot-accelerating activity of the phosphatidyl ethanolamine fraction from various sources. Fig. 1 shows the unsaturated fatty acids seen on paper chromatograms after hydrolysis of the preparations shown in Table I.

TABLE I

RECALCIFIED PLASMA CLOTTING TIMES OF PHOSPHATIDYL ETHANOLAMINE FROM VARIOUS SOURCES

Source of phosphatidyl ethanolamine	*Concentration* (mg %)*				
	100	*30*	*10*	*1*	*Control*
			Time (sec)		
Synthetic dimyristoyl and distearoyl compounds	145	155	170	210	290
Egg	125	135	143	170	290
Soybean	112	122	132	155	290
Rabbit appendix	89	95	101	115	290
Human blood platelets	75	79	86	101	290

* Concentration of initial phosphatide dispersion.

Fig. 1A shows the fatty acid composition of soybean phosphatidyl ethanolamine. For the preparation of the chromatogram, a relatively large amount of fatty

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acid was applied to paper to show the complete absence of acids migrating slower than oleic or faster than linolenic acid. No saturated fatty acids were observed on chromatograms after bromination. It was observed that the three spots seen in addition to the "oxidation" spots migrated with oleic, linoleic, and linolenic acids. After catalytic hydrogenation only stearic acid was detected in keeping with the initial chromatographic identification. The alkali-isomerization technique gave a value of 91% for linoleic acid content of the sample. Paper-chromatographic comparisons of the fatty acids released by acid hydrolysis and by moccasin venom showed the fatty acids to be distributed rather evenly between the α'- and β-positions. The data thus demonstrate this preparation to be principally dilinoleylphosphatidyl ethanolamine.

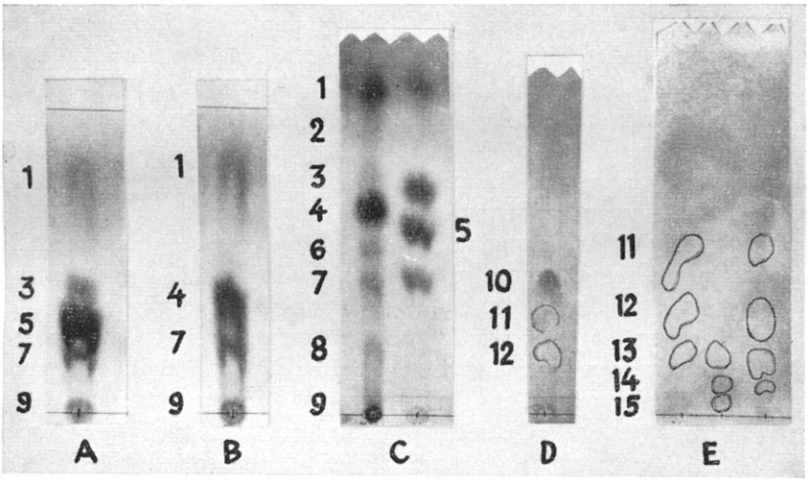

Fig. 1. Fatty acids of the phosphatidyl ethanolamine fraction from silicic acid columns: A. prepared from 500 μg soybean hydrolysate; B. prepared from 600 μg rabbit appendix; C. 600 μg human blood platelet (left) and 80 μg each of known fatty acids (right); D. platelet-saturated fatty acids; E. platelet fatty acids after hydrogenation (right) and known samples (center, left). Spots identified as: 1. oxidized fatty acid material; 2. unknown; 3. linolenic acid; 4. arachidonic acid; 5. linoleic acid; 6. unknown; 7. oleic acid; 8. unknown; 9. oxidized fatty acid material; 10. brominated fatty acids; 11. palmitic acid; 12. stearic acid; 13. arachidic acid; 14. behenic acid; and 15. lignoceric acid.

Rabbit appendix was found to contain two unsaturated fatty acids, arachidonic and oleic (Fig. 1B). That the major acid was arachidonic acid was shown by chromatography of the original compound, the production of arachidic acid by reduction, and the absorption peak characteristic of tetraenoic acids after alkali isomerization. Arachidonic acid accounted for virtually all of the unsaturated fatty acids of the sample determined by alkali isomerization. Small peaks at 346 and 374 mμ indicated the presence of traces of penta- and hexaenoic acids.

The principal unsaturated fatty acid found in platelet phosphatidyl ethanolamine was arachidonic acid. Strips C to D of Fig. 1 illustrate some of the chromatographic findings with the preparation. The unsaturated fatty acids found in the platelet preparation are shown to the left and authentic compounds to the right. The chromatogram was developed by allowing solvent to drip over the end of the

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paper. This type of run shows clearly that arachidonic acid, the major platelet spot, migrates between linoleic and linolenic acids. The presence of small amounts of oleic, linolenic and two unknown acids is also shown. Fig. 1D shows the saturated fatty acids detected in the sample after bromination. The spots migrate with authentic palmitic and stearic acids, and paper chromatographic comparisons of graded amounts of these acids indicated that the saturated fatty acids, contributed largely by phosphatidyl serine, represented approximately 15% of the total. Fig. 1E shows the fatty acid composition after hydrogenation. The two rows of spots to the left are authentic compounds and it can be seen that arachidic and behenic acids were produced after hydrogenation of platelet fatty acids.

