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ACTION OF HIGHLY PURIFIED PHOSPHOLIPASES ON BLOOD PLATELETS

EVIDENCE FOR AN ASYMMETRIC DISTRIBUTION OF PHOSPHOLIPIDS IN THE SURFACE MEMBRANE

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Summary

1. Phospholipase C (phosphatidylcholine choline phosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* is unable to produce either lysis or phospholipid hydrolysis in intact pig platelets, whereas sphingomyelinase C (sphingomyelin choline phosphohydrolase) from *Staphylococcus aureus* can degrade more than 55% of platelet sphingomyelin without producing lysis. Degradation of glycerophospholipids by *B. cereus* phospholipase C can be achieved after or during sphingomyelin hydrolysis by sphingomyelinase C. Simultaneous breakdown of both sphingomyelin and glycerophospholipids is also brought about by the separate action of *Clostridium welchii* phospholipase C. In both cases, the production of ceramides and diacylglycerols in the platelet membrane is followed by aggregation and cell lysis.

2. Phospholipase A_2 (phosphatide acylhydrolase, EC 3.1.1.4) from *Naja naja* hardly degrades phospholipids in intact pig platelets suspended in albumin-containing medium. In the absence of albumin, phospholipase A_2 induces extensive aggregation of platelets followed by lysis, both of which are inhibited by aspirin or *o*-phenanthroline. The small amount of glycerophospholipid hydrolysis observed under these conditions is increased by subsequent incubation in the presence of sphingomyelinase C without causing lysis.

3. Isolation and analysis of plasma membranes from pig platelets, which were treated first with sphingomyelinase C under non-lytic conditions, allows to estimate that 63% of the pig platelet phospholipids are located in the plasma

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membrane. The distribution of phospholipids between the two halves of the plasma membrane has been deduced by using this value and by comparing the degradative action of *N. naja* phospholipase A_2 plus *S. aureus* sphingomyelinase C on lysed and intact platelets. It is concluded that 46% of the plasma membrane phospholipids, comprising 91% of sphingomyelin, 40% of lecithin, 34% of phosphatidylethanolamine and less than 6% of phosphatidylserine, form the outer half of the plasma membrane. A similar phospholipid distribution is proposed for the human platelet plasma membrane.

4. Investigating the action of seven purified phospholipases on intact platelets (both from human and pig) has revealed that membrane phospholipids are available to enzymatic breakdown only if the phospholipase can produce degradation in monolayers spread at an initial surface pressure of at least 34 dynes/cm. By comparing these results with previous data obtained on human red cells (Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 83–96), it is concluded that the lipid packing in the platelet surface membrane is comparable with a lateral surface pressure close to 34 dynes/cm.

Introduction

Phospholipids represent a major constituent of the platelet plasma membrane [1–5]. Besides their structural function, they have been shown to participate in various platelet responses, such as providing a phospholipid-water interface to interacting coagulation factors [6], and their possible involvement in the platelet release reaction as storage site of arachidonic acid, a precursor in prostaglandin biosynthesis [7–10]. Moreover, occurrence of release reaction upon hydrolysis of platelet phospholipids by phospholipase C from *Clostridium welchii* also suggests a possible role of phospholipids in these events [11,12]. However, while several studies dealt with their chemical composition [1–4] or their metabolism [13,14], the information on the structural arrangement of the phospholipids in the platelet plasma membrane is still fragmentary.

Previous studies using phospholipases have shown that phospholipids are asymmetrically distributed between the interior and the exterior half of the red cell membrane [15–18]. Extending the first proposition of Bretscher [19], it was found that the majority of the choline-containing lipids form the outer monolayer of the membrane, whereas 80% of the phosphatidylethanolamine and all the phosphatidylserine molecules are located in the inner monolayer. Furthermore, comparison of the action of various purified phospholipases towards intact red cells and lipid monolayers allowed an estimation of the lateral surface pressure in the erythrocyte membrane [18,20].

Although the asymmetric distribution of phospholipids is presumably common among other biological membranes, some qualitative and quantitative variations might be expected between cells displaying different physiological functions. A non-uniform distribution of phosphatidylethanolamine and phosphatidylserine in the platelet surface membrane has recently been proposed by Schick et al. [21], based on labelling of intact and lysed cells with trinitro-

benzene sulphonate. This result is at variance with the observation of Otnaess and Holm [22] who used phospholipase C from *Bacillus cereus* to obtain information on the sidedness of the phospholipids in the human platelet membrane.

The present study deals with the action of a number of highly purified phospholipases on both the pig and the human platelet membrane, in order to determine phospholipid distribution in the plasma membrane and to provide further evidence for a role of phospholipids in platelet physiology. Furthermore, the ratio of phospholipid content between plasma and intracellular membranes is deduced from analysing isolated membranes from sphingomyelinase-treated platelets. Finally, the compression state of the phospholipids in the platelet plasma membrane is detected by comparing the action of different phospholipases on intact platelets with their action on red cells and phospholipid monolayers.

Materials and Methods

Phospholipases

The following enzymes were purified as described elsewhere [18]: Phospholipase A_2 from porcine pancreas, bee venom (*Apis mellifica*), and *Naja naja* venom, respectively; phospholipase C from *Bacillus cereus* and *Clostridium welchii*; sphingomyelinase C from *Staphylococcus aureus*. Phospholipase A_2 from *Crotalus adamanteus* was purified by affinity chromatography, essentially according to the method of Rock and Snyder [23].

Platelets

Pig blood was collected from the slaughterhouse, human blood was from healthy volunteer donors. Acid citrate dectrose (ACD) was used as an anticoagulant [24]. In some experiments, "aspirin-treated" platelets were obtained by dissolving acetylsalicylic acid in the ACD solution (500 mg per liter of blood). Suspensions of washed platelets were prepared as described by Ardlie et al. [25], with osmolarity adjusted to 340 mosM in the case of pig platelets [26]. Final suspension was usually in Tyrode buffer (pH 7.4) containing 1 mM $MgCl_2$, 5 mM glucose and 0.35% bovine serum albumin, and is referred to as "complete system". Omission of glucose and albumin, or addition of other factors will be reported in the text. Suspensions were adjusted to 10 mg platelets/ml (wet weight), which corresponded to 0.2—0.25 μmol lipid phosphorus/ml. The isolation procedure was done at room temperature in siliconized glassware (Siliclad).

Treatment of intact platelets with phospholipases

Incubations were started by adding platelet suspensions to siliconized tubes containing various amounts of phospholipases. Final concentrations of $CaCl_2$ were as follows: 2.5 mM for phospholipase C from *C. welchii* or *B. cereus* and sphingomyelinase C from *S. aureus*; 5 mM for phospholipase A_2 from pig pancreas, *Cr. adamanteus* or bee venom; 10 mM for phospholipase A_2 from *N. naja*. The phospholipase C preparations were equilibrated with Zn^{2+} after the purification procedure. Incubations were carried out for 30 min at 37° C with gentle stirring, followed by centrifugation at 3500 × *g* for 7 min at room temperature.

