Solubilization of Fibrin s by Phospholipids.* (28157)

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One of the most widely used procedures for detection and estimation of fibrinolytic and plasminogen activator activities is the fibrin plate method of Astrup and Mullertz(1), employing heated and unheated plates, respectively(2). Despite the widespread acceptance of this procedure for both clinical and research purposes, insufficient attention is often paid to the precise condition under which fibrin formation occurs. Factors such as concentration and ionic strength of the fibrinogen solution and presence or absence of calcium ions affect the type of fibrin formed (3,4). Thus, in the absence of calcium, fibrin s (urea-soluble fibrin) is produced; this is less resistant to lysis by plasmin than fibrin i (urea-insoluble fibrin) formed in the presence of calcium(4,5). Formation of fibrin i results from the action of fibrin-stabilizing factor (FSF), a thermolabile component of plasma requiring calcium for its activity (3). In addition to urea, other substances such as histamine dihydrochloride, monochloroacetic acid, guanidine hydrochloride and bile salts solubilize fibrin s, whereas none of these agents dissolves fibrin i(6,7).

During a recent study of the effects of phospholipids on the fibrinolytic mechanism, it was observed that some phospholipids possessed what at first appeared to be fibrinolytic activity, but what on further investigation was found to be an ability to solubilize certain types of fibrin preparations. It is the purpose of this paper to report some of these observations and to discuss their practical significance.

Methods and materials. Bovine fibrinogen, a purified product with greater than 90% clottability, was obtained from Pentex, Inc., Kankakee, Ill. Fibrinogen solutions (0.8%) were prepared in different pH 7.4 buffers of varying ionic strength. The solutions were filtered through glass wool to remove any insoluble material. Dry sodium chloride was added to the fibrinogen solutions to increase their ionic strengths when desired.

Thrombin (Parke-Davis, topical) was made up in 0.85% sodium chloride to contain 100 units/ml and stored at –40°C until used.

Plasmin was obtained from human plasminogen prepared by the method of Kline(8). It was derived from plasminogen by streptokinase activation and precipitation at pH 2.0 in 1 M sodium chloride(9). The plasmin was dissolved in 50% glycerol and stored at –40°C in 0.5 ml aliquots having an activity of 10 casein units/ml.

Phosphatidyl inositol (PI), phosphatidyl serine (PS), and phosphatidyl ethanolamine (PE), all purified preparations from bovine cephalin(10), were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. A lecithin (LE) fraction was prepared from acetone-washed, mixed soybean phosphatides (asolectin) according to the solvent fractionation procedure of Folch(11). The phospholipid preparations were emulsified in distilled water by means of a 5 minute treatment at room temperature in an MSE ultrasonic vibrator at a frequency of 20 kilocycles/second. The resulting emulsions were usually used immediately, although emulsions stored at 4°C for a period of up to 7 days produced essentially the same results.

Fibrin s plates were prepared by clotting 7.0 ml volumes of the different fibrinogen solutions with 1.5 ml of thrombin in 100 × 15 mm Petri dishes. To obtain fibrin i plates this procedure was modified to provide for the presence of 0.01 M calcium chloride. Thirty microliters of the substance to be tested were placed on the fibrin surface and the plates incubated at 37°C. The areas of the lysed or solubilized zones were estimated as the products of 2 perpendicular diameters. Each experiment was carried out in triplicate

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and the data reported are the averages from at least 3 different experiments.

Tyrosine was determined as described by Ratnoff and Menzie(12).

Results. Attempts to determine whether or not the action of certain phospholipids on fibrin s involves a solubilization of fibrin rather than actual lysis proved difficult. In the case of urea and bile salts, both of which dissolve fibrin s, solubilization can be demonstrated by dialyzing out the solvent and allowing the fibrin clot to reform. In the case of the phospholipids this was not possible, since they are not removed from the system by dialysis. However, it was found that they could be extracted with ether following saturation of the system with sodium chloride, and that subsequent dialysis to remove excess salt resulted in reformation of fibrin. The solubilization of fibrin s was also indicated by the observation that, upon dissolution of the clot, there was no significant increase in trichloroacetic acid (TCA)-soluble tyrosine as is always found when a clot is lysed by plasmin or other proteolytic enzymes. Results of such an experiment are presented in Table I.

Only phosphatidyl inositol (PI) and phosphatidyl serine (PS) solubilized fibrin s at suitable ionic concentrations; the effect was not observed with either phosphatidyl ethanolamine (PE) or lecithin (LE) (Table II). The presence of 0.01 M calcium during the clotting of fibrinogen completely prevents the dissolution of fibrin by PI and PS, presumably because it results in the formation of fibrin i. These findings are essentially similar to those reported by Shulman and coworkers for various inorganic salts(6) and by Fleming *et al.* for bile salts and detergents(7). The degree of solubilization produced by both PI and PS progressively decreases as the ionic strength of the system increases (Table II). Furthermore, it appears that this is a general relationship, independent of the particular buffer employed (Table III).

