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ORGANIZATION OF PHOSPHOLIPIDS IN HUMAN RED CELL MEMBRANES AS DETECTED BY THE ACTION OF VARIOUS PURIFIED PHOSPHOLIPASES*

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SUMMARY

1. The action of eight purified phospholipases on intact human erythrocytes has been investigated. Four enzymes, e.g. phospholipases A_2 from pancreas and *Crotalus adamanteus*, phospholipase C from *Bacillus cereus*, and phospholipase D from cabbage produce neither haemolysis nor hydrolysis of phospholipids in intact cells. On the other hand, both phospholipases A_2 from bee venom and *Naja naja* cause a non-haemolytic breakdown of more than 50 % of the lecithin, while sphingomyelinase C from *Staphylococcus aureus* is able to produce a non-lytic degradation of more than 80 % of the sphingomyelin.

2. Phospholipase C from *Clostridium welchii* appeared to be the only lipolytic enzyme tested, which produces haemolysis of human erythrocytes. Evidence is presented that the unique properties of the enzyme itself, rather than possible contaminations in the purified preparation, are responsible for the observed haemolytic effect.

3. With non-sealed ghosts, all phospholipases produce essentially complete breakdown of those phospholipids which can be considered as proper substrates for the enzymes involved.

4. Due to its absolute requirement for Ca^{2+}, pancreatic phospholipase A_2 can be trapped inside resealed ghosts in the presence of EDTA, without producing phospholipid breakdown during the resealing procedure. Subsequent addition of Ca^{2+} stimulates phospholipase A_2 activity at the inside of the resealed cell, eventually leading to lysis. Before lysis occurs, however, 25 % of the lecithin, half of the phosphatidylethanolamine and some 65 % of the phosphatidylserine can be hydrolysed. This observation is explained in relation to an asymmetric phospholipid distribution in red cell membranes.

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* Phospholipases used: phospholipase A_2 (phosphatide acyl-hydrolyse, EC 3.1.1.4) from pig pancreas, bee venom, *Naja naja*, and *Crotalus adamanteus*, respectively. Phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* and *Clostridium welchii*. Sphingomyelinase C (sphingomyelin cholinephosphohydrolase) from *Staphylococcus aureus*. Phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) from Savoy cabbage.

5. It is concluded that the ability of the various phospholipases to attack the red cell membrane is dependent upon: (i) substrate specificity of the phospholipases, (ii) sidedness of the phospholipids when only one side of the membrane is exposed to phospholipase action, and (iii) compression state of the membranous lipid layer. The latter point is dealt with in more detail in the accompanying paper (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).

INTRODUCTION

The distribution of phospholipids between the exterior and interior region of the erythrocyte membrane can be investigated by the use of purified phospholipases [1–4]. It can be argued that enzymatic breakdown of phospholipids, obtained by the action of phospholipases on intact red cells, is restricted to those phospholipids which are located at the outside of the membrane. Enzymatic action on non-sealed ghosts will lead to hydrolysis of phospholipids at both sides of the membrane. Moreover, trapping of phospholipases (without cofactor) inside resealed ghosts followed by addition of cofactor, will result in enzymatic degradation of those phospholipids which are present at the inside of the membrane.

A non-uniform distribution of phospholipids between the outside and the inside of the red cell membrane has first been proposed by Bretscher [5–7], based on the observation that the relatively non-permeant label formylmethionylsulphone methylphosphate fails to tag phosphatidylethanolamine and phosphatidylserine in intact cells, whereas both phospholipids are readily labelled in ghosts. These observations were essentially confirmed by Gordeski and Marinetti [8] using the non-penetrating probe 2,4,6-trinitrobenzene sulphonate, and very recently by Whiteley and Berg [9] upon amidination of the outer and inner surfaces of the red cell membrane.

Studies using phospholipases also favour the view that such an asymmetric arrangement of phospholipids occurs in the erythrocyte membrane. Details of these studies were recently reported by Colley et al. [1], Verkleij et al. [2] and Zwaal et al. [3]. The combined action of *Naja naja* phospholipase A_2 and sphingomyelinase C from *Staphylococcus aureus* towards intact human erythrocytes results in a degradation of half of the total phospholipid complement of the membrane, without producing lysis of the cells. Since essentially all the phospholipids of the membrane are readily degraded in ghosts by this combined enzyme action, it was concluded that the phospholipid fraction degraded in intact cells, which contains the majority of the choline-containing phospholipids (lecithin and sphingomyelin) and some one-fifth of the phosphatidylethanolamine, forms the outer monolayer of the human red cell membrane. Recently, quantitative data obtained from studies concerning phospholipase action on intact red cells [10] and sealed inside out ghosts [11] seem to support this conclusion.

