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Thromboplastin. I. Phospholipid Moiety of Thromboplastin.* (25409)

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Howell(1) and McLean(2) established that the phospholipid factor necessary for formation of thromboplastin is an unsaturated cephalin. The question, however, remained whether PE† by itself, or PS by itself, their mixtures, or mixtures with lecithin are necessary. Poole and Robinson(3), O'Brien (4), and Rouser, White and Schloredt(5) maintain that PE is the active substance. Fischer and Hecht(6), however, showed that the purest PE is actually inactive. Even more confusing are opinions on activity of PS. According to Maltaner and Rapport(7), PS is active if rabbit, not avian plasma, is used in the test. Barkhan, Newlands and Wild(8) concluded from experiments on human brain PLs that PS is probably an inhibitor. Barkhan, Silva da Costa and Tocantins(9) found that PS is clearly inhibitory in buffered, saline suspension. Silva, Turner and Tocantins(10) assayed PS as an anticoagulant by using Na deoxycholate in the test suspension. Contrary to these results, Troup and Reed (11) found that of various PLs only PS gives maximal thromboplastic effect. Marcus and Spaet(12) confirmed this, stating that PS isolated from platelets could replace the platelets in thromboplastin generation. Several authors claimed that the coagulation activity is not exercised by a single PL, but by interaction of at least 2. The following authors state that either presence of lecithin is indispensable, or that lecithin increases activity of the corresponding cephalin fractions: Zunz and La Barre(13) "cytozymin"; Gracia and Levene(14) cephalin; Rapport(15) a cephalin fraction consisting mostly of PS; Therriault, Nichols and Jensen(16) very pure PS.

Methods and materials. Testing. One reason for the diametrically opposite results of different authors is the fact that they used entirely different and often dubious test methods. We found that the original Thromboplastin Generation Test of Biggs, Douglas and McFarlane(17) gives a reliable picture of activity of PL preparations, and this method was, therefore, used exclusively. PLs used, raw lecithin, protagon, and cephalin were prepared from fresh calf brain according to Klenk and Böhm(18) and Debuch(19). Lecithin was purified by chromatography on alumina(20), and cephalin was separated into 5 fractions by the method of Folch(21). Fraction III (molar ratio N : P = 0.95) contained not only PS, but also small amounts of PE, lyso-PE, mono phosphatidyl inositol, and diphosphatidyl inositol, as described by Therriault, Nichols and Jensen(16). Fraction IV (molar ratio N : P = 1.1) contained PE and PS in the proportion of 4 : 1, whereas fraction V (molar ratio N : P = 1.1) was almost pure PE with a trace of PS. Total PLs from egg were prepared from fresh egg yolks by the method of Rhodes and Lea(20). The PLs were dialyzed, and the dialyzate contained comparatively large quantities of free ethanolamine and O-phosphoryl serine, but no serine. Upon evaporation, the dialyzate left a residue which was chromatographed on silicic acid paper with phenol-water. Three ninhydrin-positive spots with Rf = 0.05 (= O-phosphoryl serine), 0.16 (= glyceryl phosphoryl serine?) and 0.73 (= ethanolamine) were obtained. We found in the total egg, PLs

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† Abbreviations used are: PL, phospholipid; PE, phosphatidyl ethanolamine; PS, phosphatidyl serine.

after dialysis 18.2% PE + lyso-PE. This corresponds to the 17.5% isolated by Rhodes and Lea(20). *Analysis of ethanolamine and serine.* The figures given above for PE and PS content of PLs we used are not absolutely correct, because the composition of the fatty acid side chains is variable. With the most recent methods of Nojima and Utsugi(22), and Dittmer, Feminella and Hanahan(23), as well as with a new method described elsewhere, we obtained good results with mixtures of pure ethanolamine and serine, but only dubious values with natural animal and especially with vegetable PLs. Thus, our values for ethanolamine and serine are only approximate. *L-α-(Dioleoyl)-PE,* obtained from Dr. Erich Baer, Toronto. By ascending chromatography on silicic acid paper with di isobutylketone, acetic acid, and water (40 : 25 : 5) only one spot, Rf = 0.73, was detectable with ninhydrin, $FeCl_3$ + sulfosalicylic acid, Sudan Black B, or Rhodamin G 6. The iodine numbers obtained by using 0.5, 1, 1, 1.5, 1.5 mg were respectively 51.3, 52.6, 54.6, 53.3, 50.8 (theor. 68).

Results. When suspensions of platelets or total brain lipids were tested in the Thromboplastin Generation Test, those containing 100 μg PLs/ml were most active. This means that the incubation mixtures contained 25 mg% PLs as the optimal concentration. In these experiments, after 3 minutes incubation an average coagulation time of 10 seconds was observed.

