Purified Platelet Phospholipids and Blood Coagulation

By John H. Ferguson, Aaron J. Marcus and A. Jean Robinson

Platelet phosphatidyserine (PS) and phosphatidylethanolamine (PE) have been successfully isolated and purified1-3,8 by applying methods worked out by Rouser and colleagues4 for the corresponding brain phospholipids. With the modern evolution5,6 of clotting tests, applicable to research on the role of phosphatides7,8 in blood coagulation, an opportunity was offered for a collaborative effort to study the purified platelet phospholipids and to compare them with brain cephalin in these test systems. A significant new bioassay has been validated and is supplemented by data concerning altering reactivities and effects of mixtures of these phosphatides. Comparisons are made between the thromboplastic enzyme methods and the other types of clotting tests which seek a solution to these complex problems.

Materials and Methods

Details of well-known routines will be omitted, but relevant information about the present systems will be given under this head and in the sections to follow. The buffer salt solution (buff. sal.) was imidazole-buffered NaCl, pH 7.3.9 Organic materials were frozen-stored at −20°C.

Human plasmas (citrated) and sera were obtained from normal individuals and one Hageman-deficient (Hag- ) subject, L.C. Factor V was BaCO3-adsorbed beef serum,11 thrombin-free, used in 1:10 dilution.

Eluate (El) was a single stock of BaSO4-citrate eluate from normal oxalated dog plasma.5 It provided, essentially, a standard prothrombin, which was free from thrombin but adequate in other needed clotting factors (particularly factors X and VII) when supplemented by factor V (above) and used in the manner to be described.

Fibrinogen. This was also obtained from a single stock, namely, Armour’s lyophilized bovine fibrinogen (“Fraction I”). The bulk powder was 10 years old and partly denatured, but yielded filtered solutions of well-stabilized “reactivity.” A routine adsorption with BaSO4 (despite the presence of citrate) removed a slight tendency for traces of clot formation, and hence resulted in very stable solutions. The final dilution, in buff. sal., assayed 2 mg./ml. thrombin-clottable protein.

Thromboplastic Enzymes

Definition. As a result of clotting experiments with trypsin in 1939, Ferguson and Erickson12 introduced this term for enzymic (presumably proteolytic) activations of prothrombin to thrombin which were dependent on calcium ions and “available” phospholipid. This is regarded as a working definition, pending still unsolved questions concerning the modes of action (cf.13). It is applicable to three reagents used in the present investigations: 1. Russell’s viper venom (cf.14), in the form of the commercial (Burroughs-
Wellcome) “Stypven,” used in a concentration of 4 μg/ml of incubate (see later). 2. Crystalline trypsin (Worthington’s), 5 μg/ml incubate. 3. Autoprothrombin C,15,16 supplied through the courtesy of Drs. Marciniak and Seegers (Wayne State University Medical College, Detroit, Mich.). It was prepared by the IRC-50 amberlite column fractionation of citrate-activated bovine thrombin. In 50 per cent glycerol solution, the stated potency was 240,000 units11 per ml. Control tests showed non-significance of the final small amounts of glycerol and trace (only) of thrombin contaminant, under the experimental conditions. Five ml of incubate contained 0.2 ml of a 1:100 dilution of the stock preparation.

Experiments. Only the tests with Stypven will be given in detail, but it is worthy of report that the tests with trypsin and with autoprothrombin C gave very similar results. The lipid requirements of all three enzymes could be supplied by very small amounts of the three test lipids: namely, cephalin, PS, or PE. This was true also for “altered” lipids, e.g., PSa (see later).

Sodium desoxycholate. (Nutritional Biochem. Corp.) one mg./ml in buff. sal. was used to suspend some of the phosphatides (see below), initially at 1 mg/ml. (lipid), but further dilutions were made with buff. sal. The use of desoxycholate is indicated thus: φ.

Phosphatides

Cephalin (Ceph) was a chloroform extract of acetone-dried human brain (autopsy), after Bell and Alton.17 After evaporation of the chloroform in an air current, a 1 per cent stock suspension was homogenized in buff. sal. and stored at -20 C. Two stock preparations A,B (see later) were used in the present studies.

Human platelet phospholipids. These were obtained from the pooled platelets (13 Gm. wet weight) of 34 normal fasting donors, processed in the New York laboratories by the recently published methods of Marcus et al.18 It is sufficient here to state that 16 mg. of phosphatidylserine (PS), in 12.5 ml. cyclohexane, and 37.5 mg. of phosphatidylethanolamine (PE), in 9.2 ml. cyclohexane (essentially the total yield, except for small aliquots used to establish the criteria of purity), were placed under nitrogen in glass vials with Teflon-lined screw caps and transported to Chapel Hill, N. C.) by air. Surrounded by dry ice during transport, the vials were subsequently kept in the freezer at -20 C. On recorded dates, numbered aliquots were removed after thawing, evaporated under purified nitrogen, tared, and homogenized in buff. sal. (or with desoxycholate φ) for 1 mg/ml. test stocks from which further dilutions were made as required.

