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Anticoagulant Activity of Phosphatidylserine Free of Lysophosphatidylserine.* (24746)

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In our earlier work(1,2) purified phosphatidylserine (PS) from pork brain was shown to be a potent anticoagulant *in vivo* and *in vitro*. However, these fractions were contaminated with lysophosphatidylserine (LPS). The present paper describes fractions free of LPS, with anticoagulant activity similar to that of the earlier fractions. This substantiates the previously recorded evidence(1,2) that anticoagulant activity is a function of naturally occurring PS.

Methods. Preparation of fractions. In our earlier method(1,2) for purification of PS from the fraction III of Folch(3) the fractions are in prolonged contact with chloroform and methanol. The method was modified by eliminating the *overnight* extraction of each fraction with chloroform and methanol, thus completing the fractionation in one day instead of eight. The extracts derived from the fraction III of Folch are designated as III-1, III-2, III-3, . . . III-8 and the residue as III-R.

Results. Paper chromatography. Fig. 1 illustrates paper chromatography of the pure serine phosphatide fractions on paper impregnated with silicic acid. The methods employed have been described(4,1). To show how purification was followed from the "cephalin" some of the cruder fractions are included. Fractions III-2 to III-8 had only one spot corresponding to PS. This spot was ninhydrin positive and appeared blue-violet under ultra-violet light (UV) in the wet state, after staining with Rhodamine 6G. The yellow center is believed to represent aldehydogenic serine phosphatide. Fraction III-R showed a spot corresponding to PS and a slow-moving component with mobility and staining properties similar to an inositol phosphatide (IP). The IP content of this fraction would explain

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† With technical assistance of Louise O'Keefe.

its relative insolubility. A PS preparation made from the same fraction III by the method of Folch‡(5) also showed a second spot which probably represents a second type of IP. Corresponding spots are found in the fraction I of Folch(3) which is known to concentrate IP. Phosphatidylethanolamine (PE) is concentrated in fraction III-1. PE was easily detected in this latter fraction, although it could not be detected in 100 μg of the Fraction III from which the III-1 was derived. Sometimes PE appears in fraction III-2 as well as in III-1. When solutions of fraction III-3 to III-8 in chloroform were kept at 4° for one month or when the fractions were

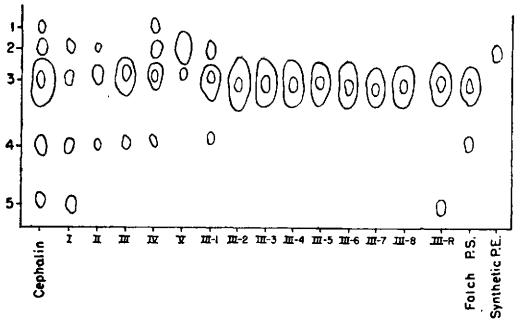

FIG. 1. *Chromatography of phospholipid fractions on paper impregnated with silicic acid.* The solvent mixture—diisobutyl ketone : acetic acid : water (40/25/5) (V/V/V)—was allowed to run ascending for 20 hr at 4°. The papers were then dried, stained with Rhodamine 6G and examined under ultra-violet light. Spots at position 2 appear yellow—phosphatidylethanolamine. Spots at position 3 appear blue-violet—phosphatidylserine (yellow centers in these spots contain aldehydogenic material). Spots at position 4 and 5 appear blue-violet, are ninhydrin negative and probably represent 2 different inositol phosphatides. Spots at position 1 appear blue-violet and remain unidentified. Synthetic PE (shown on extreme right) was L-dioleylphosphatidylethanolamine kindly provided by Dr. Erich Baer. Cephalin fraction was prepared by method of Folch(3) from pork brain. Fractions I, II, III, IV, V prepared from this cephalin by method of Folch(3). Fractions III-1 to III-8 and III-R prepared from fraction III by method described in text and in references(1,2). Fraction labelled Folch PS is a PS fraction from pork brain prepared exactly as described by Folch(5). One hundred μg of each fraction was applied to the paper in 10 μl of chloroform solution.

‡ PS fractions prepared as described by Folch(5) sometimes contain small amounts of PE as revealed by paper chromatography. Since PE has clot accelerating activity, this could explain the fact that the PS of Folch does not always have anticoagulant activity comparable to our PS fractions.