In all experiments serial dilutions of PL suspensions from 1,000 to 10 mg/ml were tested. Our report presents only results with levels of 100 mg/ml.

Synthetic PE is very difficult to emulsify in saline or in 0.3% pluronic F 68 acid (a polymer of ethylene sulfate). However, excellent and very stable emulsions were obtained with lecithin (1 : 1) in saline or imidazol buffer, pH 7.2. But even these emulsions had no activity (Table I, 1-4). Our total PL preparation from fresh eggs could be easily emulsified and was also inactive (Table I, 5). Fraction V certainly lacked the activity of whole platelet or brain PLs, but it was more active than the synthetic PE or egg PLs (Table I, 6). That fraction V was not completely inactive appears due to a minute amount of PS which it contains. It was demonstrated as follows that this may actually be the case: Chloroform solutions of different quantities of fraction III (mainly PS) were evaporated together with those of synthetic PE, and these mixtures of 2, 5, 10, and 15% PS with PE were tested in saline (Table I, 7-10). Even though these percentages of PS may not be quite accurate, due to difficulties of analytical determination of serine in PLs, we may conclude that fraction V contained about 2% PS (corresponding to 0.13% serine). Furthermore, it is evident that mixtures containing about 10% PS with PE are the most active. Fraction IV with 20% PS (Table I, 11) is more active than fraction V (Table I, 6). However, higher concentrations of PS than contained in fraction IV do not enhance activity, since fraction III (Table I, 12) is even less active than fraction IV (Table I, 11). A mixture of 3 parts of fraction III with 7 parts of fraction V gives practically the same coagulation time as fraction IV, because both contain about 20% PS in 80% PE (Table I, 13). Another preparation gave practically the same result (Table I, 14), and was also highly ac-

TABLE I. Activity of PS in Mixtures with PE. (Coagulation time in sec.)

Exp. No.	Incubation time in min. 1	2	3	4	5	6
1	160	142	138	135	130	115
2	160	138	135	125	100	95
3	220	210	130	125	130	102
4	102	100	95	89	65	48
5	68	59	43	39	40	54
6	27.5	17	16	20	20.5	25
7	22	15	14.5	17.5	18.5	19
8	12	11.5	12	13	15	16
9	11	8.5	10.2	12	13	15
10	12	11.5	10.5	11	12.4	14
11	14	9.5	10	10.2	11	15
12	14	11.2	12.5	13.8	13.9	14.5
13	9.2	8.2	7.5	9	11.5	12.8
14	9	8	7.5	6.4	8.5	12.5
15	15	9.5	10.8	14	17.5	18

1. Synthetic L-α-(Dioleoyl)PE in saline. 2. Same plus lecithin (1:1). 3. Same plus lecithin (1:1) in imidazol buffer. 4. Same in 0.3% pluronic F 68 acid. 5. Egg PLs. 6. Folch V. 7. 2% PS (brain), 98% synthetic PE. 8. 5% PS (brain), 95% synthetic PE. 9. 10% PS (brain), 90% synthetic PE. 10. 15% PS (brain), 85% synthetic PE. 11. Folch IV (20% PS). 12. Folch III (70% PS). 13. Folch III + V (3:7). 14. Repetition of 13 with new preparation. 15. Same as 14, diluted 1:10.

tive at levels as low as 10 μg/ml (Table I, 15).

Separation of PE from PS is difficult, particularly when only traces of PS are present. This appears to be one of the reasons why PE has often been taken for the thromboplastin PL factor. In the yolk of fresh eggs there is no PS. In agreement with this we found no thromboplastic activity, contrary to findings of others(3).

Lecithin is inactive (Table II, 1) as are also mixtures of lecithin with synthetic PE (Table I, 2, 3). Mixtures of lecithin and our fraction V (Table II, 2) have the same minimal activity as fraction V component alone. Lecithin, however, is known to influence strongly the activity of PS, as Therriault, Nichols and Jensen(16) have shown. It must be emphasized that optimal concentrations for mixtures of PS with lecithin (phosphatidyl choline), as well as PS with PE, are the same, namely, around 10% PS (Tables II, 4 and I, 9).

This fact supports the theory that the active PL is not phosphatidyl choline, PE, or PS alone, but a complex existing in nature or formed in organic solvents in stoichiometric proportions. PLs from platelets and brain contain PS, PE, and phosphatidyl choline in optimal proportions(12,24) and, therefore, possess highest activity.