Lipid inhibitor (LI), according to Tocantins et al.19 was a preparation of Dr. M. J. Silver’s20 pork brain “phosphatidylserine,” kindly supplied by the Philadelphia workers some 9 months previously. The 1 mg/ml. suspension in desoxycholate (φ) was preserved at 4 C. (refrigerator).

Clotting Tests

Group I: Two-stage (thrombin-forming) tests5,6 on eluate prothrombin (El), using the thromboplastic enzymes described under Materials. Details of the Stypven test are given later.

Group II: Partial thromboplastin time (PTT) tests, after Langdell et al.,21 in which human citrated plasmas were recalciﬁed in the presence of the various lipids.

Group III: Thromboplastin generation tests (TGT). In the original22 test, platelets, calcium, adsorbed plasma, and serum (diluted) were found to generate a “thromboplastin”-like (intrinsic) activity, subsequently tested on a substrate plasma in the manner of the Quick test. In the Pool-Robinson23 modification, a more dilute, more stabilized, and more sensitive technic is used. The various phosphatides (see Materials) were substituted for platelets in these test systems, using normal human plasmas (adsorbed) and sera.

Two-stage (thrombin-forming) tests

Principle: When a preparation of prothrombin is first incubated with suitable activators, and then samples of the incubate are removed at intervals and tested on a standard
fibrinogen, the progressively shortening clotting times afford a measure of the thrombin generation. When it can be demonstrated repeatedly that an identical "end point" (measured by the optimal, or shortest, clotting time reached) is obtainable with a variety of methods of activation, it is a valid conclusion that this end point quantitates the prothrombin conversion, or thrombin yield. That the thrombin yield depended upon "available" phosphatide concentration was shown in 1938 by Ferguson, who used brain cephalin and the crude prothrombins of the Howell era. Eluate prothrombins have been used in the Chapel Hill laboratories for a number of years, employing systems activated (a) "intrinsically" (i.e., by factors in the blood fractions themselves), or (b) "extrinsically" (i.e., by addition of tissue thromboplastin or various thromboplastic enzymes). The basic method can be modified to study individual clotting factors and preliminary studies have been reported on a number of phosphatide materials. The role of the phospholipid is appropriately designated prothromboplastic. The new advances of the present study are (a) the testing of chemically pure PS and PE (under the conditions to be described), and (b) the validation of a genuinely quantitative bioassay and its application to pertinent problems concerning the reactivities of phosphatides in these clotting tests.

Preliminary experiments showed: (1) The concentrations of thromboplastic enzymes, cited under Materials, were well suited for study of their lipid requirements in these two-stage systems. (2) The needed lipid, with Stypven, trypsin, or autoprothrombin C, could be provided by Ceph, PS, or PE \(\phi\). (3) "Altered" lipid reactivities could be studied (see later). (4) Less than 1 \(\mu g./ml\) amounts of the phosphatides could be accurately quantitated. (5) Optimal activations, in the Stypven system, occurred with about 20-30 \(\mu g./ml\) Ceph or with concentrations of PS and PE \(\phi\) of a similar order. (6) With up to 200 \(\mu g./ml\) PS, or 100 \(\mu g./ml\) PE \(\phi\), no real evidence of inhibitory effects was seen. At such concentrations turbidity is such as to raise questions about the physical state of the suspensions. Stocks of the pure platelet phospholipids were too limited for further pursuit of this line of enquiry.

Stypven-eluate (two-stage) tests

Since the success of the method depends on the technic and its controls, these will be given in some detail. A stable room temperature, 25 C. (± 3 C), is satisfactory.

**Method.** Incubate 5 ml mixture in buffered saline containing 0.1 ml eluate, 0.1 ml factor V, 0.1 ml phosphatide (varying concentrations), 0.2 ml Stypven (1:10,000), and 0.5 ml 0.15 M CaCl\(_2\). Test 0.2 ml samples of incubate at successive (1 minute) intervals with 0.2 ml fibrinogen, timing clotting with a stopwatch. Thrombin formation is followed from the first minute of incubation to the end point (shortest clotting-time reached), which is determined with the greatest possible accuracy. In the following examples, end points are given in seconds, and the corresponding incubation periods in minutes.

**Controls.** (1) There is negligible activation when no phosphatide is added, e.g., 233"-304" (20-30 min.). (2) There is a very minor "intrinsic" activation when phosphatide is added without Stypven, e.g., for 0.7 \(\mu g./ml\), (a) Ceph 92.8", (b) PS 71.1". (both in 25 min.).

