

Thromboplastic activity of phosphatidylethanolamine from natural and synthetic sources*

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SUMMARY

Improvements are described in the preparation of phosphatidylethanolamine from hen's egg and in the preparation of intermediates used in the total synthesis of racemic dioleoyl-phosphatidylethanolamine. Both preparations have been found to be potent accelerators in the Hicks-Pitney test and moderately active in the thromboplastin generation test.

There is still some controversy about the thromboplastic activity of phosphatidylethanolamine. Kuhn and Klesse (1, 2) reported that the synthetic *L*- α -(dioleoyl)-phosphatidylethanolamine of Baer and Buchnea (3) shortened the clotting time of their lung thromboplastin preparation from which lipid had been removed. Their test system was similar to that used by Rapport and Maltaner (4). Later, they found activity in a two-stage thrombin generation test (5). Rouser (6) reported that a sample of *L*- α -(dioleoyl)-phosphatidylethanolamine, which had been freshly prepared by Baer and Buchnea (3), was active in the recalcified plasma test system. Slotta (7) later reported that the synthetic product was inactive in the thromboplastin generation test of Biggs et al. (8). Later, Slotta and Deutsch (9) found some activity in a more impure synthetic *L*- α -(dioleoyl)-phosphatidylethanolamine. Troup et al. (10) reported that Baer's synthetic dioleoyl phosphatidylethanolamine was completely inactive in the thromboplastin generation test. Barkhan et al. (11) found the same synthetic product to be highly active in the thromboplastin generation test, but only if it was solubilized in sodium desoxycholate solutions.

In Slotta's paper (7), it is reported that the synthetic product used had an iodine number of 53 instead of the theoretical 68, although it showed only one spot in paper chromatographs. This suggests that some of the

preparations may have been contaminated with stearoyl-oleoyl-phosphatidylethanolamine, reduction of the double bond occurring during the removal of phthaloyl groups by hydrazine. This has been discussed by deHaas and coworkers (12, 13), who introduced a milder procedure for dephthaloylation than that employed by Baer and Buchnea. This possibility adds more confusion to the question of the thromboplastic activity of phosphatidylethanolamine.

Malkin (14) has outlined methods for the preparation of a racemic (dioleoyl)- α -phosphatidylethanolamine. While we followed the method of Bevan et al.,¹ a very detailed account of this general procedure with significant improvements has since appeared from the laboratory of deHaas (13). We report here the results of our studies of the thromboplastic activity and other characteristics of this phospholipid.

The preparation of egg phosphatidylethanolamine has been improved by using the DEAE cellulose acetate procedure of Rouser et al. (15). The properties of the natural material from egg sources have been compared with those of the synthetic phosphatidylethanolamine.

EXPERIMENTAL METHODS

Paper Chromatography. The method of Marinetti (16) was used with silicic acid impregnated Whatman 3 MM paper, and the solvent system diisobutylketone-

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¹ Bevan, Malkin, and Counsell, unpublished work.

acetic acid-water 40:25:5. Amounts of 300–400 μg were ordinarily applied.

Column Chromatography. Cellex D (Bio-Rad Laboratories, Richmond, California) was washed with distilled water by decantation until free of fine particles. This required eight washings with 35 volumes of water. After four washings with methanol, the material was dried and converted to DEAE cellulose acetate according to Rouser et al. (17). The universal indicator of British Drug Houses was found to be the most useful for testing the neutrality of the methanol washes used to remove the acetic acid from the product. Some commercial samples of DEAE cellulose are equally effective or more so than Cellex D for separating phosphatides, but yield samples of phosphatidylethanolamine that are inactive in the thromboplastin generation test. It is thought that this may be caused by impurities in the DEAE cellulose (probably amines) that may be inhibitory in blood coagulant systems. This occurred with Selectacel (Carl Schleicher and Schuell, Inc.).

Whatman cellulose powder was washed as described by Rouser et al., and dried.