TABLE I. Trichloroacetic Acid (TCA)—Soluble Tyrosine Released from Fibrin s During (a) Lysis by Plasmin, and (b) Solubilization by Urea, Phosphatidyl Inositol, and Phosphatidyl Serine.

Test substance	Increase in TCA—soluble tyrosine over control (μg/ml)	(%)
Plasmin (10 casein units/ml)	7.3	1.7
4 M urea	.2	.1
1% phosphatidyl inositol	.6	.1
1% phosphatidyl serine	.4	.1

Time of incubation: 16 hr.
Buffer: 0.005 M imidazole; ionic strength (fibrin plate): .041.

TABLE 2. Effect of Ionic Strength on Solubilization of Fibrin s by Various Phospholipids.

Buffer concentration	Ionic strength (fibrin plate)	Solubilizing agent	Area solubilized (mm²) 6 hr	24 hr
.005 M	.041	PI	80	178
"	"	PS	156	540
"	"	PE	0	0
"	"	LE	0	0
"	.085	PI	80	115
"	"	PS	115	178
"	"	PE	0	0
"	"	LE	0	0
"	.131	PI	13	51
"	"	PS	13	64
"	"	PE	0	0
"	"	LE	0	0
"	.176	PI	0	13
"	"	PS	0	7
"	"	PE	0	0
"	"	LE	0	0
.250 M	.766	PI	0	0
"	"	PS	0	0
"	"	PE	0	0
"	"	LE	0	0

Concentration of phospholipid solution applied: 1%.
Buffer: Imidazole.

TABLE III. Effects of Various Buffers at Different Ionic Strengths on Solubilization of Fibrin s by Phosphatidyl Inositol.

Buffer	Ionic strength (fibrin plate)	Area solubilized (mm²) 6 hr	24 hr
Imidazole	.041	80	178
(.05 M)	.176	0	13
Tris	.068	80	204
(.05 M)	.176	0	13
Barbital	.047	115	258
(.03 M)	.176	0	29
Borate	.036	146	314
(.005 M)	.176	0	39

Concentration of phosphatidyl inositol applied: 1%.

Discussion. The mechanism by which PI, PS or other substances dissolve fibrin s is unknown. Of the phosphatides tested, only those with acidic characteristics are effective. It may be primarily this property which promotes rupture of the secondary hydrogen bonds holding the fibrin strands in the loose

network of the fibrin s clot. When calcium is present, fibrin-stabilizing factor (present in most fibrinogen preparations) gives rise to fibrin i by cross-linking these fibrin strands and thereby making them resistant to the action of solubilizing agents. The data suggest that there may be additional, and possibly physiological, substances whose fibrin s solubilizing ability has not yet been reported. Therefore, to avoid possible misinterpretation of fibrinolytic activity data obtained with the fibrin plate method it would appear essential that a) only fibrin i preparations be used in such tests, and b) the ionic concentration of the fibrinogen solution used be carefully standardized and controlled.

Summary. Solubilization of bovine fibrin s preparations by phosphatidyl inositol and phosphatidyl serine has been observed whereas phosphatidyl ethanolamine and lecithin did not have this ability. The solubilizing effect did not occur with preparations of bovine fibrin i. The extent of solubilization progressively diminished with increasing ionic concentration of the system, but was not significantly influenced by the type of buffer. The importance of using only fibrin i preparations in the fibrin plate assay for fibrinolytic or activator activities and the need for careful control of the ionic strength of the assay system are stressed.

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Isolation and Properties of a Human Enterovirus, Non-Pathogenic for Monkey Kidney Cell Cultures and Suckling Mice.* (28158)

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During studies on the association of enteroviruses with human diarrheal disease a virus was isolated on April 14, 1959, in human amnion (FL line) cell cultures from the stool specimen of a 9-month-old male child. The virus proved to be non-pathogenic for monkey kidney cell cultures and for suckling mice. It could not be identified as any of the recognized human enteroviruses. The original specimen (in the form of a rectal swab) was sent to the laboratory by accident; the patient was actually admitted to the Children's Hospital, University of Pittsburgh Health Center, because of an acute upper respiratory illness of undetermined etiology. Due to absence of diarrheal disease acute and convalescent serum specimens were not obtained.

Materials and methods. Source of viruses and immune sera. Poliomyelitis Type 1-3, Coxsackie A 7, 9, B 1-6, ECHO 1-27 viruses were kindly supplied by Dr. D. S. Yohn; ECHO 28 virus and immune serum by Dr. W. J. Mogabgab; E 59 (JV 10) virus and immune serum by Dr. W. C. Branche; Coxsackie A 21 (Coe) virus and immune serum by Dr. E. H. Lennette; the Pett virus(20) by Dr. J. A. Kasel, and Coxsackie A 2,

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