**Results with phosphatides.** On plotting end points (sec.) against phospholipid concentration \(\mu g./ml\) incubate on log-log paper, essentially rectilinear plots are obtained over the concentration range suitable for bioassay. Figure 1 shows typical results. The "standards" were I: Ceph A and II: \(\phi\) Ceph (with desoxycholate), tested between 1.0 and 0.1 \(\mu g./ml\). The "unknowns" were III: \(\phi\) PE (with desoxycholate) and IV: PS (No. 2). These four plots are clearly parallel. Hence they are all assayable in terms of one another. The desoxycholate makes a difference, so the appropriate standard must be chosen. Calculation: For each test, the observed end point clotting-time of amount (N) of the unknown is read off the appropriate standard as the amount \(\mu g./ml\) of cephalin with equivalent reactivity (i.e., same end point). The assay value, as percentage \(\%\) of the standard, is: (Ceph Eq. \(\div N\) x 100).

Platelet phosphatidylethanolamine \((\phi\) PE) assayed close to 30 per cent, namely 28-33 per cent for three tested concentrations (0.4, 0.8, 2.0 \(\mu g./ml\)). Platelet phosphatidylserine
Figs. 1 (above) and 2 (below).

Fig. 1.—Bioassay of phosphatidylethanolamine (PE φ) and phosphatidylserine (PS:2), using cephalin (Ceph. A) standards. PS* was an altered preparation (non-assayable).

Fig. 2.—Variations in cephalin standards.

(PS) was close to 20 per cent, namely 17–22 per cent for four tested concentrations (0.1, 0.4, 1.0, 2.0 μg./ml.). PS* was an altered phosphatidylserine (see later) which gave a plot with a different slope and was, therefore, no longer assayable.

Suitability of cephalin (Ceph) standards. As shown in figure 2, three Ceph A concentrations (0.16, 0.30, 0.70 μg./ml.) proved most useful for defining the straight line log-log reference plot. Dotted line "X" (fig. 2), plotted in the next log cycle, shows that higher concentrations of Ceph A deviated from the standard slope.

Replicate tests on the same occasion, and similar tests on different occasions (over a 3-month period) established a fixed reproducibility of the results, within a small (± 5–10 per cent, estimated) experimental error, and with few exceptions (see below). Plot 1 of figure 2 averages 27 "standard" testings. The exceptions fall into two patterns. In 2A, 2B,
the plots remain parallel to the standard, suggesting mere change in test conditions. It is a routine in many bioassays to run a standard-of-the-day and make a small correction for any change in conditions. The presence of desoxycholate is one example of “conditions” causing a parallel shift, as previously shown in figure 1.

In 3 and 4, the plots are divergent, invalidating the assays. In 3, the cephalin was weak and may have been incompletely homogenized. Plot 4 is a composite of five very similar experiments, all of which used weak cephalin suspensions that were intentionally exposed to air for periods up to 8 days at 4°C. (refrigerator). Although “standard” at the start, they altered to this distinctly hyper-reactive state, after a period under what were believed to be oxidative conditions. These observations constitute a pilot study of the reactivity of crude brain cephalin, in the Stypven test system, which opens up some new lines of enquiry.

Data on purified platelet phospholipids. With only a few milligrams of purified phospholipids available, and the instability problems which appeared, the following conclusions are to be regarded as a preliminary definitive study:

1. The original cyclohexane solutions, held under nitrogen at −20°C, except for brief thawing (sampling) periods, gave assayable aliquots over a limited period (days-weeks).

2. In Ceph Eq’s, the best validated assays were those from figure 1, previously cited as φ PE 30 per cent, PS 20 per cent. Other data confirmatory of these values were obtained on at least one subsequent occasion before significant alteration.

3. Deteriorative alterations, indicated by reduced reactivity, were evident in later testing, e.g. (a) after 10 days, PE φ aliquots assayed only 5 per cent, and (b) after 10 weeks, PS assayed only 8 per cent.

4. Anomalous hyperreactivity, presumably due to oxidative change (according to published absorption spectrophotometry data), was observed in the following cases:
   (a) Weak aerated suspensions of Ceph A, see above (fig. 2, line 4): also Ceph B (see 5).
   (b) PS suspensions proved very sensitive to this type of alteration. Plot PS, of figure 1 and figure 4B, is a typical example. These were retests, 72 hours later, of the same 1 mg./ml. suspension which had given the valid assay (20 per cent) of figure 1, line PS: 2. Referred to Ceph A, as if valid (which they are not), PS “pseudoassays” (%) for four respective concentrations (μg./ml.) gave: 380 per cent (0.1), 230 per cent (0.3), 108 per cent (0.7), 96 per cent (1.0). These divergent results are obviously meaningless as an assay but they do serve to illustrate the striking anomalous hyperreactivity. At 25°C, such changes could occur in a few hours and cause deviation from linearity in the data plots.
   (c) The PE stock had deteriorated (see 3. above) before the possibility of a similar type of alteration could be investigated with this lipid. A nearly 10 per cent assay 2 months after 3(a) (above) might be questionable. PE is known to oxidize readily.