In the accompanying paper [12], the action of various phospholipases towards mono-molecular films of phospholipids has been described. The present study deals with the action of the same enzymes on intact human erythrocytes, and the possible relation between these actions and those on phospholipid monolayers. Furthermore, the effects of phospholipase C from *Clostridium welchii* on intact cells, and the action of pancreatic phospholipase A_2 inside resealed ghosts is studied in more detail, in

order to provide further evidence for an asymmetric distribution of phospholipids in red cell membranes.

MATERIALS AND METHODS

Purification of phospholipases

Pure phospholipase A_2 from porcine pancreas was prepared as described by de Haas et al. [13]. Phospholipase A_2 from bee venom (*Apis mellifica*), purified according to Shipolini et al. [14], was a gift of Dr C. A. Vernon to Dr G. H. de Haas. Phospholipase A_2 from *Naja naja* venom (Koch Light) was purified as described by Cremona and Kearney [15], with the exception that gel filtration on Sephadex G-75 was replaced by gel filtration on Sephadex G-100 (column 4×180 cm) at room temperature, using as elution buffer: 50 mM Tris–HCl (pH 7.4), 5 mM $CaCl_2$, 50 vol.% of glycerol. Phospholipase A_2 from *Crotalus adamanteus* (Koch Light) was partially purified by gel filtration on Sephadex G-100 in 50 vol.% glycerol, as described above for *N. naja* phospholipase A_2.

Phospholipase C from *Bacillus cereus* was prepared as described previously [16, 17], with the exception that the bacteria were grown in a fermentor (5 l culture in a 7-l vessel) under continuous aeration (2 l/min). The foam produced during growth, which appeared to contain virtually all the phospholipase C activity, was collected from the air-outlet pipe on the fermentor vessel. After condensing the foam by cooling to 4 °C, the bacteria were spun down and the supernatant was used as the starting material for purification. The main advantage of the foaming technique appears to be a much higher production of phospholipase C by the bacteria, combined with a 3–4-fold increase in specific activity in the starting material.

Phospholipase C from *C. welchii* was purified from a lyophilized culture filtrate (purchased as Type I from Sigma). The dry material (200 mg) was dissolved in 1 ml of 50 mM Tris-HCl (pH 7.4), 5 mM $CaCl_2$, 50 vol.% of glycerol and fractionated by gel filtration on Sephadex G-100 (column: 2×140 cm), equilibrated and eluted with the same buffer. The active fractions were pooled and concentrated as described before for *B. cereus* phospholipase C [17]. The concentrated enzyme preparation was further purified by repeating the gel filtration procedure on the same Sephadex G-100 column.

Sphingomyelinase C was purified from log phase cultures of *S. aureus* (strain 269 HH), grown for 16 h at 37 °C by rotation in a Todd-Hewitt (Difco) medium (30 g/l) at low oxygen tension in an atmosphere containing 10 % CO_2 by vol. After removal of the cells by centrifugation (45 min, $5000 \times g$), the supernatant was adjusted to 77 % saturation with solid $(NH_4)_2SO_4$ and allowed to stand overnight at 4 °C. The precipitate was collected by centrifugation (30 min, $5000 \times g$) and dissolved in water (as concentrated as possible). The solution was subsequently dialyzed against 50 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$, 50 vol.% of glycerol and applied to Sephadex G-100 (column: 4.2×210 cm), equilibrated and eluted with the same buffer. The active fractions were pooled and concentrated as described before for *B. cereus* phospholipase C [17]. The concentrated enzyme preparation was dialysed against 50 mM Tris-HCl (pH 9.0), 5 mM $CaCl_2$, 50 vol.% of glycerol, and filtered through DEAE-Sephadex A-25 (column: 2×6 cm), equilibrated and eluted with the same buffer. The active fractions were adjusted to pH 9.25 with Tris and filtered through

CM-Sephadex C-25 (column: 2×6 cm), equilibrated and eluted with the same buffer (pH 9.25). Active fractions were pooled, adjusted to pH 7–8, and concentrated if necessary. Due to a p*I* around 9.1 [18–20] for sphingomyelinase C, there is no binding of the enzyme to the two ion-exchangers used under the conditions described.