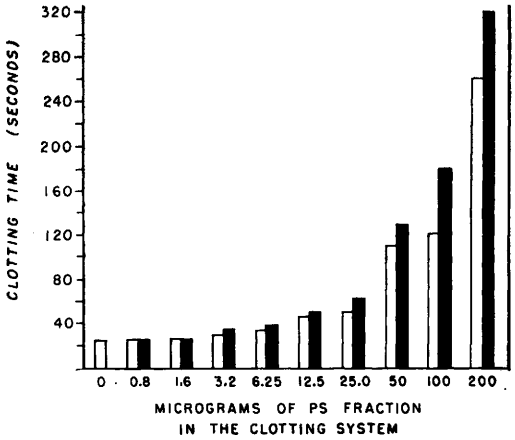

FIG. 2. *Anticoagulant activity of serine phosphatide fractions free of LPS and those containing LPS.* Unshaded bars represent clotting times produced by a solution of fraction III-4 prepared as described in this paper. Shaded bars indicate clotting times produced by a solution of a fraction III-5 prepared by the older method(1,2) and containing some LPS. Fractions tested in the fully solubilized form(2). Dilutions from original 2 mg /ml solution containing sodium desoxycholate (2 mg/ml) were made with 0.9% sodium chloride solution buffered at pH 7.4 with 0.05 M imidazole. Clotting tests were performed by adding 0.1 ml amounts of the following to siliconized tubes and noting clotting time at 37°: normal, human, citrated plasma; PS solution (or buffered saline control); human brain thromboplastin; 0.02 M $CaCl_2$ solution.

stored dry under nitrogen at −15° a second ninhydrin positive spot was noted in each, after paper chromatography. This spot corresponded to the LPS described elsewhere(1). It is evident that LPS is an artefact generated from PS during prolonged contact with chloroform and methanol, or storage after briefer treatment with these solvents.

Anticoagulant activity. Anticoagulant activity of pure PS fractions was compared to that of fractions previously described(1,2) which contained small amounts of LPS. The results (Fig. 2) show that pure PS had anticoagulant activity of the same order of magnitude as PS fractions containing LPS. The slightly longer clotting times produced by the fractions containing LPS may be due to increased solubilization of PS by LPS. The inhibiting effect of PS on formation of plasma thromboplastin (Fig. 3) and on thromboplastin itself after it is formed (Fig. 4) is noteworthy. There is little doubt that PS actually inhibits thromboplastin *formation* because of

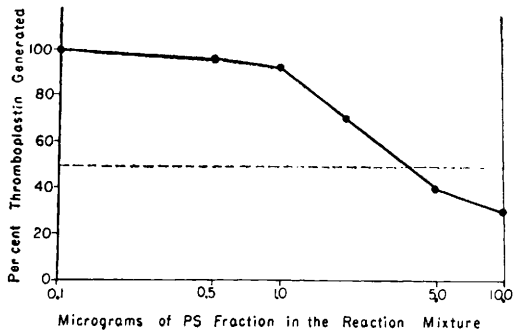

FIG. 3. *Inhibition of thromboplastin generation by purified serine phosphatide fraction.* Reaction mixture: 0.3 ml suspension of normal human platelets; 0.3 ml 20% normal, human, citrated plasma adsorbed with $Al(OH)_3$; 0.1 ml PS solution or control; 0.3 ml 10% normal human serum; 0.3 ml $CaCl_2$ solution, 0.02 M. Mixture incubated at 37° in siliconized tubes. At timed intervals 0.1 ml portions of mixture were added to 0.1 ml normal human plasma followed by 0.1 ml of 0.02 M $CaCl_2$ solution in glass tubes. Shortest clotting time obtained for a PS test solution was converted to per cent thromboplastin generated from dilution curve based on control reaction mixture(6). These values are shown by unbroken line. Broken line indicates greatest amount of thromboplastin generated by similar concentrations of sodium desoxycholate when buffered saline solution was employed instead of platelet suspension. This control was included since PS was dissolved with aid of sodium desoxycholate as described elsewhere(2) and indicates that inhibition can not be due to this solubilizing agent. With platelets in the system, the mixture containing sodium desoxycholate gives 100% thromboplastin generation. All dilutions were made with 0.9% sodium chloride solution, buffered at pH 7.4 with imidazole.

the order of magnitude of quantities of PS required to produce similar clotting times when PS is added immediately to the thromboplastin generating mixture or when it is added after full thromboplastin generation has occurred. For example, in another series of tests in which the control clotting time (full thromboplastin generation, no PS added) was 13 seconds, 0.8 μg of PS produced a clotting time of 16 seconds when PS was added immediately to the generating mixture; when PS was added to the reaction mixture after full thromboplastin generation, 80 μg was necessary to produce the same clotting time. When 80 μg of PS was added immediately to the generating mixture, clotting time was 335 seconds. Comparison of clotting times must be limited to those obtained in individual experiments, because of the variability normally found in the thromboplastin generation test (6) and because PS affects both rate and amount of thromboplastin formation(7). Within individual experiments, the quantities of PS required to produce similar delays in clotting, when added after full thromboplastin generation, varied between 10 and 100 times as much as that required when PS was added to the generating mixture, at its start.