5. Ceph B requires special mention. Compare figures 3 and 1 to show that it had a different and greater reactivity than Ceph A. It was learned that the new “standard” had been overexposed to the air current during evaporation.
of the chloroform. Nevertheless, as shown for PS (No. 3) in figure 3, and for some other lipid in figure 4B, Ceph B could serve as a valid standard in these cases, where the log-log data plots were parallel. It seemed more than a coincidence that the two-month old stock of PS:3 assayed 20 per cent in Ceph B equivalents, which was an identical value to the original Ceph A equivalents before any alteration was detected. It is an important point, technically, that the "standard" may be replaced by another one which, according to the parallel plots, has undergone the same type and degree of alteration as the lipid to be tested.

6. Sodium desoxycholate, in preliminary experiments, did not influence the cephalin-Stypven test results when added separately to the incubation mixture in final concentrations below 10 μg./ml. Ceph A φ, initially suspended in desoxycholate and subsequently diluted with buff. sal., showed about twice the reactivity of the simple saline suspension (fig. 1). With PS φ, the reactivity was increased about eightfold as compared with suspensions omitting the desoxycholate. With PE, poor activity without desoxycholate indicated incomplete homogenization. Effects of desoxycholate, therefore, are interpreted as a "condition" of the test, rather than as an "alteration" of the

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**Fig. 3.—Assay of phosphatidylserine (PS:3), in terms of Ceph. B, EPA, and ETY.**

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lipid, but are presumably related to the physical state of dispersion. On several occasions, PS and PSφ were simultaneously assayed against Ceph and Cephφ, respectively. Reference of each to the corresponding standard gave identical assay values.

Reactivities of Mixtures of Phospholipids in the Stypven-Eluate Test System

Cephalin is a relatively crude mixture of phosphatides. The finding that it was considerably more active, weight-for-weight, in validated assay comparisons with the best available purified platelet phospholipids, invited exploration of the results obtainable with mixtures of phosphatides.

Method. It is implicit that the end points of the two-stage technic measure the amounts of prothrombin converted to thrombin. With suboptimal amounts of phosphatides we are dealing with incomplete activations, which, in all probability, represent equilibrium reactions. Additional frames of reference are available if we can quantitate these equilibria. This requires correlation of the end point clotting-times with the amount (percentage) of prothrombin activated, or of the potential (optimal = 100 per cent) thrombin yield. Figure 3 illustrates how this was accomplished.

Equivalent prothrombin activations (EPA). Serial dilutions of eluate (with factor V, previously mixed), corresponding to 100, 70, 40, 20, and 10 per cent of the original (100 per cent) strength, were maximally activated with optimal (20 µg./ml.) Ceph A in the usual Stypven test system. Figure 3, line EPA, shows that the log-log plot of these end point clotting-times against the above percentages of "prothrombin" was precisely rectilinear. These data were confirmed on other occasions.

Equivalent thrombin yields (ETY). In other experiments, full-strength (100 per cent) eluate was maximally activated to "thrombin," using 20 µg./ml. ml. Ceph A in the Stypven test system. Then various dilutions (percentage-wise) were immediately retested on the same standard fibrinogen. Figure 3, line ETY, gives our best experimental data. These showed variability in repeated tests, however, which was attributed to the well-known experimental difficulty of verifying the so-called inverse law in actual practice. This is discussed on page 18 of ref. 5. The ETY plot of figure 3 obeys the inverse law, but its divergence from the EPA plot is unexplained. Notwithstanding these reservations, the ETY results will be cited as essentially confirming the main conclusions drawn from the more reliable EPA data.

EPA data on individual phosphatides. Before going into the question of mixtures, EPA data for the individual phosphatides are presented in figure 4 for varying concentrations (µg./ml.) of lipid. The log-log plots are all rectilinear and fall into two groups—4A and 4B—corresponding to the two cephalin standards. Whenever the plots are parallel it is valid to assay the reactivity of one phosphatide in terms of any other member of the group. In the following analyses of mixtures, both cephalin equivalents (Ceph Eq.) and phosphatidylserine equivalents (PS Eq.) are cited to permit a double-check of the results.

It must be emphasized that the EPA plot is unique for each individual
phosphatide. It is not permissible to attempt the simple addition of the EPA's for different lipids. What is valid is the conversion of two lipid reactivities to the equivalents of just one of them, or of the same third lipid, and then the addition of the EPA's (or ETY's) has real significance.