Glyceryl Iodide. The difficulties in preparing this substance have been discussed by Rosenthal and Geyer (18). Their method is also difficult because of the cumbersome procedure used to remove the sodium iodide from the product, but this is easily accomplished by precipitation as lead iodide.

To 150 g of freshly distilled 1-chloro-2,3-propanediol was added 240 g of powdered dry sodium iodide and 1 liter of methyl isobutyl ketone. This mixture was refluxed for 7 hr. After cooling and filtering, the filter cake was washed with ethyl acetate. The solvent mixture was treated with enough of a solution of lead acetate (350 g of the trihydrate in 1 liter of water) to precipitate all of the iodide as lead iodide. The aqueous layer was separated and discarded after filtering out the lead iodide. The solution was then washed with 10% sodium thiosulfate, dried, and distilled *in vacuo* below 40°. The residue crystallized and was recrystallized from chloroform-*n*-butyl chloride; yield: 134 g, m.p. 46–47°.

Dioleoylglyceryl Iodide. Oleic acid is most conveniently purified by the method of Rubin and Paisley (19). A commercial oleic acid from British Drug Houses (Ealing Corp., Cambridge, Mass.) was purified only by the acid soap method because its composition was similar to the material used by Rubin and Paisley for this part of their procedure. Dioleoylglyceryl iodide was made as described for the saturated analogs by Hessel et al. (20). The product was an oil, which was purified by treatment with methanol, cooling to –15°, and decanting the methanol. The iodide crys-

tallized in the cold, but became an oil again at room temperature, $n_D^{29.5} = 1.4780$.

Anal. Calcd. for $\text{C}_{39}\text{H}_{71}\text{IO}_4$: I, 17.36. Found: I, 18.0, 17.53.

2-Iodophthalimide. 2-Bromophthalimide (Eastman Kodak Co.) was refluxed with a 10% excess of dry sodium iodide in dry acetone for 2 hr. It was cooled, filtered, and evaporated, and the iodide was crystallized from methanol, m.p. 92–93°. Phthalimidoethyl dibenzyl phosphate was made from the iodide and silver dibenzyl phosphate as described by Bevan et al.¹ It was converted into the crystalline salt of phthalimidoethylmonobenzyl phosphate and then to the silver salt of this by the methods of Bevan. Combination of the silver salt with dioleoylglyceryl iodide gave the dioleoylglyceryl phthalimidoethylmonobenzyl phosphate.¹ The benzyl and phthaloyl groups were removed by the method of deHaas and vanDeenen (12). The synthetic phosphatidylethanolamine was purified by chromatography on a silicic acid column as described by Baer and Buchnea (3), or, with better results and in higher yields, by the use of a column of DEAE cellulose acetate as described below for natural phosphatidylethanolamine.

The synthetic phosphatidylethanolamine showed only one spot when chromatographed on silicic acid paper in diisobutyl ketone-acetic acid-water, when up to 200 μg was applied. A trace of a second spot with the mobility of phosphatidic acid was seen when 400 μg was applied.

Anal. Calcd. for $\text{C}_{41}\text{H}_{78}\text{O}_8\text{NP}$: C, 66.18; H, 10.57; N, 1.88; P, 4.16; iodine No. 68.

Found: C, 65.64; H, 10.50; N, 2.00; P, 4.10; iodine No. 69.

Separation of Egg Phosphatides. All operations were conducted under nitrogen. The yolks of four eggs were dried by three extractions with 250 ml of acetone. The phosphatides were then extracted with chloroform-methanol 2:1 using three extracts of 250 ml. The combined chloroform extracts were then washed with $\frac{1}{6}$ volume of 1% potassium chloride and then with $\frac{1}{6}$ volume of the equilibrated wash mixture of chloroform-methanol-water 3:48:47 described by Folch and coworkers (21, 22). The chloroform solution was dried with filter paper or by passing through a column of washed Whatman filter paper powder. The chloroform solution was evaporated.