The supernatants were collected and lysis was determined by measuring lactate dehydrogenase activity. Usually, the pellets from non-lytic incubations were used for phospholipid analysis.

Treatment of lysed platelets with phospholipases

Platelets were lysed by sonication for 2 min as described by Baenziger and Majerus [27]. Incubations were carried out in the same way as for intact platelets, followed by centrifugation at 100000 × *g* for 60 min (4°C). The pellets were used for phospholipid analysis.

Phospholipid analysis

Enzymatic activity was inhibited by addition of EDTA and *o*-phenanthroline (final concentrations 15 mM and 4 mM, respectively), prior to extraction of the lipids according to Reed et al. [28]. The extracts were taken to dryness under reduced pressure and the residue was dissolved in 100 µl chloroform/methanol (1 : 1, v/v). The phospholipids were separated by two dimensional thin layer chromatography using the procedure of Broekhuyse [29] and determined as phosphorus after destruction with 70% $HClO_4$ at 190°C by a modification [30] of the procedure of Fiske and Subbarow. Percentage degradation of the phospholipids by phospholipases was determined as described previously [17]. Since phosphatidylserine was not always separated from phosphatidylinositol they were routinely analyzed as one fraction. This fraction mainly consists of phosphatidylserine, because phosphatidylinositol is a minor component.

Preparation of membranes from sphingomyelinase C-treated and control platelets

Platelet suspensions in complete system were incubated with or without sphingomyelinase C (1 IU/100 mg platelets) as described above. Platelet pellets were directly suspended in 5 volumes of cold 0.1 M KCl, 0.05 M Tris, 0.002 M EDTA (pH 6.5 with 1 N HCl) and disrupted by sonication in ice for two periods of 15 s with one interval of 15 s for recooling, using a B12-Branson Sonifier with an output of 100 W. The following procedures were carried out at 4°C. The homogenates were diluted five fold with Tris/KCl/EDTA buffer and centrifuged at 15000 × *g* for 10 min. The microsomal fraction, which also contains the plasma membranes, was obtained by centrifuging the 15000 × *g* supernatant at 100000 × *g* for 60 min and resuspended in 2 ml Tris/KCl/EDTA buffer for further fractionation on linear sucrose gradients (19 to 46%, w/v) in 10 mM Tris/2 mM EDTA (pH 7.4). Sucrose gradient fractionation was carried out using the SW 27 rotor (Beckman) at 24000 rev./min for 14 h. Eleven fractions were collected by pipetting from the top, diluted three fold with Tris/KCl/EDTA buffer and pelleted by centrifugation at 100000 × *g* for 60 min. The pellets were suspended in 0.3 ml Tris/KCl/EDTA buffer and used for further analysis.

Enzyme assays

Lactate dehydrogenase (EC 1.1.1.27) was measured as described by Wroblewski and La Due [31], except that 0.1% Triton X-100 was included in the phosphate buffer. This produced total lysis of platelet suspensions [32],

and did not interfere with the enzyme assay. *N*-Acetyl-β-glucosaminidase (EC 3.2.1.30) was assayed by the method of Day et al. [33]. The same procedure was applied to the determination of phosphodiesterase (EC 3.1.4.1) activity, except that bis (*p*-nitrophenyl) phosphate (Sigma) was used as a substrate and incubation was carried out in 0.1 M ammonium acetate buffer (pH 5.5) in the absence of Triton [4,34].

Other analyses

Protein was determined by the method of Lowry et al. [35], using bovine serum albumin as a standard. Sucrose concentration in the fractions from the gradients was determined with a refractometer (Bausch and Lomb) at 20°C.

Results and Discussion

General effects of different phospholipases on intact platelets

When phospholipases are used as probes for detecting phospholipid distribution in cell membranes, it is essential to find conditions where hydrolysis of phospholipids in intact cells occurs without lysis. The effects of different phospholipases on human erythrocytes have been shown to be dependent on type, substrate specificity, and surface pressure characteristics of each enzyme [15–18]. Also incubation conditions, such as those influencing energetic metabolism of the cells, may influence their behaviour towards phospholipases [36–38]. Therefore, a preliminary investigation was undertaken using intact pig platelets, in order to screen the effects of the various phospholipases under different conditions (see Table I).

With respect to the C-type phospholipases, phospholipase C from *B. cereus* produced neither lysis nor hydrolysis of phospholipids, whereas sphingomyelinase C from *S. aureus* hydrolyzed 55–60% of sphingomyelin (corresponding to 12% of the total phospholipids) without causing lysis. Hydrolysis of sphingomyelin rendered glycerophospholipids susceptible to the action of *B. cereus* phospholipase C, but simultaneous formation of ceramides and diacylglycerols was accompanied by lysis. The same result was found with phospholipase C from *C. welchii*, which is known to hydrolyze both sphingomyelin and lecithin [18]. Furthermore, irreversible aggregation occured when both choline-containing phospholipids were hydrolyzed. It has been previously reported that *C. welchii* phospholipase C induces release reaction in human platelets, which is presumably responsible for the observed aggregation [11,12]. At the concentrations used, the process of secretion occured rapidly and was completed within 5 min, whereas cell lysis started more slowly afterwards. It is probable that release reaction occurs also during combined action of *S. aureus* sphingomyelinase C and *B. cereus* phospholipase C. This would suggest that secretion is due either to the formation of both ceramides and diacylglycerols or to the production of diacylglycerols alone, sphingomyelinase C being necessary only to make glycerophospholipids available to *B. cereus* phospholipase C. Apparently, the production of ceramides alone is not sufficient to induce secretion. However, the mechanism by which release reaction is triggered was not further investigated and remains as yet unknown. Thus, except for specific responses inherent to platelet functions, C-type phospholipases have similar actions on

TABLE I

EFFECTS OF DIFFERENT PHOSPHOLIPASES ON PIG PLATELETS UNDER VARIOUS CONDITIONS

Aggregation was checked from macroscopic appearance of aggregates after 5 minutes. Lysis and hydrolysis less than 5% are reported as (—). Hydrolysis is expressed as percent of total phospholipid and was studied only quantitatively when no lysis occurred. For combined action of *N. naja* phospholipase A_2 and *S. aureus* sphingomyelinase C, phospholipase A_2 was added at zero time and sphingomyelinase C after 5 min. For further details see the text. BSA, bovine serum albumin.