When, owing to alteration, as in the case of PS*, again presented in the new frame of reference (fig. 4B), the plots are not parallel, true equivalents cannot be obtained and the "pseudoassay" of a single concentration of lipid is of questionable validity in the further analyses.

Results on mixtures. Table 1 analyzes two mixtures of PS and Ceph B, the valid assay equivalents of which may be obtained from figures 3 and 4B.
Table 1.—Analyses of Mixtures of Cephalin (Ceph) and Phosphatidylserine (PS)

<table>
<thead>
<tr>
<th>Test</th>
<th>P-Lipid (µg./mL)</th>
<th>C.T. (sec.)</th>
<th>Ceph. Eq. (µg./mL)</th>
<th>PS. Eq. (µg./mL)</th>
<th>EPA %</th>
<th>ETY %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td>Ceph (0.3)</td>
<td>35.2&quot;</td>
<td>0.3</td>
<td>(1.4)</td>
<td>45</td>
<td>54</td>
</tr>
<tr>
<td>B.</td>
<td>Ceph (0.16)</td>
<td>44.0&quot;</td>
<td>0.16</td>
<td>(0.77)</td>
<td>33</td>
<td>44.5</td>
</tr>
<tr>
<td>C.</td>
<td>PS (0.7)</td>
<td>45.8&quot;</td>
<td>(0.145)</td>
<td>0.7</td>
<td>31.5</td>
<td>43</td>
</tr>
<tr>
<td>D.</td>
<td>Ceph (0.3)</td>
<td>31.1&quot;</td>
<td>(0.42)</td>
<td>(2.0)</td>
<td>53</td>
<td>62</td>
</tr>
<tr>
<td>E. a</td>
<td>expected</td>
<td>(30.7&quot;)</td>
<td>0.43</td>
<td>54</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>E. b</td>
<td>expected</td>
<td>(30.5&quot;)</td>
<td>2.1</td>
<td>54.5</td>
<td>63.5</td>
<td></td>
</tr>
<tr>
<td>F.</td>
<td>Ceph (0.16)</td>
<td>34.5&quot;</td>
<td>(0.315)</td>
<td>(1.5)</td>
<td>46</td>
<td>56</td>
</tr>
<tr>
<td>G. a</td>
<td>expected</td>
<td>(35.0&quot;)</td>
<td>0.305</td>
<td>45</td>
<td>55.5</td>
<td></td>
</tr>
<tr>
<td>G. b</td>
<td>expected</td>
<td>(35.0&quot;)</td>
<td>1.47</td>
<td>45</td>
<td>55.5</td>
<td></td>
</tr>
</tbody>
</table>

The data include: (a) concentrations in µg./mL, (b) clotting-time end points (sec.), (c) cephalin B equivalents (Ceph Eq.), (d) phosphatidylserine equivalents (PS Eq.), (e) EPA, (f) ETY (see above). The "found" values were read directly from the reference curves. The "expected" values were computed from additions of the common (same lipid) equivalents, as stated above.

The results clearly show very close agreements of the "found" and "expected" values, both for the two equivalents and for the EPA and ETY values. This would not be expected if there were any potentiation or antagonism (inhibition). These analyses, therefore, permit the important conclusion that these weak assayable phosphatides simply summate their prothromboplastic reactivities in a strictly quantitative manner.

Table 2.—Mixtures of Phosphatides

<table>
<thead>
<tr>
<th>Test</th>
<th>Mixture</th>
<th>EPA (%)</th>
<th>ETY (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Ceph (0.3), PE φ (3.0)</td>
<td>53</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>&quot;found&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&quot;expected&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Ceph (0.3), PS (0.3)</td>
<td>53</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>&quot;found&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&quot;expected&quot; (a)</td>
<td></td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>&quot;expected&quot; (b)</td>
<td></td>
<td>61</td>
</tr>
<tr>
<td>3.</td>
<td>PS φ (0.3), PE φ (3.0)</td>
<td>45</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>&quot;found&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&quot;expected&quot; (a)</td>
<td></td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>&quot;expected&quot; (b)</td>
<td></td>
<td>55</td>
</tr>
</tbody>
</table>

The additional conclusion, therefore, is that good correspondence of "found" and "expected" values is further validation of the assays, and, conversely, when the agreement is poor.
The question of inhibitors (antithromboplastic)? With the very weak phosphatide suspensions in the present test mixtures, it is still possible that we could be diluting out, and so missing, an inhibitor. It was necessary, therefore, to explore the sensitivity of the test system to the presence of a known antithromboplastic inhibitor. Such was available in the LI preparation described under Materials. In a previous report this material was shown to possess both activator (prothromboplastic) and inhibitor (antithromboplastic) properties.