In preparing columns, a 10-cm high layer of washed cellulose powder was used as support and on top of this was put 50 g of DEAE cellulose acetate. This column was then washed with methanol until neutral; it was then washed with chloroform and the egg phosphatides were applied in chloroform. The load was 30 mg of phosphatide/g of DEAE cellulose.

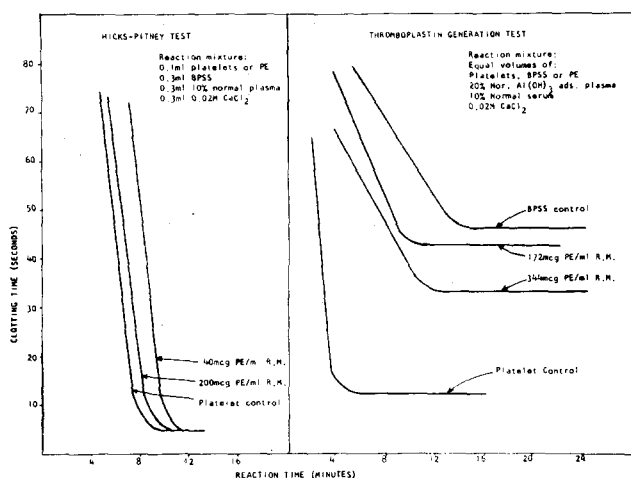


FIG. 1. Accelerating activity of egg phosphatidylethanolamine in the Hicks-Pitney test and thromboplastin generation test. At timed intervals (abscissa), 0.1 ml of the reaction mixture was added to 0.1 ml of normal human citrated plasma and 0.1 ml of 0.02 M CaCl_2 was added immediately. The clotting times are recorded on the ordinate.

After exhaustive elution with chloroform (a), the column was eluted with 4% methanol in chloroform 1 (liter) (b), 8% methanol in chloroform (1 liter) (c), and 12.5% methanol in chloroform (d). Paper chromatography of the material obtained after evaporating the eluates showed that (a) contained glycerides, lecithin, and sphingomyelin; (b) contained pure lecithin; (c) was a mixture of lecithin and phosphatidylethanolamine; and (d) was pure phosphatidylethanolamine. The pure phosphatidylethanolamine had an iodine No. 102-104; P, 4.00%; N, 1.79%. The yields of pure lecithin in (b) and pure phosphatidylethanolamine in (d) varied in different batches depending on the losses in (a) and (c). In order to obtain a quantitative separation of phosphatidylethanolamine, it was necessary to rechromatograph fraction (c). We did not recover the large amount of lecithin lost in fraction (a) since we were only interested in phosphatidylethanolamine. The total yield of phosphatidylethanolamine was 180 mg/egg yolk weighing 14 g, and the yield of lecithin in fraction (b) was 0.5-1.3 g/egg yolk.

When the products of total hydrolysis of fractions (b) and (d) were analyzed by paper chromatography, the only hydrolysis products detected were choline in (b) and ethanolamine in (d).

The location of the minor phosphatides in the chromatograph on DEAE cellulose was as follows:

The presence of sphingomyelin in fraction (a) was established by paper chromatography and was in agreement with the data given by Rouser et al. (17)

in their separations of beef brain phosphatides. These investigators found that sphingomyelin was eluted with lecithin by low concentrations of methanol in chloroform. Rouser has also shown that phosphatidylserine and inositol phosphatides are retained by the DEAE cellulose column and are not eluted by chloroform-methanol mixtures. We did not observe these phosphatides in any of our paper chromatographs of the chloroform-methanol eluates.