Phospholipases (IU/100 mg platelets)	Incubation conditions	Aggregation (after 5 min)	Lysis (after 30 min)	Hydrolysis (after 30 min)
Phospholipase C *B. cereus* (40)	complete system	—	—	—
Sphingomyelinase C *S. aureus* (1)		—	—	12
Sphingomyelinase C *S. aureus* (1) + phospholipase C *B. cereus* (40)		+	+	+
Phospholipase C *C. welchii* (1.5)		+	+	+
Phospholipase A_2 *N. naja* (40)	complete system	—	—	—
	decreasing osmolarity	—	+	+
	minus glucose	—	+	+
	minus glucose, minus BSA	—	+	+
	minus BSA	+	+	+
	minus BSA, aspirin-treated platelets or minus BSA, plus *o*-phenanthroline	—	—	5–6
Sphingomyelinase C *S. aureus* (1)	minus BSA, plus *o*-phenanthroline	—	—	12
Phospholipase A_2 *N. naja* (20) + sphingomyelinase C *S. aureus* (0.5)	minus BSA, plus *o*-phenanthroline	—	—	25

platelets as on red cells and the results suggest a relative abundance of sphingomyelin at the exterior of the plasma membrane. This is in disagreement with the work of Otnaess and Holm [22], who reported an extensive degradation of phospholipids by *B. cereus* phospholipase C and a limited hydrolysis by *S. aureus* sphingomyelinase C. The possible reasons for this discrepancy will be discussed below.

Phospholipase A_2 from *N. naja*, which degrades 68% of the red cell lecithin without causing lysis, did not produce any significant breakdown of phospholipids in platelets incubated in complete system. Hydrolysis could be obtained at osmolarities below 200 mosM, but this is accompanied with lysis and subsequent breakdown of glycerophospholipids present in the cell interior. Omitting glucose in the suspending medium and preincubating the cells at room temperature for 3 to 5 h was also followed by lytic degradation of phospholipids upon addition of phospholipase A_2. This occured both in the absence and presence of albumin, suggesting that lysis cannot be attributed to removing the split products by albumin. This phenomenon has been described for red cells, but higher concentrations of "fatty acid free" albumin were used [39]. When albumin was omitted, *N. naja* phospholipase A_2 induced extensive platelet aggregation, followed by cell lysis. The use of aspirin-treated platelets or addition of 2 mM *o*-phenanthroline to the medium suppressed both effects, although some phospholipid hydrolysis occured. Non-lytic degradation of glycerophospholipids by *N. naja* phospholipase A_2 could be promoted by subsequent addition of sphingomyelinase C.

The interaction of phospholipase A_2 with platelets thus appears to be more complex than with red cells. Swelling of the platelets in a hypotonic buffer or decreasing their energetic metabolism both result in an increased fragility of the platelets, with a concomitant increase in phospholipid susceptibility to phospholipase A_2. It should be recalled that previous studies from this laboratory on enzymatic degradation of erythrocyte phospholipids were done with fresh cells collected in glucose-containing medium [15—18]. Other workers [36—38] have shown that depleting the cells of ATP make them more susceptible to phospholipase actions and accelerates the process of lysis. From a practical point of view, this implies to use cells as intact as possible, particularly in the case of cells as fragile as platelets.

Free arachidonic acid can be converted by platelets into endoperoxides, which are very potent triggers of release reaction [40]. This process is inhibited both by aspirin [40] and *o*-phenanthroline [41]. Our results suggest that phospholipase A_2 might release arachidonic acid from platelet phospholipids in amounts sufficient to induce secretion and aggregation, provided that energetic metabolism of the cells is maintained at a normal level. Neither aspirin nor *o*-phenanthroline modify the results of phospholipid hydrolysis by sphingomyelinase C. Therefore, in order to inhibit both aggregation and lysis, either aspirin or *o*-phenanthroline were included in the following experiments. Using the experience from previous studies on red cells [17], hydrolysis of platelet glycerophospholipids could be increased by combining *N. naja* phospholipase A_2 and *S. aureus* sphingomyelinase without producing lysis of the cells. These experiments are described in the next section.

Degradation of phospholipids in intact and lysed platelets

Differences in lytic and non-lytic action of sphingomyelinase C and phospholipase A_2 on pig platelets are shown in Figs. 1 and 2. In intact platelets, *S. aureus* sphingomyelinase C was found to degrade 65—67% of sphingomyelin without producing lysis. No further breakdown was observed upon increasing enzyme concentration per cell. With lysed platelets, however, around 90% of sphingomyelin became susceptible to hydrolysis by sphingomyelinase C.

The difference in behaviour between intact and lysed cells was more pronounced in the case of *N. naja* phospholipase A_2. Only a small amount of lecithin and phosphatidylethanolamine was hydrolysed in intact platelets, even at concentrations as high as 100 IU/100 mg platelets, whereas 90—95% of all the glycerophospholipids were degraded in sonicated platelets.

The degradative action of *N. naja* phospholipase A_2 on intact cells could be increased by subsequent addition of sphingomyelinase C. It has been found that addition of sphingomyelinase C before or together with phospholipase A_2 was lytic for red cells, whereas the reverse order of addition produced non-lytic degradation of phospholipids [17]. Similar effects were found with platelets. As depicted in Fig. 3, extensive degradation of phospholipids available at the platelet surface could be achieved, provided that the following conditions were observed: energetic metabolism of the cells was maintained at a sufficient level by increasing glucose concentration from 5 mM to 25 mM and the platelets were preincubated with phospholipase A_2 for 5 min followed by two successive additions of sphingomyelinase C at 0 and 15 min, respectively. In this way, platelets could be incubated for periods over 60 min, without significant lysis either of phospholipase-treated or control cells. Moreover, it was verified that no release of either *N*-acetyl-β-glucosaminidase or nucleotides occured. Hydrol-

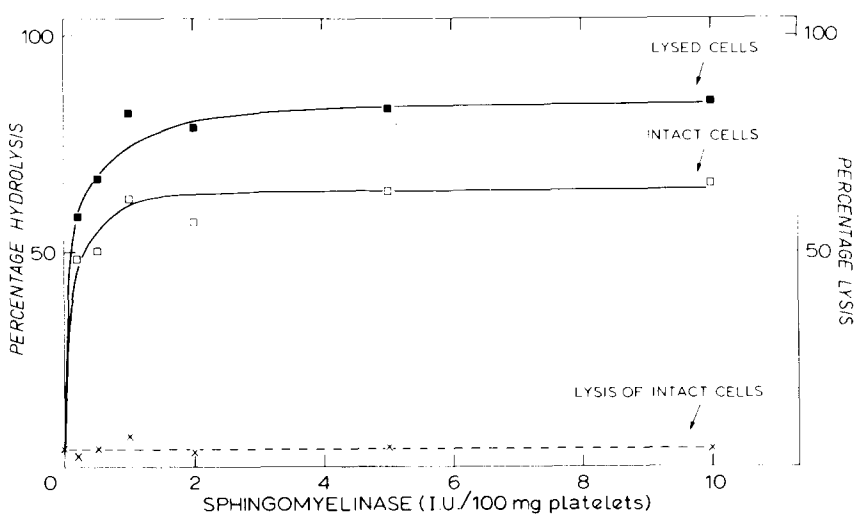

Fig. 1. Sphingomyelin hydrolysis in intact and lysed pig platelets by increasing amounts of *S. aureus* sphingomyelinase C per wet weight of platelets. Aspirin-treated platelets were suspended in Tyrode buffer without albumin and incubated for 30 min at 37°C either immediately (intact cells) or after sonication (lysed cells).