The new tests, illustrated in figure 5, are EPA percentages for:

(a) increasing concentrations of PS alone (the solid line);
(b) "expected" summations (indicated: X) of: (1) 0.3 PS + 0.3 LI, (2) 0.3 PS + 0.6 LI, (3) 0.6 PS + 0.3 LI (µg./ml.);
(c) "found" values (indicated: ●) for the actual mixtures.

The last were all less than expected, and the effects of increasing the LI from (1) to (2) are emphasized by connecting these two points with the interrupted line in figure 5. The method distinctly reveals inhibitory effects of the antithromboplastic lipid in as little as 0.3–0.6 µg./ml., which is a high degree of sensitivity.

The absence of any indication of inhibitory effects with the platelet PS or PE preparations may be regarded as physiologic evidence for the high purity of these phospholipid products. It remains to be proved whether the inhibitor demonstrated in the brain “phosphatidylserine,” or LI, may be due to some contaminant.

Partial thromboplastin time (PTT) tests, etc. The data shown in table 3 were obtained with the cooperation of Dr. S. G. Iatridis (Visiting Scientist, from Athens, Greece) in our Chapel Hill laboratories. The tests on normal plasmas confirm previous findings, including those from the New York laboratories. A number of concentrations of the phosphatides was tested, but what reactivity is “quantitated” in this type of testing is not apparent. The most important qualitative finding was confirmation of the activity of PE. A mixture of PS and PE (test 6) gave results which might have been expected, and which do not suggest any potentiation or inhibition. Whether the somewhat longer clotting-time in tests 3A, 5A are evidence of any “inhibition”
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Table 3.—PTT Tests on Normal and Hageman-deficient (Hag-) Human Plasmas

<table>
<thead>
<tr>
<th>Test</th>
<th>Plasma</th>
<th>P-Lipid (μg.)</th>
<th>PTT (sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>normal</td>
<td>0</td>
<td>270.5&quot;</td>
</tr>
<tr>
<td>2.</td>
<td>normal</td>
<td>Ceph (60)</td>
<td>123.7&quot;</td>
</tr>
<tr>
<td>3. A.</td>
<td>normal</td>
<td>PE (50)</td>
<td>129.0&quot;</td>
</tr>
<tr>
<td></td>
<td>B.</td>
<td>PE (20)</td>
<td>125.8&quot;</td>
</tr>
<tr>
<td></td>
<td>C.</td>
<td>PE (10)</td>
<td>164.8&quot;</td>
</tr>
<tr>
<td>4.</td>
<td>normal</td>
<td>Ceph (60)</td>
<td>112.8&quot;</td>
</tr>
<tr>
<td>5. A.</td>
<td>normal</td>
<td>PS (500)</td>
<td>146.4&quot;</td>
</tr>
<tr>
<td></td>
<td>B.</td>
<td>PS (100)</td>
<td>102.2&quot;</td>
</tr>
<tr>
<td></td>
<td>C.</td>
<td>PS (50)</td>
<td>90.5&quot;</td>
</tr>
<tr>
<td></td>
<td>D.</td>
<td>PS (25)</td>
<td>99.6&quot;</td>
</tr>
<tr>
<td></td>
<td>E.</td>
<td>PS (10)</td>
<td>114.0&quot;</td>
</tr>
<tr>
<td>6.</td>
<td>normal</td>
<td>PE (25); PS (25)</td>
<td>91.8&quot;</td>
</tr>
<tr>
<td>7.</td>
<td>Hag-</td>
<td>Ceph (60)</td>
<td>1000&quot;</td>
</tr>
<tr>
<td>8.</td>
<td>Hag-</td>
<td>PE (50)</td>
<td>1000&quot;</td>
</tr>
<tr>
<td>9.</td>
<td>Hag-</td>
<td>PS (50)</td>
<td>1000&quot;</td>
</tr>
</tbody>
</table>

is debatable. They could indicate a nonspecific physical hindrance in such turbid mixtures. As anticipated, platelet PS and PE resembled cephalin in giving very prolonged PTT’s on the Hageman-deficient plasma. We also performed some experiments with a new method,25 which failed to show that 25 μg./ml. PS or PE could modify the Hageman factor activation (cf.26).

**Thromboplastin generation (TGT) tests**

**Methods.** These are detailed in the original references22,23 and the technics are widely recognized.

**Results,** with the “modified” TGT, are presented in tables 4 and 5. On a number of occasions, within a limited range of concentrations, test data (log-log plots) paralleled the standard Ceph A sufficiently for significant bioassays. Numerical values are given in the tables. Comments will be limited to the following:

1. These TGT assays, for the most part, could not be correlated with the styphven test results, showing that the “reactivities” depend upon the particular clotting test system.
2. PE was very weak, but undeniably showed some effect, in the “modified” TGT (table 5), as compared with its complete failure to act in the “original” method.18
3. “Altered” phosphatides, e.g., PS* (see earlier), never showed anomalous hyperreactivity in the TGT, but only a loss in activity (table 4).
4. In the modified TGT, no “fixed” standard was found, and it was necessary to rely upon a “standard-of-the-day.”
5. Full controls were run in these TGT tests, and when desoxycholate was used, the final concentrations were shown not to interfere with the tests.