Assuming a chain length of 22 carbons for penta- and hexaenoic acids, the alkali-isomerization technique gave the following percentage distribution of unsaturated fatty acids: linolenic acid, 3.6%; arachidonic, 72%; pentaenoic, 6.2%; and hexaenoic, 18.2%. Enzymic degradation indicated the unsaturated fatty acids to be relatively evenly distributed between the α'- and β-positions.

The presence of more pentaenoic and hexaenoic acids in the platelet preparation is in keeping with the ready loss of clot-accelerating activity by oxidative changes on standing exposed to air, and the greater difficulties encountered in the isolation of total clot-accelerating activity from silicic acid columns[1].

Egg phosphatidyl ethanolamine was found to have only unsaturated fatty acids in the α'-position and principally saturated fatty acids in the β-position in keeping with the early work of CHARGAFF AND COHEN[7] on mixed egg phosphatides and RHODES AND LEA[8] on egg phosphatidyl ethanolamine isolated from silicic acid columns. Also in agreement with RHODES AND LEA was the finding of some very highly unsaturated fatty acids. These acids have not been studied extensively since it appeared that the asymmetric distribution of the fatty acids was largely responsible for the relatively low thromboplastic activity of egg phosphatidyl ethanolamine. These findings emphasize the fact that the presence of unsaturated fatty acid alone is not always enough to produce thromboplastic activity, but that the acids must occupy both the α'- and β-positions in the molecule to produce highly active thromboplastic phosphatide.

DISCUSSION

The data presented show that as the degree of unsaturation of symmetrically distributed fatty acids of phosphatidyl ethanolamine increases, the thromboplastic activity increases. This has been shown by the isolation of a series of phosphatidyl ethanolamines containing fatty acids of varying degrees of unsaturation.

The fact that a water-soluble product formed by hydroxylation of active phosphatide is inactive in recalcified plasma[1] has been taken to indicate that the reaction requires a colloidally dispersed phosphatide. An aqueous dispersion of water-insoluble phosphatide will be formed in such a way that the hydrophobic carbon chains will interact with each other and be bound by van der Waal's forces, leaving the ionic groups projecting into the polar aqueous phase. Evidently the size and shape of the phosphatide molecules will influence the manner in which molecules interact with each other and the medium. It is evident that the phosphatide molecules containing only unsaturated fatty acids in the *cis*-form are folded back

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upon themselves to form a molecule that is shorter and greater in diameter than the molecule containing the corresponding saturated fatty acid. Construction of phosphatide models shows clearly that as the degree of unsaturation of the fatty acids is increased, a more nearly spherical shape is attained. On the other hand, the carbon chains of phosphatides containing only saturated fatty acids exist in a more extended form and it seems probable that such molecules will interact to form a platelet-type structure as visualized by McBAIN[9]. The growth of a platelet type structure is not limited as is that of spherical micelles and thus a larger particle size can be attained. These concepts of micelle formation are in keeping with current theoretical and experimental observations[10,11].

It is known that phosphatide must interact with plasma factors to produce the complete thromboplastic complex. The information available makes it seem probable that both calcium ion and a plasma factor must interact with phosphatide. If one visualizes an active complex as being formed by the linking of an acidic phosphatide to a negatively charged acidic group on a protein, *e.g.*, by the divalent calcium ion, the mode of action of a number of inhibitors can readily be visualized. The formation of such a complex held together by ionic bonds could be prevented by various acidic and basic compounds by interaction of the inhibitory substance with one or more of the constituents of the active complex. Thus acidic compounds such as heparin, sulfated polysaccharide, and cerebroside sulfate[12,13] could inhibit by binding to calcium with the formation of an inactive complex. On the other hand, basic materials such as dyes and the inhibitory cetyltrimethylammonium ion can be visualized as being inhibitory by virtue of interaction with either the acidic phosphatide or protein constituents of the complex and thus preventing the formation of active complex.

There is some evidence that certain anticoagulants can interact with phosphatides and lipoproteins. CHARGAFF *et al.*[14,15] have observed that heparin and other anticoagulants disrupt lipoproteins. It has been observed in this laboratory that cetyltrimethylammonium ion is a powerful anticoagulant and when used in excess can also aid in the formation of a finely dispersed colloidal preparation with dimyristoyl or distearoyl phosphatidyl ethanolamine. The finely dispersed synthetic phosphatide preparations so prepared inhibit coagulation.

There is no information available to indicate how the hypothetical active complex could accelerate coagulation. Such a complex could possess enzymic activity or be effective in removing an inhibitor from some other active substance. The work of WENCKERT AND NILSSON[16] suggests that phosphatide might function to inactivate a heparin-like anti-thrombin substance split from prothrombin in the formation of thrombin.