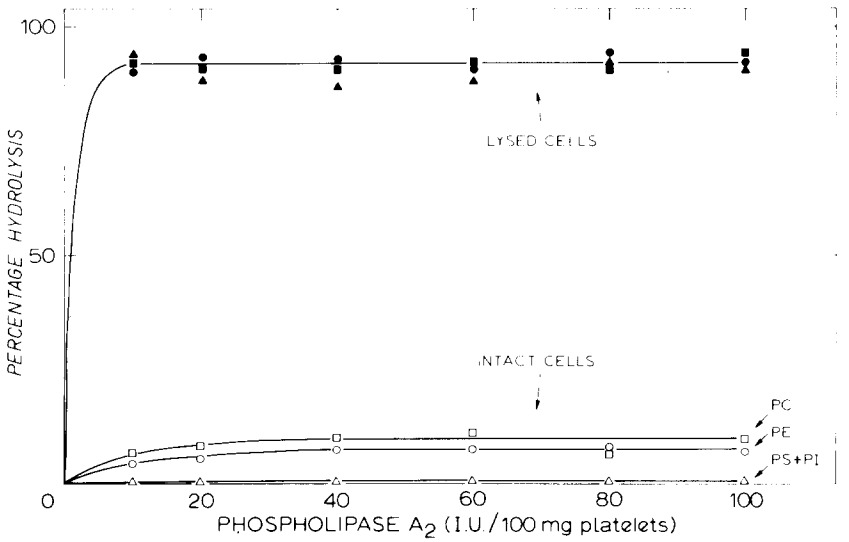

Fig. 2. Glycerophospholipid hydrolysis in intact and lysed pig platelets by increasing amounts of *N. naja* Phospholipase A_2 per wet weight of platelets. Incubation conditions were identical to those under Fig. 1. Hydrolysis is expressed as percent of each phospholipid class. Lysis is not represented but was the same as in Fig. 1. Abbreviations: PC, lecithin (□ or ■); PE, phosphatidylethanolamine (○ or ●); PS, phosphatidylzerine; PI, phosphatidylinositol (PS + PI: △ or ▲).

ysis of phospholipids leveled off after 45 min and was not increased by further incubation for 30 min. Sphingomyelin hydrolysis was the same as obtained with sphingomyelinase C alone, whereas both lecithin and phosphatidylethanolamine degradation was markedly higher than with phospholipase A_2 alone.

A similar picture was obtained with human platelets and detailed results are given in Table II. The phospholipid composition was roughly the same in both

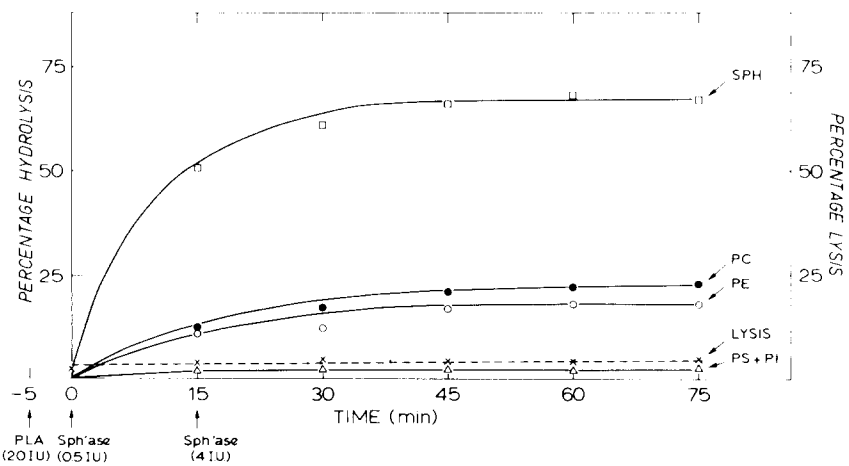

Fig. 3. Non-lytic degradation of pig platelet phospholipids during prolonged incubations with *N. naja* phospholipase A_2 (PLA) and *S. aureus* sphingomyelinase C (Sph'ase). Number of units (IU) are expressed per 100 mg platelets (wet weight). Aspirin treated platelets were suspended in Tyrode buffer without albumin. Glucose concentration was 25 mM. For further details see the text. Abbreviations: SPH, sphingomyelin. For the others, see Fig. 2.

TABLE II

NON-LYTIC DEGRADATION OF PHOSPHOLIPIDS BY PHOSPHOLIPASE A_2 (*N. NAJA*) AND SPHINGOMYELINASE C (*S. AUREUS*) IN HUMAN AND PIG PLATELETS

The values are expressed as percentage of total lipid phosphorus ± S.D. and are the means of 14 experiments for pig platelets and 8 experiments for human platelets. The numbers in parenthesis refer to the percent degradation of the corresponding phospholipid classes. Incubations were performed for 45 to 75 min in presence of 2 mM *o*-phenanthroline or with aspirin-treated platelets. For further details see the text.

	Pig platelets		Human platelets	
	Phospholipid composition	Hydrolysis	Phospholipid composition	Hydrolysis
Sphingomyelin	22.1 ± 1.3	14.9 ± 1.1 (67)	18.1 ± 0.2	13 ± 0.4 (72)
Phosphatidylcholine	35.0 ± 1.7	7.7 ± 1.7 (22)	37.7 ± 0.7	8.5 ± 0.8 (23)
Phosphatidylserine + phosphatidylinositol	15.2 ± 1.6	0.6 ± 0.8 (4)	16.1 ± 1.0	0.2 ± 0.2 (1)
Phosphatidylethanolamine	27.8 ± 1.8	5.8 ± 1.2 (21)	28.1 ± 0.4	3.3 ± 0.4 (12)
Total phospholipid	100	28.9 ± 2.9	100	25.0 ± 1.2

species, except that pig platelets have a slightly higher content of sphingomyelin at the expense of lecithin. It is noteworthy that the same qualitative difference has been found for red cells from both species [42]. With respect to phospholipid hydrolysis, sphingomyelin degradation was the most extensive (around 70%), whereas smaller amounts of lecithin and phosphatidylethanolamine disappear, the latter phospholipid being more exposed in pig platelets. Hydrolysis of phosphatidylserine and phosphatidylinositol remained very low and was not significant. The maximal non-lytic degradation corresponded to 25% and 29% of human and pig platelet phospholipid, respectively. This indicates that these fractions are present in the outer half of the platelet surface membrane. This is supported by the absence of either lysis or release reaction during phospholipase action and by the fact that prolonged incubation does not result in further degradation of phospholipids. In contrast, incubation of lysed platelets with the two phospholipases resulted in almost complete degradation of all phospholipid classes. The observation that a small fraction of the phospholipids remains refractory to phospholipases presumably reflects the difficulty to obtain completely "open" platelets, even in distilled water. It is not excluded either, that some granules remain intact, keeping their inside masked for phospholipase action.