**Discussion**

Knowledge of the role of phosphatides in blood coagulation goes back to Wooldridge,27 Howell28,29 and Zak30 associated the activity with “cephalins.”
rather than with "lecithins," and the need for unsaturated fatty acids in the molecule was pointed out in that era. Folch\textsuperscript{31} fractionated conventional cephalin into several phospholipids, but incompletely, since we\textsuperscript{32} were able to detect (pro)thromboplastic activity in all of Folch's original fractions with the sensitive two-stage technic. Slotta\textsuperscript{33} has refined these preparative methods in the case of brain phosphatides, and our\textsuperscript{7} tests with his materials have been reported.

The present data are limited to brain cephalin and a single batch of purified\textsuperscript{18} platelet phospholipids (PS and PE). The results depend on the technologies, but the new method of bioassay and its validations, together with the critical analyses of prothromboplastic (and antithromboplastic) reactivities in phosphatide mixtures are significant advances within the framework of these technologies.

A detailed review of the extensive current literature will not be attempted, since this is well summarized in the three references\textsuperscript{34-36} selected as representative for their particular contributions exemplifying the newer biochemical methodologies. The divergent clotting test results in the literature and in the present investigations may partly depend on questions of biochemical purity and characterization of specific phospholipids, but they are largely due to use of various clotting test systems. "Specificity," therefore, relates not only to the lipids, but also to the other factors they have to work with.

Since phosphatidylserine (PS) works well in all the cited test systems, the weight of evidence strongly suggests that it plays a major role in blood coagula-

### Table 4.—Modified TGT (*Thromboplastin Generation Test*), with Cephalin (Ceph) or Phosphatidylserine (PS)

<table>
<thead>
<tr>
<th>Date</th>
<th>P-Lipid</th>
<th>1.0</th>
<th>0.5</th>
<th>0.25</th>
<th>0.2</th>
<th>0.125</th>
<th>0.1</th>
</tr>
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<tbody>
<tr>
<td>1-29-62</td>
<td>Ceph</td>
<td>26.0&quot;</td>
<td>28.6&quot;</td>
<td>33.8&quot;</td>
<td>39.8&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PS</td>
<td>41.4&quot;</td>
<td>42.4&quot;</td>
<td>45.6&quot;</td>
<td>53.8&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ceph. Eq.</td>
<td>10.5%</td>
<td>18.6%</td>
<td>27.6%</td>
<td>28.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-12-62</td>
<td>Ceph</td>
<td>23.0&quot;</td>
<td>27.4&quot;</td>
<td>34.0&quot;</td>
<td>41.8&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PS</td>
<td>43.0&quot;</td>
<td>47.0&quot;</td>
<td>57.9&quot;</td>
<td>70.0&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ceph. Eq.</td>
<td>7.3%</td>
<td>10.2%</td>
<td>10.0%</td>
<td>9.8%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-19-62</td>
<td>Ceph</td>
<td>20.4&quot;</td>
<td>24.0&quot;</td>
<td></td>
<td>34.6&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PS</td>
<td>49.0&quot;</td>
<td>57.0&quot;</td>
<td>70.1&quot;</td>
<td>87.8&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ceph. Eq.</td>
<td>2.3%</td>
<td>2.4%</td>
<td>2.35%</td>
<td>1.9%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 5.—Modified TGT, with Phosphatidylethanolamine (PE$\phi$)

<table>
<thead>
<tr>
<th>PE$\phi$ Conc. Date</th>
<th>160 $\mu$g./ml</th>
<th>80 $\mu$g./ml</th>
<th>40 $\mu$g./ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-11-62</td>
<td>34.6&quot;</td>
<td>0.093%</td>
<td></td>
</tr>
<tr>
<td>1-25-62</td>
<td>36.2&quot;</td>
<td>0.075%</td>
<td></td>
</tr>
<tr>
<td>3-26-62</td>
<td>34.8&quot;</td>
<td>0.063%</td>
<td></td>
</tr>
<tr>
<td>3-27-62</td>
<td>32.8&quot;</td>
<td>0.065%</td>
<td></td>
</tr>
</tbody>
</table>
tion. A clotting role for phosphatidylethanolamine (PE) seems to depend on special circumstances. It works well in the PTT, but plasma recalcification times are affected by so many variables that these results leave unanswered questions regarding specificities. PE also gives excellent activations of prothrombin in the “thromboplastic enzyme” systems, but these could very well have special specificities. The failure of PE to work in the “original” TGT surprised previous workers. That we were able to pick up a weak, but unequivocal, PE reactivity with the more sensitive “modified” TGT could suggest the need for some special factor, which is perhaps not quite completely removed in preparing the reagents for the thromboplastin generation test. May we not be overlooking some factor(s), as yet unidentified, but possibly removed during the plasma adsorption?