The relative inactivity of phosphatidyl serine as a thromboplastic phosphatide probably arises at least in part from the fact that it is made up of saturated fatty acids and unsaturated fatty acids with at most one or two double bonds.

Our data relating to an increase in thromboplastic activity with increase in unsaturation of phosphatidyl ethanolamine are in apparent disagreement with the findings of POOLE AND ROBINSON[17]. These investigators used the Russell viper-venom test system to show that synthetic and egg phosphatidyl ethanolamines were about equally active. The differences appear to be due to two major factors. We have shown that a number of compounds inactive in recalcified plasma are active in the

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venom system. Since egg phosphatidyl ethanolamine, even when freshly prepared, is relatively inactive and will lose activity on standing, it is possible to account for the findings of POOLE AND ROBINSON by assuming that their egg phosphatidyl ethanolamine had lost a great deal of activity and in the fast-clotting venom system appeared about as active as the synthetic phosphatides.

In our hands fresh rabbit-brain total-phosphatide extracts have about the thromboplastic activity of mixed soybean phosphatides. The reason for the relatively low thromboplastic activity of brain phosphatide is to be found in the fatty acid composition of the phosphatidyl ethanolamine. DEBUCH[18] found that 70% of brain phosphatidyl ethanolamine was in the aldehyde form (plasmalogen, acetalphosphatidyl ethanolamines) and only 30% in the fatty acid diester form. We have shown the plasmalogen form to be inactive and it is interesting in this regard that the aldehyde components are saturated[19]. DEBUCH further showed that plasmalogen-free phosphatidyl ethanolamine contained 48.8% saturated fatty acids and 34.9% weakly unsaturated acids, thus the phosphatidyl ethanolamine itself was relatively highly saturated and hence relatively inactive.

It is interesting to compare the data on the fatty acid composition of phosphatidyl ethanolamine to similar data on lecithin. DEBUCH[20] has shown that a soybean lecithin contained principally linoleic acid, only unsaturated fatty acids in the α'-position, and about 40% saturated fatty acid present in the β-position. HANAHAN[21] has shown that liver lecithin contains unsaturated fatty acids in the α'-position and saturated fatty acid in the β-position.

SUMMARY

1. Phosphatidyl ethanolamine from several sources has been compared for clot-accelerating ability in a recalcified plasma test system.

2. The thromboplastic activity of phosphatidyl ethanolamine has been found to increase as the degree of unsaturation of symmetrically distributed fatty acids in the molecule increases.

3. The importance of unsaturation of the fatty acids appears to be related to the size and shape of the colloidal particle of phosphatide dispersed in water.

4. The formation of an active complex composed of phosphatide micelle, calcium ion, and a third plasma factor, is postulated and discussed.

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Addendum

Since this manuscript was submitted for publication, a monograph by P. F. HJORT (*Scand. J. Clin. & Lab. Invest.*, 9 (1957) suppl. 27) has come to our attention. HJORT presents data that strongly indicate that a phospholipid-containing particle is united through calcium ion to a plasma protein in the sequence of reactions leading to the clotting of blood plasma in the presence of brain thromboplastin.

VOLUME CHANGES IN OVALBUMIN AND BOVINE SERUM ALBUMIN ON ADDING ACID

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Polypeptide chains undoubtedly pack in certain more or less well-defined patterns in native protein molecules. If denaturation consists in the partial or complete replacement of these regular patterns by a more random arrangement of the chains, then one should expect that denaturation will be accompanied by characteristic volume changes that should parallel, at least in a general way, the changes in other properties, such as the optical rotation, which are also affected by the chain configuration. Several workers have, in fact, noticed that the destruction of the native configuration in proteins is accompanied by a decrease in the volume of the protein solution amounting to several hundred ml/100,000 g of protein[1-4]. The apparent volume occupied by a protein molecule in solution is also influenced by the charged groups present in the molecule because of electrostriction of the solvent surrounding the charged groups; if the configuration changes are accompanied by known changes in the charged groups of the protein, however, the effects of the latter can be taken into account by suitable model experiments[5,6].

The measurements reported in the present communication were performed with Carlsberg dilatometers of the type described by LINDERSTRØM-LANG[7], with the modifications suggested by JOHANSEN AND THYGESEN[8] and by JOHANSEN[9].

The dilatometers consist of an inverted "V", one arm containing the protein solution and the other containing the acid. The space above the solutions is filled with kerosene and a graduated capillary is attached by means of a suitably greased[8] ground-glass joint at the vertex of the "V". The inverted "V" is inserted into a well-controlled thermostat ($\pm$ 0.001°) and allowed to stand until thermal equilibrium has been reached. If the bath is warmer than room temperature, as will generally be the case, the kerosene meniscus will be at the top of the capillary tube and must be brought down to a suitable point in the graduated region by placing the inverted "V" momentarily in water slightly warmer than the thermostat, soaking up the excess kerosene that flows out of the top of the tube, and replacing in the thermostat. After thermal equilibrium has been re-established and the kerosene meniscus has reached a constant level, the two solutions are mixed.

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