The results of the successive action of phospholipase A_2 and sphingomyelinase C strongly suggest an asymmetric phospholipid distribution in the platelet surface membrane. However, with intact cells, the nonhydrolysed phospholipid classes do not only represent phospholipids at the plasma membrane interior, but also phospholipids from intracellular membranes. A more accurate detection of the phospholipid asymmetry in the plasma membrane is only possible if the distribution ratio of the phospholipids between plasma and intracellular membranes is known. This ratio can be detected by analysing plasma membranes from sphingomyelinase C-treated and control cells, as described below.

Isolation of membranes from sphingomyelinase C-treated and control platelets

Results of enzymatic activities in the subcellular fractions of pig platelets are given in Table III. Microsomes isolated from nontreated platelets were enriched 3.3 times in phosphodiesterase, a platelet plasma membrane marker [4,5], and 3.4 times in *N*-acetyl-β-glucosaminidase, used as "granular" marker [33]. The enrichment in phosphodiesterase was slightly less in the case of sphingomyelinase C-treated platelets whereas no significant change was observed for *N*-acetyl-β-glucosaminidase.

Distribution of enzyme activities over the sucrose gradients shows that both are clearly separated from each other (Fig. 4). Phosphodiesterase peaked usually in fractions 4 to 6, corresponding to densities of 24—28% sucrose (w/v), while the highest specific activities of *N*-acetyl-β-glucosaminidase were found at densities of 36—38% sucrose (w/v). Profiles of enzyme activities were essentially the same in both gradients, although membranes from treated platelets had somewhat lower specific activities of phosphodiesterase and were more contaminated by *N*-acetyl-β-glucosaminidase (see also Table III).

The fraction of each gradient having the highest specific activity in phosphodiesterase with minimal contamination by *N*-acetyl-β-glucosaminidase (generally fraction 4 or 5) was taken for phospholipid analysis (Results are shown in Table III). It is clear that in the case of nontreated platelets, sphingomyelin content was significantly increased in the membranes as compared to the homogenate. In contrast, membranes from sphingomyelinase C-treated platelets had a lower sphingomyelin content than the corresponding homogenate. Percent of total phospholipid hydrolysed increased from 11.8% (54% of sphingomyelin) in total platelets to 18.6% (71% of sphingomyelin) in platelet membranes. The microsomal fraction shows an intermediate picture, which presumably reflects contamination with plasma membranes.

TABLE III

ENZYME ACTIVITIES AND SPHINGOMYELIN CONTENT IN SUBCELLULAR FRACTIONS ISOLATED FROM NON-TREATED AND SPHINGOMYELINASE C-TREATED PIG PLATELETS

For details see the text. Enzyme specific activities are expressed in μmol of product released/h/mg protein ± S.D. (mean of 3 experiments). Sphingomyelin is expressed as percent of total lipid phosphorus present in each fraction ± S.D. and is the mean of 5 experiments for the homogenate and the membrane fraction and 3 experiments for the microsomal fraction. Enzyme enrichment is given in parenthesis.

		Phospho-diesterase	*N*-acetyl-β-glucosamini-dase	Sphingomyelin	
				Present	Hydrolysed
Homogenate	non treated	0.20 ± 0.03	1.16 ± 0.18	21.8 ± 1.8	
	treated	0.20 ± 0.04	1.18 ± 0.26	10.0 ± 2.1	11.8
Microsomes	non treated	0.66 ± 0.08 (3.3)	3.97 ± 1.69 (3.4)	22.5 ± 0.1	
	treated	0.46 ± 0.01 (2.3)	4.33 ± 0.56 (3.7)	8.7 ± 2.2	13.8
Membranes	non treated	1.08 ± 0.05 (5.4)	0.53 ± 0.25 (0.46)	26.1 ± 2.3	
	treated	0.79 ± 0.04 (4.0)	1.02 ± 0.11 (0.86)	7.5 ± 2.8	18.6

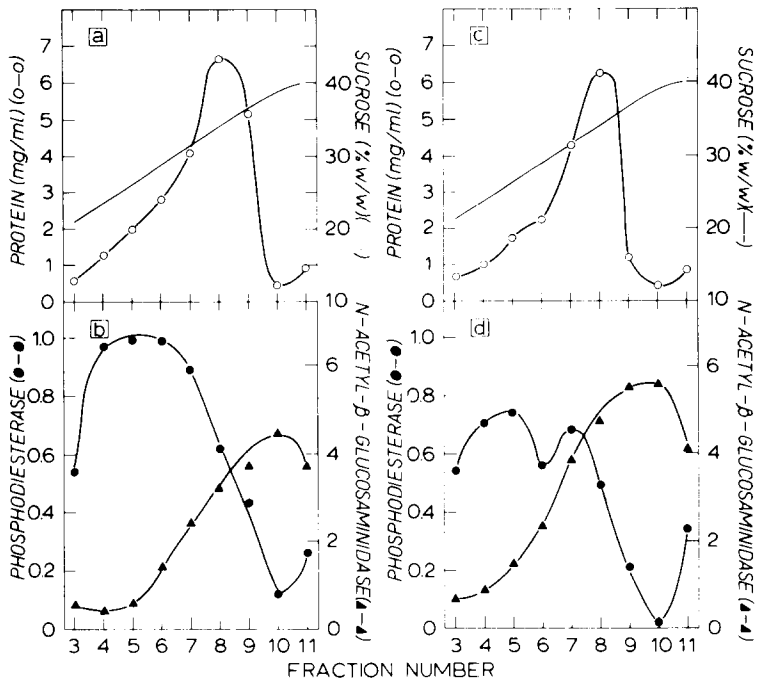

Fig. 4. Distribution of protein and enzymatic activity after fractionation on sucrose gradients of pig platelet microsomes. Enzyme activities are expressed in μmol product released/h/mg protein. The preparation procedure is described under Materials and Methods. Fractions 1 and 2 contained no appreciable amounts of particular protein and were not further analysed. (a) and (b), non-treated platelets; (c) and (d), sphingomyelinase C-treated platelets.

The preparation of membranes from sphingomyelinase C-treated and control platelets was carried out to find a simple and reproducible method, which results in membrane fractions of similar and acceptable purity. Moreover, sphingomyelinase C activity is blocked during the fractionation procedure by addition of EDTA in all the solutions used during the membrane preparation. Although brief sonication periods were used to disrupt the platelets, lysis was almost complete (over 90% of lactate dehydrogenase solubilised), whereas only some 10% of *N*-acetyl-β-glucosaminidase appeared in the supernatants. The same results have been reported by Baenziger and Majerus [27]. The buffer employed in the lysis of the platelets was the same as described by Broekman et al. [43], except that EDTA replaced citrate. No actomyosin gel transformation occured under these conditions, which allowed a preliminary fractionation. Despite a lower recovery in membrane material, this step was found to be essential, since direct fractionation of the total homogenate on sucrose gradients gave membrane fractions of insufficient purity, with respect to phosphodiesterase enrichment. It has been reported by Salganicoff et al. [44] that the microsomal fraction is enriched in both plasma membrane (as shown here by phosphodiesterase) and *N*-acetyl-β-glucosaminidase, currently used as a "granular" marker [4,5,33,44].