In a limited series of tests with mixtures of the phosphatides in the modified TGT, no significant potentiations or inhibitions could be detected. The inhibitory (antithromboplastic) activity shown in the stypven-eluate tests with certain brain “phosphatidylserine” preparations (cf. 7) was not observed with the pure platelet PS. It could, therefore, be due to some unidentified contaminant. Biochemical criteria of purity are discussed in the references cited, dealing with the phospholipids used for the present studies. Everything points to specific reactivities of both PS and PE in the sensitive enzyme clotting test systems, in which these reactivities may now be quantitated. The reactivities of “oxidized” PS (see Results) may provide additional information, since it loses activity in the TGT but gains potency in the Stypven tests. Clearly, more investigations are needed both on the phospholipid alterations and on the factors with which the lipids work. An obvious suggestion is to explore a variety of enzymes.

Autoprothrombin C, blood “thrombokinase” according to Milstone,37 and the so-called intermediate product I according to workers38,39 with the thromboplastin generation test may have significant points in common. These are prepared with different technologies, but have the natural source material of the blood itself. Each of these products needs further study as to enzyme-like properties and relationships to specific phospholipids. Further experimentation with autoprothrombin C will appear in another publication.40 The idea that phospholipid reactivities are related to a proteolytic role of some prothrombin-converting enzyme must be advanced with caution. It will require much more understanding of enzymatic roles in the clotting mechanism before the true significance of the phospholipids can be understood. The biochemistry of blood coagulation is an exceedingly complex problem.

**SUMMARY**

Pure platelet phosphatidylserine (PS) and phosphatidylethanolamine (PE) are compared with brain cephalin (Ceph) in clotting-tests; (1) two-stage, using (a) stypven, (b) trypsin, (c) autoprothrombin-C; (2) PTT (partial thromboplastin time); (3) TGT (thromboplastin generation test); all showing “prothromboplastic” activity, except PE in the unmodified TGT. In validated bioassays (1a), log-log plots of endpoint clotting-times against phosphatide
concentrations are rectilinear and parallel. End points related to equivalents of prothrombin-activated (EPA), or thrombin yield (ETY), make possible computation of reactivities in mixtures. Unlike brain PS ("antithromboplastic"), platelet P-lipids show no inhibitor but summate quantitatively with each other or with Ceph. Alterations in lipid reactivities include: (i) decrease during storage, (ii) increase in desoxycholate, (both assayable), (iii) anomalous ("oxidative") increase, in thromboplastic enzyme tests, invalidating bioassays. TGT's (3) do not show (iii), but can quantitate certain reactivities. Besides suggesting the basis for a physiologic thromboplastic role of platelets, these methods offer means to explore biochemical bases of reactivities in identifiable lipids. These may be partly related to certain enzymes or intermediates in blood clotting reactions.

**SUMMARIO IN INTERLINGUA**

Pur plachettal phosphatidylserina (PS) e phosphatidylethanolamina (PE) es comparate con cephalina de cerebro in tests de coagulation, (1) hi-phasic, con le uso de (a) stypven, (b) trypsina, e (c) autoprothrombina-C, (2) tempore de thromboplastina partial, e (3) test de generation de thromboplastina. Omnes monstra activitate "prothromboplastic," excpete PE in le non-modificate test de generation de thromboplastina. In validate bioessayos (1a), curvas loglog pro terminal tempores de coagulation como function del concentrationes de phoshatido es rectilinee e parallel. Punctos terminal relate attitude a equivalentes de rendimento prothrombino-activate o del rendimento de thrombina rende possibile le computation de reactivitates in mixturas. Per contrasto con PS de cerebro ("antithromboplastic"), phospholipidos plachettal non monstra un inhibitor sed face summation quantitative le unes con le alteres o con cephalina de cerebro. Alterationes in le reactivitates lipidic es (i) declino in magasinage, (ii) augmento in desoxycholate—amb isto essayabile—, e (iii) augmento anormal ("oxydative") in tests de enzyme thromboplastic, lo que rende bioessayos invalidate. Tests de generation de thromboplastina non monstra (iii), sed illos pote quantitar certe reactivitates. A parte le facto que iste methodos suggere le base pro un physiologic rolo thromboplastic del plachettas, illos offere medios pro explorar le bases biochimic de reactivitates in identificabile lipidos. Isto pote esser partialmente relateate con certe enzymas o intermediarios in reactiones de coagulation de sanguine.

**REFERENCES**

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Purified Platelet Phospholipids and Blood Coagulation

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