# Relation of Blood Thromboplastin Activity to Its Total Phospholipid Content.* (27258)

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The important role of phospholipids in the clotting mechanism has been appreciated for many years. Various phospholipids have been investigated for their thromboplastic activity or for their ability to enhance coagulation(1-9). It now seems apparent that the ability of various phospholipids to support coagulation *in vitro* depends on their ability to form a colloidal suspension which, in turn, is influenced by the unsaturation of the fatty acids on the $\alpha'$ and $\beta$ side chains(6), by pH, oxidation, ionic strength and temperature(4).

Our data relate the phospholipid content of blood thromboplastin to its thromboplastic activity.

*Methods and materials.* Blood from normal fasting human subjects was collected with siliconized equipment. A 20 ml sample was distributed among 3 tubes; 5 ml for oxalated plasma, 10 ml mixed with EDTA and 5 ml without anticoagulant for serum.

Blood thromboplastin was prepared from human blood by the method of Spaet and Cintron(10). Plasma and serum were separated and frozen at –20°C for use the next day. Dilutions of the thromboplastin reagents were made with 0.85% saline buffered to pH 7.3 with imidazole buffer. Platelets were obtained by differential centrifugation of blood containing EDTA, washed 3 times with 0.85% saline, and concentrated 3-fold in buffered saline. They were frozen at –20°C for use the next day. One ml of barium sulfate adsorbed human plasma diluted 1:5, one ml of serum diluted 1:10 and one ml of 0.025 M calcium chloride were placed in each of 3 glass centrifuge tubes. These were mixed by tilting and allowed to incubate for 15 minutes in a water bath at 37°C. At this time, a filmy clot was usually present and was removed with a wooden applicator stick. Occasionally, an additional 5 minutes of incubation was necessary for clot formation to occur. One ml of the platelet suspension then was added to each tube and the mixture incubated for 5 minutes. The thromboplastic activity thus generated was checked by adding 0.1 ml of the mixture to recalcified human plasma and obtaining a clotting time. The tubes containing the thromboplastin mixture were centrfiuged at 4°C at 18,500 rpm for 30 minutes. The supernatant was discarded, and the button of thromboplastin was suspended in 4 ml of buffered saline containing 0.025 M calcium chloride by grinding with a glass stirring rod. The tubes were centrifuged as before and the washing process repeated one additional time. After the final centrifugation, the buttons of thromboplastin were each resuspended in one ml of buffered saline-calcium chloride solution and pooled to give a total volume of 3 ml. The thromboplastic activity of these pooled thromboplastin suspensions was checked as previously described. A normal activity, measured by adding the thromboplastin to a substrate of recalcified human plasma, was denoted by a clotting time of 10-13.9 seconds. During the washing procedure thromboplastic activity very frequently was lost. That is, before centrifugation a good activity was obtained, but after repeated centrifugation and washing, final thromboplastic activity was markedly reduced. In other procedures formation of thromboplastin was inhibited by reducing the pH of the buffering saline to 4.5 and 3.0.

Three ml of this thromboplastin was analyzed in duplicate for total phospholipid content by the method of Marinetti(11,12) in 54 patients. In control procedures phospholipid content of whole plasma was measured after diluting it 1:2, 1:4 and 1:10 prior to analysis. The phospholipid content showed a linear de-

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cline with dilution from 174 μg% to 18 μg% with an accuracy of ± 0.8 μg%.

Total phospholipid content of the preincubation mixture for preparing the thromboplastin was investigated in 31 patients. Barium sulfate treated plasma, serum, calcium chloride and platelets in the proper proportions for generation of blood thromboplastin were mixed according to Spaet and Cintron (10), but the generation of any thromboplastic activity was prevented in an aliquot by quickly transferring 3 ml of the mixture in a 50 ml conical centrifuge tube and immediate addition of 12 ml of hot ethanol (60°C) followed by 4 ml of chloroform. The rest of the analytical procedure then was carried out.

Finally, the phospholipid content of the button obtained by centrifugation of the precursor substances without thromboplastin formation was determined in an additional 23 subjects. The ingredients for preparation of thromboplastin were mixed, but thromboplastin formation was prevented by substitution of 0.85% saline for the calcium chloride solution. After addition of platelets and incubation, the total absence of thromboplastic activity was established, the mixture centrifuged, washed, resuspended and phospholipid analysis completed. The results were compared with analyses of thromboplastin made from the same ingredients.

*Results.* Fig. 1 shows total phospholipid content of the formed blood thromboplastin plotted against its thromboplastin activity in seconds. Phospholipid concentration has a definite correlation with amount of thromboplastic activity. The total phospholipid content quickly falls with decreasing activity but soon reaches a plateau and remains there despite decreasing thromboplastin activity. The total phospholipid appears to contribute significantly to the thromboplastic activity only when the concentration is above 30 μg of lipid phosphorus per 3 ml of thromboplastin.