It is remarkable that the distribution of both protein and enzymatic activities over the gradient did not change markedly in the case of sphingomyelinase

C-treated platelets. In agreement with other investigators [4,5], non-treated membranes were more than 5 times enriched in phosphodiesterase, while contamination by *N*-acetyl-β-glucosaminidase remained low. The degree of purity was slightly less in the membranes from sphingomyelinase C-treated platelets. Despite some variation in marker enzymes, the purest fraction of each gradient should represent the same membranous material. This allows a direct comparison of their phospholipid composition, assuming that the isolated fraction is representative of the total platelet plasma membrane.

Phospholipid distribution in the pig platelet membrane

Degradation of 71% of sphingomyelin found in the membrane fraction does not completely correspond to that part of this phospholipid present in the outer layer, since sphingomyelinase C concentration was kept low which resulted in an incomplete degradation of available sphingomyelin: only 54% of sphingomyelin was hydrolysed in the total pig platelets, whereas percentage breakdown leveled off at 65—67% with higher concentrations of sphingomyelinase (see Fig. 1). Nevertheless, this result can be used to estimate the fraction of total pig platelet phospholipid present in the plasma membrane. The rational of this estimation is schematically shown in Fig. 5. Since sphingomyelin hydrolysis occured under non-lytic conditions and sphingomyelinase C activity was blocked immediately after the incubation, one can postulate that the degraded sphingomyelin is coming from the phospholipid pool of the plasma membrane. When hydrolysis is expressed as percentage of total phospholipid of either platelet homogenate or platelet plasma membrane, one can deduce the amount of phospholipids present in the platelet membrane relative to the total platelet. Such a calculation indicates that 63% of pig platelet phospholipids would be located in the plasma membrane. Despite some variations in

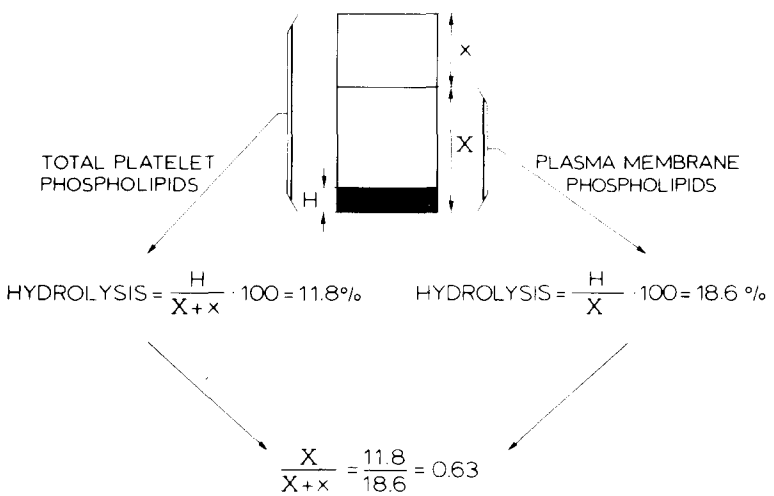

Fig. 5. Estimation of the amount of phospholipid present in the plasma membrane. *X*, total amount of phospholipid present in the plasma membrane; *x*, total amount of phospholipid present in the intracellular organelles; *H*, amount of phospholipid degraded by the sphingomyelinase-C under non-lytic conditions.

phospholipid composition or extent of sphingomyelin hydrolysis between different platelet preparations, the calculated ratio was found to be reproducible, values varying from 62 to 64% in 5 experiments. This relatively high amount might be in accordance with the morphology of blood platelets, whose membrane surface is considerably increased by numerous tortuous channels constituting the open canalicular system [45].

The estimation presented in Fig. 5 can be used also for any other phospholipid degraded under non-lytic conditions. The ratio $X/(X + x)$ being known, it becomes possible to calculate for each phospholipid class the percentage hydrolysis in the platelet membrane itself (H/X) from the percentage hydrolysis measured in the total platelet, $H/(X + x)$. The results of such a calculation are compiled in Table IV. It is clear that 29% degradation of total phospholipid measured in pig platelet suspensions corresponds to 46% of phospholipid hydrolysed in the membrane. It is concluded that the plasma membrane phospholipid fraction, which comprises 91% of sphingomyelin, 40% of lecithin, 34% of phosphatidylethanolamine and less than 6% of phosphatidylserine (+ phosphatidylinositol), forms the outer half of the surface membrane (Fig. 6). The inner half would then be relatively rich in glycerophospholipids, roughly consisting of equal amounts of lecithin, phosphatidylethanolamine and phosphatidylserine. The distribution of sphingomyelin is similar to that found for human red cells. In contrast, glycerophospholipids show a somewhat different pattern, since a smaller amount of lecithin but more phosphatidylethanolamine is present at the outside of the platelet membrane. It is likely that the asymmetric distribution of the phospholipids in the human platelet membrane is also similar to that from pig, phosphatidylethanolamine may be somewhat more confined to the membrane interior (compare Table II).

Recently, Schick et al. [21] reported evidence for an asymmetric distribution of phosphatidylethanolamine and phosphatidylserine in the platelet membrane using trinitrobenzene sulphonate (TNBS) to label aminophospholipids. Their data are in good agreement with the present experiments using phospholipases, since with intact human platelets no phosphatidylserine reacted with

TABLE IV

NON LYTIC DEGRADATION OF PHOSPHOLIPIDS BY PHOSPHOLIPASE A_2 AND SPHINGOMYELINASE C IN THE PIG PLATELET MEMBRANE

Phospholipid composition is the mean of 5 experiments (± S.D.).

	Phospholipid composition	Phospholipid degradation	
	% of total phospholipid	% of total phospholipid *	% of phospholipid class
Sphingomyelin	26.1 ± 2.3	23.7	91
Phosphatidylcholine	30.4 ± 2.0	12.2	40
Phosphatidylserine + phosphatidylinositol	16.1 ± 1.6	1.0	6
Phosphatidylethanolamine	27.3 ± 2.3	9.2	34
Total phospholipid	100	46.1	

* Calculated as described in the text from data of Table II (column 2).