Solubilization of the Phosphatides. The samples of phosphatidylethanolamine were stirred into a paste with a 0.05 M imidazole buffer, pH 7.8, and the paste was diluted slowly with the buffer, with continual stirring, to a final concentration of 2 mg/ml. A similar technique was used with the preparations containing sodium desoxycholate, which was dissolved in the buffer, pH 7.5, at a concentration of 2 mg/ml together with 0.85% sodium chloride.

RESULTS

Activity in Clotting Systems. The thromboplastin generation test (8) and Hicks-Pitney test (23) were used to study the activity of the two samples of phosphatidylethanolamine. The accelerating activity of both the synthetic and egg phosphatidylethanolamine preparations was greater when measured in the Hicks-Pitney test than in the thromboplastin generation test. The synthetic preparation was more active than the egg preparation in both tests; that is, smaller concentrations produced an equivalent acceleration.

In the Hicks-Pitney test, the addition of the egg preparation at a concentration of 200 $\mu\text{g}/\text{ml}$ of reaction mixture (Fig. 1) produced rapid and complete generation of thromboplastic activity similar to that of the platelet control. Complete thromboplastic activity was still obtained when the concentration was reduced to 40 $\mu\text{g}/\text{ml}$ of reaction mixture; however, a slightly longer reaction period was required. In the thromboplastin generation test, relatively large amounts of the egg preparation (172 $\mu\text{g}/\text{ml}$ of reaction mixture) produced minimal changes in the activity curve as compared to the control with buffered saline (PBSS) alone. This control contained no phosphatide or platelets. The amount of thromboplastic activity formed was increased by doubling the concentration to 344 $\mu\text{g}/\text{ml}$ of reaction mixture, but the rate of formation of thromboplastin was not significantly affected. The concentration used in this test was the maximum obtainable under the conditions of the test. Neither the rate nor the amount of activity formed was comparable to the platelet control.

The synthetic phosphatidylethanolamine had activity in the Hicks-Pitney test comparable to that of the platelet control and at lower concentrations than the egg preparation (Fig. 2). At 40 $\mu\text{g}/\text{ml}$ of reaction mixture, the platelet and phosphatidylethanolamine curves were almost alike (Fig. 2). In the thromboplastin generation test, 120 μg of phosphatidylethanolamine/ml of reaction mixture, the maximum concentration obtainable, markedly increased the rate and amount of thromboplastic activity formed as compared to the buffered saline control, but the mixture was still less active than the system accelerated by platelets. Smaller concentrations of phosphatidylethanolamine produced proportionately less activity in both rate and amount.

Since the fractions described were dispersed in buffered saline, the maximum concentrations that could be added to the test mixtures were limited by the degree of dispersion in the diluent. Fractions solubilized in solutions of sodium desoxycholate gave results equivalent at similar concentrations to those in which the fraction was dispersed in buffered saline, provided that the concentration of sodium desoxycholate did not exceed 100 $\mu\text{g}/\text{ml}$ of reaction mixture. The use of concentrations of sodium desoxycholate in excess of this value resulted in inhibition of rate and amount of thromboplastic activity formed, as measured in the platelet and buffered saline controls. Large amounts of phosphatidylethanolamine solubilized in equivalent concentrations of sodium desoxycholate then resulted in decreased rate and amount of thromboplastic activity formed, although the concentration of phosphatidylethanolamine was great enough to produce acceleratory activity when suspended in buffered saline.

At present, the decreased accelerating activity of phosphatidylethanolamine in the thromboplastin generation test as compared to the Hicks-Pitney test cannot be explained. The mechanisms by which the two systems produce thromboplastic activity have not been clearly defined and the explanation probably will be found in the differences inherent in the reaction mixtures.