Discussion. The purified serine phosphatide fractions described here consist mainly of PS in the diester form and contain some aldehydogenic serine phosphatide. Anticoagulant activity of these fractions must be due to PS since destruction of the aldehydogenic

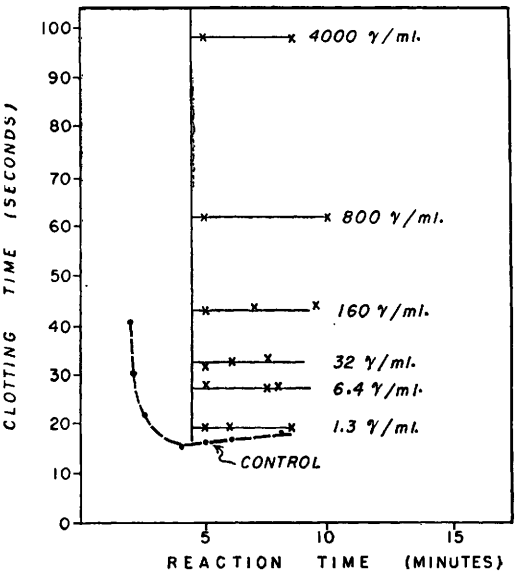

FIG. 4. *Inhibition of thromboplastic activity by purified serine phosphatide fraction after completion of generation of thromboplastin.* Reaction mixture: 0.2 ml suspension of normal human platelets; 0.2 ml 20% normal, human, citrated plasma adsorbed with $Al(OH)_3$; 0.2 ml 10% normal human serum; 0.2 ml $CaCl_2$ solution 0.02 M. Reaction mixture incubated at 37° for 4.5 min. This allowed for full thromboplastin generation as shown by clotting time of the control (broken line). Then, as indicated by vertical line, 0.2 ml of a suspension in buffered saline solution of purified serine phosphatide fraction was added to reaction mixture. Incubation at 37° was continued. Thirty sec. later (and at intervals to 10 min. thereafter) 0.1 ml of final mixture was added to 0.1 ml of normal, human, citrated, platelet-poor plasma, followed by 0.1 ml of 0.02 M $CaCl_2$ solution. Clotting times were noted (indicated by × in chart). Concentration of PS in reaction mixture is indicated alongside each curve. Control curve represents clotting times of same mixture without PS.

component does not cause loss of activity(1). The fact that such fractions have anticoagulant activity *in vitro* slightly lower than PS fractions containing small amounts of LPS argues in favor of the solubilizing effect of LPS on PS.§ The inhibitory activity of the fraction III of Folch in the thromboplastin generation test reported by Barkhan, Newlands and Wild(11) can, in the light of the findings reported here for purified serine phosphatide fractions, be attributed to PS.

Summary. Serine phosphatide fractions free of lysophosphatidylserine have been prepared from brain tissue. The strong anticoagulant activity of these fractions is ascribed to a naturally occurring serine phosphatide in the diester form which has the capacity to block formation of plasma thromboplastin as well as neutralize its effect after it is formed.

§ The importance of complete solubilization of phospholipids to obtain optimal anticoagulant activity has been mentioned elsewhere(2,8,9)) and is discussed at length in a review of lipid anticoagulants (10).

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Porphyrin Formation by Tissues from Laying Hens Fed Nicarbazin.* (24747)

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The brown color of a hen's egg is due to protoporphyrin deposited on the shell while it is in the uterine portion of oviduct(1). Uterine glandular tissue from laying hens has considerable capacity for transforming δ-amino-levulenic acid (ALA), an intermediate in the porphyrin cycle(2,3), into porphyrins *in vitro*(4). It is well established that decrease in shell color can be produced by feeding the coccidiostat nicarbazin(5), a complex of 4,4′-dinitrocarbanilide and 2-hydroxy-4,6-dimethylpyrimidine (DNC-HDP), to laying hens(6,7,8). However, a quantitative relationship between dietary level of nicarbazin and resultant decrease in shell porphyrins has not been established. Also, it is not known whether nicarbazin, of which DNC is the active component(9), prevents deposition of shell protoporphyrin by inhibiting protoporphyrin synthesis.

Methods. Hens which lay brown eggs, New Hampshires and Crossbreeds, were kept in single cages of 3-deck batteries and housed in air-conditioned room at 72 ± 2°F. Medicated rations were prepared by adding nicarbazin to open-formula breeder diet used as basal ration. All rations were fed *ad lib.* A value for egg shell porphyrin concentration was established for each hen by averaging values for all egg shells laid during 5-day period prior to feeding experimental diets. This value served as control to which subsequent values, obtained while hens were on medica-

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