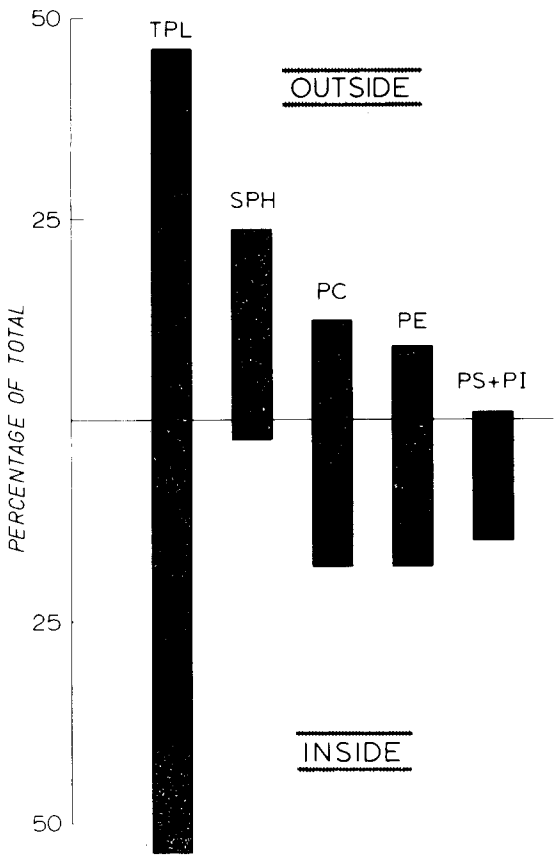

Fig. 6. Proposed distribution of phospholipids between inner and outer layer of the pig platelet surface membrane. Abbreviations: TPL, total phospholipid. For the others, see Figs. 2 and 3.

TNBS, whereas labelling of phosphatidylethanolamine leveled off at 12—17%. In contrast, Otnaess and Holm [22] found a completely different picture upon treatment of human platelets with *B. cereus* phospholipase C and *S. aureus* sphingomyelinase C. Only 20—25% of sphingomyelin was found to be hydrolysed by sphingomyelinase C, whereas phospholipase C was able to degrade 50—75% of phosphatidylethanolamine, 20—50% of lecithin and 20—25% of phosphatidylserine, representing 30—45% of total phospholipid. The possible reasons for such a discrepancy are not completely clear. With respect to the effect of sphingomyelinase C, the authors provide little information on the details of the experimental conditions. It should be mentioned, however, that this enzyme preparation has been reported to exhibit a powerful lytic activity towards human platelets [46], and to have a low specific activity (2 IU/mg), almost 1000 times smaller than the sphingomyelinase C preparation used in the present experiments [47]. Therefore, it is conceivable that the amounts of enzyme used by Otnaess and Holm were insufficient to give complete degradation of sphingomyelin at the platelet membrane exterior (compare also Fig. 1). There seems to be a remarkable difference between the phospholipase C preparation from *B. cereus* used by Otnaess and Holm and the preparation used in

our laboratory, in that the latter fails to produce phospholipid breakdown in intact pig and human platelets. This might be due to strain differences in *B. cereus*. However, the authors do not report to have blocked the enzymatic activity by addition of EDTA and *o*-phenanthroline after the incubation procedure but prior to extraction of the phospholipids. We have occasionally found that phospholipases A_2 and C are not immediately blocked upon mere addition of methanol and chloroform to the incubation mixture (activity might even be enhanced with methanol). Therefore, it is conceivable that the reported phospholipid breakdown by *B. cereus* phospholipase C occured during the extraction procedure and not during incubation. This would explain why no changes in platelet aggregation were enregistered directly after incubation with phospholipase C [22].

We have reported earlier [18] that pure *B. cereus* phospholipase C is intrinsically unable to attack the lipids in intact red cells, because the maximal surface pressure up to which the enzyme can act is lower than the surface pressure of the lipids in the erythrocyte membrane. The same experimental approach was used to determine the lateral surface pressure of the lipids in the plasma membrane of human and pig platelets.

Estimation of the surface pressure of the platelet membrane

In addition to revealing a non-random distribution of phospholipids in the membrane, phospholipases can also provide information on the compression state of the lipids in the native membrane. This is based on the observation that one group of phospholipases produces phospholipid breakdown in intact erythrocytes, whereas the other group of phospholipases fails to exert its action on intact cells [18]. These two groups of enzymes can also be distinguished in studies dealing with the activity of phospholipases towards monomolecular

TABLE V

AVAILABILITY OF PHOSPHOLIPIDS TO DIFFERENT PHOSPHOLIPASES IN INTACT PLATELETS AND ERYTHROCYTES

Incubations were carried out for 30 min in the absence of albumin, with aspirin-treated platelets or in presence of 2 mM *o*-phenanthroline (except for *B. cereus* and *C. welchii* phospholipase C). N.D.: Not determined.

Phospholipases (IU/100 mg platelets)		Maximal surface pressure (dynes/cm) *	Hydrolysis (% of total phospholipid)		
			Pig platelets	Human platelets	Human red cells **
Phospholipase A_2	Pig pancreas (40)	16.5	—	N.D.	—
	Cr. adamanteus (40)	23.0	—	—	—
Phospholipase C	*B. cereus* (40—80)	31.0	—	—	—
Phospholipase A_2	*N. naja* (40)	34.8	6	6	20
	Bee venom (40)	35.8	7.5	N.D.	19
Sphingomyelinase	*S. aureus* (10)	>40	14.2	13	20
Phospholipase C	*C. welchii* (8) ***	>40	+	+	+

* Taken from Demel et al. [20].

** Taken from Zwaal et al. [18].

*** Degradation produces lysis.

films of phospholipids, spread at an air-water interface at various initial surface pressures [20]. Those phospholipases which fail to exert their action on intact cells are also unable to hydrolyse lipids when injected under a monolayer of phospholipids spread at an initial surface pressure above 31 dynes/cm. On the other hand, those phospholipases which are able to attack the intact red cell membrane can produce phospholipid degradation in monolayers with an initial surface pressure of at least 34 dynes/cm. From these observations it has been concluded that the packing of the phospholipids at the exterior layer in the erythrocyte membrane is comparable with a lateral surface pressure of 31—34 dynes/cm.

It is most striking that the same distinction can be made between the two groups of phospholipases using blood platelets (see Table V). It is emphasized, however, the the degradative action of both *N. naja* and bee venom phospholipase A_2 is limited towards platelets as compared to erythrocytes. This suggests that the surface pressure in the platelet membrane might be somewhat higher than in the erythrocyte membrane, and very close to 34 dynes/cm.

Concluding remarks

In spite of the different origin of platelets and erythrocytes, the asymmetric distribution of the phospholipids appears to be rather similar. This can be taken to support the notion that the phenomenon of lipid asymmetry is wide spread in nature. One of the most unsatisfactory aspects of the observed membrane asymmetry is that both its origin and physiological significance are still unknown. In the case of platelets, it would be tempting to hypothesize a relation between the relative abundance of phosphatidylethanolamine on the platelet surface and its procoagulant activity. However, some preliminary experiments in our laboratory do not seem to support this view.

De Pierre and Dallner [48] have given evidence that the cytoplasmic surface of intracellular membranes of hepatocytes is rich in phosphatidylserine. Assuming a similar distribution of phosphatidylserine in platelets, this would imply that the majority of the phosphatidylserine molecules is facing the cytoplasm, both in plasma and intracellular membranes. It is the generally received opinion that Ca^{2+} induces release reaction, which is accompanied by the fusion of granules with the plasma membrane. Such a fusion process might be enabled by the location of phosphatidylserine on the opposing surfaces of the membranes to be fused, since this phospholipid has been shown to be active in fusion processes in the presence of Ca^{2+} [49—51].