On analyzing this observation of decreasing thromboplastin activity with decreasing phospholipid content, it seems evident that there are 2 distinct possible causes. One is that the increased activity and phospholipid content are directly related to a higher phospholipid content of the precursor ingredients

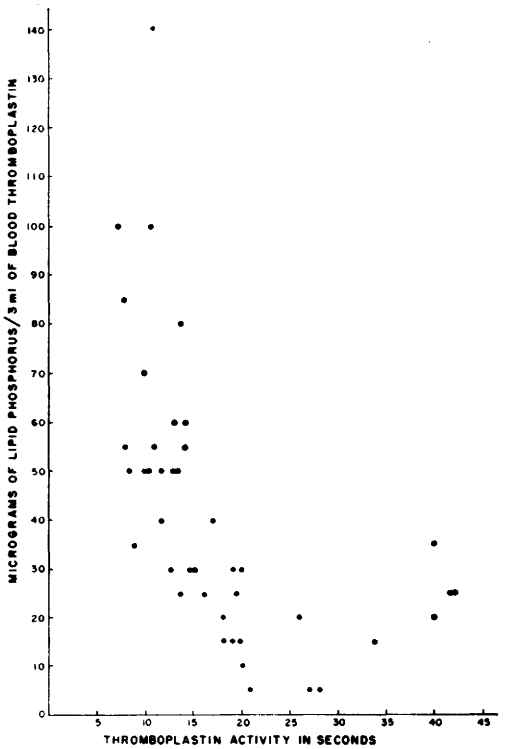


FIG. 1. Lipid phosphorus content of thromboplastin is plotted against thromboplastin activity. As thromboplastin activity decreases, there is a decrease in total lipid phosphorus content.

and do not indicate a true correlation of phospholipid content of blood thromboplastin and its activity. The other is that loss of phospholipid is associated with a loss of activity during the washing process of preparing the thromboplastin suspension. These possibilities are explored in the following tables.

Table I shows the results obtained when total phospholipid content of the preincubation mixture is compared with total phospholipid found in the thromboplastin. The average of total lipid phosphorus in the preincubation mixtures associated with normal thromboplastic activity was 113.8 μg% and 106 μg% in those associated with abnormal thromboplastic activity. Yet the average in the thromboplastins with normal activity was 41.1 μg% and only 22.8 μg% in the abnormal. Essentially the same results are obtained when centrifugation of the precursor substances is carried out (Table II). Data

in both tables demonstrate that total phospholipid content of the precursor substances does not correlate at all with phospholipid content of the blood thromboplastin.

The higher yield of phospholipid found in active thromboplastins suggests that a loss of activity is coincident with a loss of phospholipid. However, as shown in Table III, a high yield of total phospholipid is obtained in samples in which thromboplastin formation was inhibited by lowering the pH. This strongly suggests that the mere presence of phospholipid is not sufficient for thromboplastic activity but that a definite pattern of coupling or certain essential phospholipids must be present to contribute significantly to the thromboplastic activity.

TABLE I. Lipid Phosphorus in Precursors and Thromboplastin as Related to Thromboplastin Activity.

| | Final thromboplastin activity in sec. | μg of lipid phosphorus | |
|---|---|---|---|
| | | Preincubation mixture | 3 ml thromboplastin |
| Normal activity | 9.6 | 132 | 24 |
| | 10.0 | 126 | 69 |
| | 10.4 | 132 | 65 |
| | 10.6 | 100 | 70 |
| | 11.4 | 104 | 45 |
| | 11.6 | 80 | 39 |
| | 11.6 | 124 | 30 |
| | 11.6 | 80 | 40 |
| | 12.0 | 120 | 39 |
| | 12.2 | 108 | 33 |
| | 12.0 | 92 | 18 |
| | 12.6 | 92 | 25 |
| | 13.0 | 148 | 44 |
| | 13.2 | 156 | 35 |
| Avg | | 113.8 | 41.4 |
| Abnormal activity | 14.0 | 124 | 40 |
| | 14.0 | 96 | 30 |
| | 14.0 | 92 | 20 |
| | 14.2 | 168 | 44 |
| | 14.6 | 132 | 56 |
| | 14.8 | 88 | 11 |
| | 16.2 | 156 | 17 |
| | 18.0 | 104 | 18 |
| | 19.4 | 96 | 17 |
| | 19.6 | 92 | 17 |
| | 19.8 | 100 | 15 |
| | 22.4 | 91 | 16 |
| | 25.8 | 86 | 23 |
| | 26.0 | 104 | 18 |
| | 27.0 | 80 | 16 |
| | 27.4 | 100 | 17 |
| | 34.6 | 92 | 14 |
| Avg | | 106 | 22.8 |