As Table III shows, very active preparations, *e.g.*, of mixtures of 20% PS in PE, are not completely inactivated, if less than 1 mg of sodium deoxycholate is present/mg PL. Test suspensions become clear, because the well-known formation of deoxycholate complexes with fatty acid groups takes place.

TABLE II. Activity of PS in Mixtures with Phosphatidyl Choline. (Coagulation time in sec.)

Exp. No.	Incubation time in min. 1	2	3	4	5	6
1	75	50	39.6	36	34.5	37
2	27	17.8	17.5	16	21	24.5
3	10.5	8.8	9.8	11	12	12.5
4	9.5	8	8.8	9.8	11.8	14
5	12	11.5	10.5	11.5	12.4	14
6	11.8	11.5	13	13.8	15	20

1. Lecithin. 2. Lecithin + Folch V (1:1). 3. Lecithin + 5% PS (brain). 4. Lecithin + 10% PS (brain). 5. Lecithin + 15% PS (brain). 6. Lecithin + 20% PS (brain).

TABLE III. Inhibition of PS + PE Mixtures by Sodium Deoxycholate. (Coagulation time in sec.)

Exp. No.	Incubation time in min. 1	2	3	4	5	6
1	26	13	8	8	9	9
2	80	60	24	14	9	9
3	47.5	35	19	11	10.5	13
4	40	45	28	15.5	10	9.4
5	360	110	95	105	100	90
6	90	85	90	90	91	88
7	120	102	95	95	93	90
8	100	88	105	100	85	93
9	130	125	125	120	130	133.5

Suspensions in imidazol buffer pH 7.2 of 1 mg/ml of fraction III + V (3:7) were used containing the following amounts in mg of sodium deoxycholate per ml of suspension: (1) none, (2) 0.2, (3) 0.5, (4) 1.0, (5) 2.0, (6) 3.0, (7) 4.0, (8) 5.0, (9) 6.0.

Generation of thromboplastin only is retarded, but after 5 to 6 minutes a potent thromboplastin is formed (Table III, 1-4).

If, however, 2 to 6 mg sodium deoxycholate/mg PL is used, pronounced and practically the same inhibition effects occur (Table II, 5-9). The threshold value lies between 1 to 2 mg deoxycholate to 1 mg PLs. Apparently below this ratio less than one deoxycholate is available for each fatty acid ester in the PLs. Evidently, a 1 : 1 relationship is necessary for maximal inhibitory effect.

Summary. Phosphatidyl ethanolamine and phosphatidyl choline are inactive in the Thromboplastin Generation Test, but they become highly active when present with about 10% phosphatidyl serine. Phospholipids from fresh eggs are inactive, because they contain no phosphatidyl serine. Sodium deoxycholate gives clear, but less active or inhibitory suspensions.

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Effect of Methyl Linoleate on Tissue Cholesterol of Normal and Vit. E-Deficient Rabbits.* (25410)

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It has been shown(1,2) that a diet high in polyunsaturated fatty acids could significantly lower serum cholesterol; whereas Vit. E-deficient diet increased serum and muscle cholesterol(3,4). Shull *et al.*(5) have shown that supplementation of Vit. E-deficient rations with certain antioxidants delayed onset of dystrophy symptoms, but did not prevent the rise in plasma and muscle cholesterol. It appears, therefore, that Vit. E and unsaturated fatty acids might be interrelated with one another in controlling cholesterol levels of various body tissues. Previous studies on the effect of Vit. E or essential fatty acids on serum or tissue cholesterol level have been made with either Vit. E or essential fatty acids alone. These have been further complicated by simultaneous feeding of cholesterol or other antioxidants. Our studies describe the effect of feeding Vit. E and methyl linoleate on cholesterol content of serum, liver, kidney, heart, spleen, brain and muscle of normal and Vit. E-deficient rabbits.

Methods. Forty-two rabbits of both sexes were divided into 4 groups and fed the following diets *ad lib.*: A. Dystrophy producing diet of Young and Dinning(6); B. Dystrophy producing diet plus 10% methyl linoleate; C. Dystrophy producing diet plus 8 mg oral alpha tocopherol acetate thrice weekly; D. Dystrophy producing diet plus 8 mg oral alpha tocopherol acetate thrice weekly plus 10% methyl linoleate. When methyl linoleate was incorporated into the diet, an equivalent weight of sucrose was deducted. After symptoms appeared(7), blood was withdrawn by heart puncture and animals were sacrificed by intravenous injection of air. Then aliquots of spleen, kidney, liver, heart, brain and muscle were removed, and approximately 100 mg (wet wt) were used for cholesterol analyses. Cholesterol was determined by the method of Herrmann(8) after slight modifications suggested by the author.

Results. Distribution of cholesterol in vari-

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