Identification of Lipids in Blood Thromboplastin.* (27913)

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Blood and tissue thromboplastin apparently differ, as the former will correct the prolonged prothrombin time seen in Stuart or labile factor deficiency while the latter is ineffective(1,2). The active portion of tissue thromboplastin has been shown to be pure lipid(3), but at present the activity of blood thromboplastin has been related only to its total phospholipid content(4). However, the mere presence of phospholipid in blood thromboplastin does not insure strong activity. Previous work(4) has shown that the total quantity of phospholipids in active, sedimented thromboplastin is less than the total quantity in the sedimented precursors. Therefore, activity is not simply a matter of quantity of phospholipid. However, very low amounts of sedimented phospholipids are found in the least active thromboplastin samples, so that there appears to be a minimal amount of phospholipid required for activity.

This paper is concerned with the lipids found in blood thromboplastin and a correlation of a gross change in these lipids with variations in thromboplastic activity.

Methods. Blood thromboplastin was prepared from human blood factors and was sedimented and washed as previously described(4). Thromboplastic activity was checked by adding 0.1 ml of the thromboplastin mixture to recalcified human plasma and measuring the clotting time. Occasionally during the washing process of preparing the thromboplastin, activity was lost so that wide variations in activity were seen.

For column chromatography, blood thromboplastin was prepared in 6 ml quantities until a total of 84 ml of active thromboplastin was collected. As each 6 ml aliquot was prepared, it was extracted as previously described(4). Each extract was placed in a common extraction flask and evaporated at 42°C under reduced pressure with a constant flow of nitrogen. Between each evaporation, the extraction flask was stored under nitrogen in a vacuum desiccator. After the total 84 ml had thus been pooled, extracted, and evaporated, the residue was extracted for 10 minutes at 42°C with 56 ml of chloroform. This was repeated for a total of 4 extractions. The extractions were pooled and made to a total volume of 250 ml with chloroform. Duplicate 1 ml samples were taken for phosphorus analysis by the modification of Marinetti(5). The remaining chloroform was evaporated under nitrogen. The lipid residue was dissolved in chloroform-methanol 1:1 and applied to a silicic acid column using the precautions of Rouser(6). The column was developed with 50 ml of chloroform as the first solvent, 100 ml of chloroform-methanol 4:1 as the second, 150 ml of chloroform-methanol 1:1 as the third, and 150 ml of methanol as the fourth. A flow rate of 1 ml/minute was maintained, and the eluent was collected in 5 ml aliquots in glass stoppered tubes. One ml aliquots were taken from each tube and analyzed for phosphorus.

The eluent from the chloroform extraction was pooled and evaporated under nitrogen at 37°C. The residue was dissolved in isoamyl alcohol and benzene 1:1 and spotted on silicic acid impregnated paper. Chromatography was performed using n-heptane and 2,6 dimethyl-4 heptanone as the solvent(7). The lipids were identified using a cholesterol standard and staining reactions as described by Marinetti(7).

After phosphorus determinations on each tube, the eluent from the chloroform-methanol 4:1 extraction was pooled and evaporated under nitrogen. The residue was dissolved in chloroform-methanol 1:1 and placed on a silicic acid, silicate, water column(6). A flow rate of 1 ml/minute was maintained using 100 ml chloroform-methanol 4:1 as the first solvent, followed with 100 ml methanol. Samples were collected in 5 ml aliquots.

The tubes containing phospholipids were evaporated under nitrogen, and the residue

* Supported by U.S.P.H.S. grant.