*Discussion.* Data similar to those presented here have been obtained by Chargaff (13). He showed a relation between clotting times of chicken plasma and the concentration of phospholipid obtained from platelets. Marcus *et al.*(7,8) and others(13) observed that phospholipids isolated from platelets could be substituted for platelets in the thromboplastin generation tests. Therefore,

TABLE II. Lipid Phosphorus in Centrifuged Precursor Substances and Thromboplastin as Related to Thromboplastin Activity.

| | Final thromboplastin activity in sec. | μg of lipid phosphorus | |
|---|---|---|---|
| | | Precursor substance | 2 ml thromboplastin |
| Normal activity | 7.2 | 120 | 95 |
| | 7.2 | 170 | 55 |
| | 7.8 | 95 | 85 |
| | 9.0 | 100 | 35 |
| | 10.0 | 135 | 50 |
| | 10.2 | 180 | 50 |
| | 10.6 | 165 | 70 |
| | 10.6 | 145 | 65 |
| | 10.8 | 160 | 45 |
| | 11.0 | 105 | 55 |
| | 11.6 | 105 | 25 |
| | 12.0 | 165 | 25 |
| | 12.8 | 90 | 50 |
| | 13.6 | 75 | 25 |
| Avg | | 129 | 52 |
| Abnormal activity | 14.2 | 85 | 30 |
| | 14.8 | 165 | 40 |
| | 16.2 | 310 | 75 |
| | 16.2 | 125 | 25 |
| | 17.2 | 150 | 35 |
| | 17.8 | 70 | 40 |
| | 18.0 | 50 | 20 |
| | 19.0 | 55 | 20 |
| | 19.6 | 125 | 25 |
| Avg | | 126 | 34.4 |

TABLE III. Phospholipid Contained when Acid Buffer Used.

| Thromboplastin activity in sec. | | μg total lipid phosphorus/3 ml thromboplastin |
|---|---|---|
| Before washing | After washing | |
| 15.4 | 20.6 | 120 |
| 18.4 | 21.0 | 175 |
| 22.8 | 21.8 | 227 |
| 22.0 | 21.8 | 235 |
| 21.2 | 22.4 | 80 |
| 15.0 | 27.0 | 80 |
| 23.6 | 32.8 | 60 |
| 28.2 | 56.2 | 130 |
| 48.6 | 57.6 | 90 |
| 37.0 | 61.0 | 110 |
| 35.0 | 80.0 | 85 |
| 24.0 | 96.0 | 75 |

it is conceivable that the relation between total phospholipids and blood thromboplastin clotting times obtained in this investigation may merely represent an artifact of centrifugation of platelets and their phospholipid material into the thromboplastin matrix.

We do not think that this is true for several reasons. First, as shown in Table I, total phospholipid content of the preincubation mixture is much greater than that found in the formed thromboplastin matrix. Also, as shown in Table II, there is no correlation between phospholipid content of the centrifugation product containing the precursor substances and thromboplastic activity or total phospholipid content of the thromboplastin. Thus, the phospholipid of the formed thromboplastin is apparently not related simply to centrifugation of platelets and phospholipid micelles.

The amount of phospholipid found in the blood thromboplastin is directly related to clotting activity of the thromboplastin, as the most active thromboplastins (*i.e.*, those with the shortest clotting time) have the greatest amounts of phospholipid. There seems to be a correlation here between loss of activity and loss of total phospholipid. However, adequate amounts of phospholipid alone do not mean that strong thromboplastic activity will be present. As shown in Table III, if initial adequate thromboplastic activity is never attained because of low pH, the amount of sedimented phospholipid is very high despite low blood thromboplastin activity. Apparently, either a certain order of linkage, certain phospholipids or the proper ratios of phospholipids must be incorporated into blood thromboplastin during generation to obtain activity. This strongly suggests that active chemical changes occur during blood thromboplastin formation. We interpret this evidence to indicate that phospholipids play an essential role in blood thromboplastin activity. The relation between amount of thromboplastin activity and phospholipid content is compatible with this concept.

*Summary.* (1) The amount of total phospholipid in blood thromboplastin was found to correlate with thromboplastic activity. (2). A sufficient concentration of the total phospholipid in formed thromboplastin appears to be critical. However, the mere presence of phospholipid does not insure strong thromboplastic activity. (3) There was no relation between amount of total phospholipid in the precursor substances, either before or after centrifugation, and total phospholipid in the blood thromboplastin. This strongly suggests that active changes occur during thromboplastin formation.

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