DISCUSSION

The separation of phosphatidylethanolamine on a DEAE-cellulose acetate column, essentially according to the procedure described by Rouser et al. (17), is very superior to the separation methods using silicic acid as described by Rhodes and Lea (24) and by Hawke (25). The difference lies in the remarkably high capacity of the columns of DEAE cellulose acetate. In egg phosphatides, the quantity of lecithin is 3-4 times

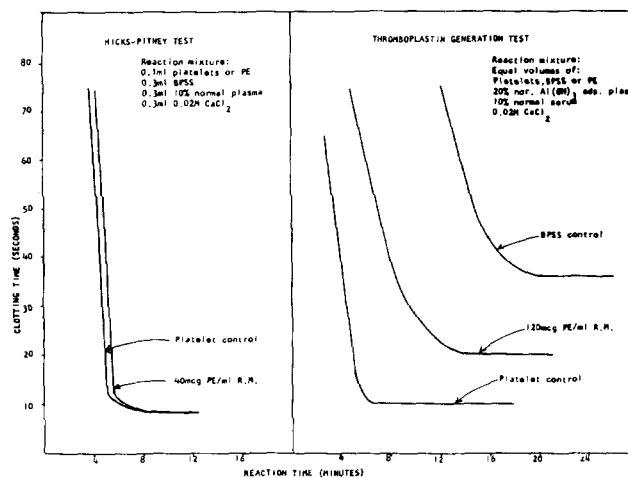


FIG. 2. Accelerating activity of synthetic phosphatidylethanolamine in the Hicks-Pitney test and thromboplastin generation test. At timed intervals (abscissa), 0.1 ml of the reaction mixture was added to 0.1 ml of normal human citrated plasma and 0.1 ml of 0.02 M CaCl_2 was added immediately. The clotting times are recorded on the ordinate.

that of the phosphatidylethanolamine. The phosphatidylethanolamine is retained by the column while the lecithin is not. The retention of the phosphatidylethanolamine is so marked that it is possible to apply 10 g of phosphatides to a column of 30 g of DEAE cellulose acetate and obtain very pure phosphatidylethanolamine.²

In the case of silicic acid columns, the lecithin is more strongly retained than the phosphatidylethanolamine, which makes it almost impossible to prepare large quantities of phosphatidylethanolamine. In addition, the DEAE cellulose column retains phosphatidylserine and inositol phosphatides so strongly, that they can only be released by glacial acetic acid or strong alkali (17). This is significant because of the statement of Slotta (7) that phosphatidylethanolamine must contain traces of phosphatidylserine for thromboplastic activity. The only possible contaminants in our egg phosphatidylethanolamine are the phosphatides that are weakly retained by the column: lecithin, lysolecithin, and sphingomyelin. Most samples of egg phosphatidylethanolamine showed only one spot when amounts of 250 μg were applied to silicic acid paper, but some samples showed minute spots running opposite lecithin and lysolecithin and it proved impossible to purify these samples further by repeated chromatography.

² Of course, such a load was not ordinarily applied and would not be adapted to the quantitative separation of phosphatidylethanolamine.

It is of interest that the clot-promoting activity of the synthetic product was observed with a racemic material and appeared equivalent to the effect obtained by Barkhan et al. (11) with the synthetic l-stereoisomer of Baer. The two preparations have also been found to behave similarly in the Hicks-Pitney test. The usual stereospecificity noted in biological effects is lacking here. This is in agreement with the ideas of Rouser et al. (26, 27), and of Wallach et al. (28), that the mechanism of the thromboplastic effect of phosphatidylethanolamine depends on the particle size and polar functional group of a micellar complex. The biological activity of the preparations of phosphatidylethanolamine described here confirms the positive findings of the authors cited above. The lower activity of egg phosphatidylethanolamine as compared with the synthetic product might be explained by the suggestion of Rouser and Schloretdt (27) that activity is associated with unsaturated fatty acids on both α and β positions. The phosphatidylethanolamine of egg contains mostly saturated acids in the β position. The exact fatty acid composition of egg phosphatidylethanolamine is given by Hawke (25). Wallach et al. (28) have studied egg phosphatidylethanolamines selected from a silicic acid column with iodine numbers much higher than the egg phosphatide studied here.

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