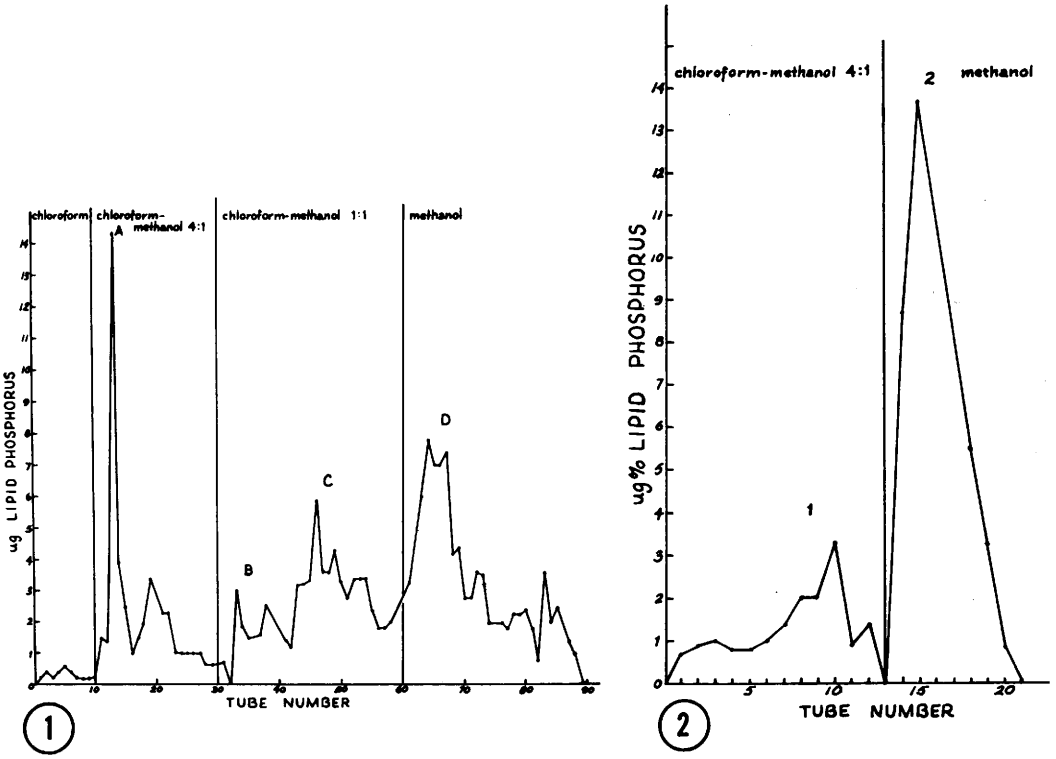

FIG. 1. Silicic acid chromatography pattern of phospholipids from blood thromboplastin. Peak A contains phosphatidyl serine and phosphatidyl ethanolamine; peak B, inositol phosphatide; peak C, lecithin; and peak D, lecithin and sphingomyelin.

FIG. 2. Silicic acid, silicate, water chromatography pattern of peak A in Fig. 1. Peak 1 contains phosphatidyl ethanolamine, and peak 2 contains phosphatidyl serine.

was dissolved in iso-amyl alcohol-benzene 1:1. A sample from each tube was spotted on silicic acid impregnated paper, and chromatography was performed using 2,6 dimethyl-4 heptanone, acetic acid, and water as a solvent system(8). The phospholipids were identified by established staining reactions(8) and by simultaneous use of standards of phosphatidyl serine and phosphatidyl ethanolamine (Nutritional Biochemicals Corp.). The standards were further purified by chromatography before use.

Silicic acid paper chromatography was performed on 70 individual 3 ml aliquots of blood thromboplastin. After determining thromboplastic activity, the lipids were extracted, and chromatography was performed. In 50 samples the solvent was 2,6 dimethyl-4 heptanone, acetic acid, and water (40:25:5), and in 8 samples, the solvent composition was changed to 40:30:3(8). In the remaining 12 patients, the solvent was n-heptane and 2,6 dimethyl-4 heptanone(7). The lipids were identified as described above.

Results. Column chromatography led to identification of phosphatidyl serine, phosphatidyl ethanolamine, inositol phosphatide, lecithin and sphingomyelin (Fig. 1, 2). Paper chromatography demonstrated that the eluent from the first 50 ml of chloroform passed through the silicic acid column contained only cholesterol. Silicic acid paper chromatography of 58 specimens of blood thromboplastin constantly demonstrated these same 5 phospholipids even though thromboplastic activity varied from 5.8 seconds to 31.6 seconds.

When examining the chromatographic pattern of the phospholipids, a spot was always noticed at the solvent edge. The identity of the lipids in this spot was determined in 12 specimens of blood thromboplastin by silicic acid paper chromatography using n-heptane and 2,6 dimethyl-4 heptanone as a solvent.

Cholesterol was present in all specimens even though the thromboplastic activity varied from 7.0 seconds to 22.8 seconds. No other lipids were present.

Discussion. The role of lipids in coagulation is becoming much more apparent. Phospholipids can react with product I(9) and substitute for platelets in the thromboplastin generation test(10,11). However, this depends to a great degree on pH, ion concentration, temperature(12), unsaturated fatty acid side chains(13), and net surface charge (14).

The data presented here show that 5 phospholipids and cholesterol are present in blood thromboplastin despite wide variations in thromboplastic activity. Thus, the presence of a single phospholipid does not insure active thromboplastin.

The possibility exists that these lipids merely represent the centrifugation of platelet lipids into the button of thromboplastin. This doubtless does occur to some extent. However, our previous work(4) has shown that active thromboplastin contains less total phospholipid than the sedimented precursor substances. Therefore there apparently is not just a simple centrifugation of lipids. In addition, cholesterol has been implicated in an indirect manner in coagulation(15,16). Perhaps its presence here may explain the findings of others showing it to accelerate coagulation.

Investigation is under way to see if there are quantitative changes in any of these lipids with variations in thromboplastic activity.

Summary. Lipids from blood thromboplastin were identified by column chromatography and paper chromatography as inositol phosphatide, lecithin, sphingomyelin, phosphatidyl serine, phosphatidyl ethanolamine, and cholesterol. Despite wide variations in thromboplastic activity in 70 samples of blood thromboplastin, there was no change in the presence of any of the lipids.

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Received October 1, 1962. P.S.E.B.M., 1962, v111.