It has been shown that during release reaction no phosphatidylserine becomes available at the outside of the surface membrane [21]. The observation of Joist et al. [26] and Sixma and Nijessen [52] that only few percent of procoagulant activity becomes available during release reaction as compared with the activity obtained upon lysis, supports the general view that negatively charged phospholipids, like phosphatidylserines, are required for maximal clot promoting activity.

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References

1 Marcus, A.J., Ullman, H.L. and Safier, L.B. (1969) J. Lipid Res. 10, 108—114
2 Nordöy, A., Day, H.J. and Lund, S. (1969) Scand. J. Clin. Lab. Invest. 23, 169—174
3 Broekman, M.J., Handin, R.I., Derksen, A. and Cohen, P. (1976) Blood 47, 963—972
4 Taylor, D.G. and Crawford, N. (1976) Biochim. Biophys. Acta 436, 77—94
5 Barber, A.J. and Jamieson, G.A. (1970) J. Biol. Chem. 245, 6357—6365
6 Marcus, A.J. (1966) Adv. Lipid Res. 4, 1—37
7 Bills, I.K., Smith, J.B. and Silver, M.J. (1976) Biochim. Biophys. Acta 424, 303—314
8 Pickett, W.C. and Cohen, P. (1976) J. Biol. Chem. 251, 2536—2538
9 Rittenhouse-Simmons, S., Russel, F.A. and Deykin, D. (1976) Biochem. Biophys. Res. Commun. 70, 295—301
10 Russel, F.A. and Deykin, D. (1976) Am. J. Haematol. 1, 59—70
11 Chap, H. and Douste-Blazy, L. (1974) Eur. J. Biochem. 48, 351—355
12 Schick, P.K. and Yu, B.P. (1974) J. Clin. Invest. 54, 1032—1039
13 Derksen, A. and Cohen, P. (1975) J. Biol. Chem. 250, 9342—9347
14 Kaulen, H.D.R. and Gross, R. (1976) Thromb. Haemost. 35, 364—376
15 Colley, C.M., Zwaal, R.F.A., Roelofsen, B. and van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 307, 74—82
16 Zwaal, R.F.A., Roelofsen, B. and Colley, C.M. (1973) Biochim. Biophys. Acta 300, 159—182
17 Verkleij, A.J., Zwaal, R.F.A., Roelofsen, B., Comfurius, P., Kastelijn, D. and van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 323, 178—193
18 Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 83—96
19 Bretscher, M.S. (1972) J. Mol. Biol. 71, 523—528
20 Demel, R.A., Geurts van Kessel, W.S.M., Zwaal, R.F.A., Roelofsen, B. and van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 97—107
21 Schick, P.K., Kurica, K.B. and Chacko, G.K. (1976) J. Clin. Invest. 57, 1221—1226
22 Otnaess, A.B. and Holm, T. (1976) J. Clin. Invest. 57, 1419—1425
23 Rock, C.O. and Snyder, F. (1975) J. Biol. Chem. 250, 6564—6566
24 Aster, R.H. and Jandl, J.H. (1964) J. Clin. Invest. 43, 843—855
25 Ardlie, N.G., Packham, M.A. and Mustard, J.F. (1970) Brit. J. Haematol. 19, 7—17
26 Joist, J.H., Dolezel, G., Lloyd, J.V., Kinlough-Rathbone, R.L. and Mustard, J.F. (1974) J. Lab. Clin. Med. 84, 474—482
27 Baenziger, N.L. and Majerus, P.W. (1974) Methods Enzymol. 31A, 149—155
28 Reed, C.F., Swisher, S.N., Marinetti, G.V. and Ede, G.V. (1960) J. Lab. Clin. Med. 56, 281—289
29 Broekhuyse, R.M. (1969) Clin. Chim. Acta 23, 457—461
30 Böttcher, C.J.F., van Gent, C.M. and Pries, C. (1961) Anal. Chim. Acta 24, 203—204
31 Wroblewski, F. and La Due, J.S. (1955) Proc. Soc. Exp. Biol. 90, 210—215
32 Kaulen, H.D. and Gross, R. (1973) Thromb. Diath. Haemorrh. 30, 199—214
33 Day, H.J., Holmsen, H. and Hovig, T. (1969) Scand. J. Haematol. Suppl. 7, 3—35
34 Koerner, J.F. and Sinsheimer, R.L. (1957) J. Biol. Chem. 228, 1039—1048
35 Lowry, O.H., Rosebrough, N.J., Farr, N.L. and Randall, N.J. (1951) J. Biol. Chem. 193, 265—275
36 Martin, J.K., Luthra, M.G., Wells, M.A., Watts, R.P. and Hanahan, D.J. (1975) Biochemistry 14, 5400—5408
37 Gazitt, Y., Loyter, A., Reichler, Y. and Ohad, I. (1976) Biochim. Biophys. Acta 419, 479—492
38 Haest, C.W.M. and Deuticke, B. (1976) Biochim. Biophys. Acta 436, 353—365
39 Gul, S. and Smith, A.D. (1974) Biochim. Biophys. Acta 367, 271—281
40 Samuelsson, B., Granström, E., Green, K., Hamberg, M. and Hammarström, S. (1975) Annu. Rev. Biochem. 44, 669—695
41 Vargaftig, B.B., Tranier, Y. and Chignard, M. (1975) Eur. J. Pharmacol. 33, 19—29
42 Nelson, G.J. (1967) Biochim. Biophys. Acta 144, 221—232
43 Broekman, M.J., Westmoreland, N.P., Cohen, P., Wilcox, E. and Raynesford, A. (1974) J. Cell. Biol. 60, 507—518
44 Salganicoff, L., Heba, P.A., Yandrasitz, J. and Fukami, M.H. (1975) Biochim. Biophys. Acta 385, 394—411
45 White, J.G. (1972) Am. J. Pathol. 66, 295—312
46 Wadström, T. and Möllby, R. (1971) Biochim. Biophys. Acta 242, 308—320

47 Wadström, T. and Möllby, R. (1971) Biochim. Biophys. Acta 242, 288–307
48 DePierre, J.W. and Dallner, G. (1975) Biochim. Biophys. Acta 415, 411–472
49 Jacobson, H. and Papahadjopoulos, D. (1975) Biochemistry 14, 152–161
50 Papahadjopoulos, D., Poste, G., Schaeffer, D.E. and Vail, W.J. (1974) Biochim. Biophys. Acta 352 10–28
51 Miller, C. and Racker, E. (1976) J. Membrane Biol. 26, 319–333
52 Sixma, J.J. and Nijessen, J.G. (1970) Thromb. Diath. Haemorrh. 24, 206–213