Phospholipase D was partially purified from Savoy cabbage according to the method of Davidson and Long [21], with the exception that the treatment with calcium phosphate gel was replaced by gel filtration on Sephadex G-100 (column: 2×140 cm), equilibrated and eluted with 50 mM Tris buffer (pH 7.6), 5 mM $CaCl_2$, 150 mM NaCl and 50 vol.% of glycerol.

Sodium dodecyl sulfate electrophoresis in polyacrylamide gels of reduced phospholipase samples was performed as described by Fairbanks et al. [22].

Phospholipase assays

Routine assays of phospholipase A_2 activities were carried out as described by Nieuwenhuizen et al. [23]. Phospholipase C activities were measured as described by Zwaal and Roelofsen [17]. Sphingomyelinase C activities were detected in a similar manner as the substrate-decrease assay, as described for *B. cereus* phospholipase C [17], with the exception that lecithin was replaced by sphingomyelin and that the assay was carried out in the presence of 0.25 mM $MgCl_2$. Assay of the substrate's decrease was also used to measure phospholipase D activities; for this purpose 0.1 M acetate buffer (pH 5.6) containing 50 mM $CaCl_2$ was used instead of Tris.

All phospholipase activities are expressed in international units (I.U.).

Treatment of erythrocytes with phospholipases

Freshly collected human erythrocytes (from acid-citrate dextrose-treated blood; cells packed for 10 min at 3000×*g*) were washed four times with 0.87 % NaCl, 0.25 mM $CaCl_2$, 0.25 mM $MgCl_2$, pH 7.4, with 0.05 M Tris. Incubations were carried out as follows: aliquots of enzyme (5–10 I.U.) were mixed with 5 ml of the isotonic saline solution, followed by addition of 0.25 ml of packed cells. Incubations with *N. naja* phospholipase A_2 contained 10 mM $CaCl_2$; those with *B. cereus* and *C. welchii* phospholipase C also contained 0.1 mM $ZnCl_2$. Incubations with phospholipase D were carried out in isotonic acetate buffer (50 mM sodium acetate, 50 mM $CaCl_2$, 30 mM NaCl, pH 5.6, with acetic acid). Unless otherwise stated, the mixtures were incubated for 1 h at 37 °C with gentle stirring, followed by centrifugation at 3000×*g* for 5 min. The supernatants were collected and percentage haemolysis was determined as described previously [24].

Treatment of ghosts with phospholipases

Human erythrocyte ghosts were incubated with phospholipases as described previously [2, 24].

Phospholipid analysis

Enzymatic activity was inhibited by addition of a mixture of *o*-phenanthroline and EDTA (final concentration : 4 and 15 mM, respectively) prior to lipid extraction of the cells or ghosts by the method of Reed et al. [25]. The extracts were taken to dryness under reduced pressure and the residue was dissolved in 150 µl chloroform methanol (1 : 1, v/v). The phospholipids were separated by two-dimensional thin-

layer chromatography using the procedure of Broekhuyse [26], and determined as phosphorus after destruction with 70 % $HClO_4$ at 190 °C by a modification [27] of the procedure of Fiske and SubbaRow. Percentage degradation of the different phospholipid classes was calculated as described previously [2, 24].

Trapping of phospholipase A_2 inside resealed ghosts

Washed human red cells (25 ml) were lysed by addition of 155 ml 10 mM NaCl, 1 mM EDTA (final osmolarity of the mixture, 60 mosM) and stirred for 5 min at 0 °C. Prior to resealing, the haemolysate was centrifuged (10 min, 12 000 × *g*, 0 °C) and two-third of the supernatant was removed, in order to reduce the volume of the ghost suspension to be resealed. The ghosts were resuspended in the remaining one-third of the supernatant, and 750 µl pancreatic phospholipase A_2 (1 I.U./µl) was added, followed by stirring for 5 min at 0 °C. The ghosts were subsequently resealed by addition of 8.4 ml 1 M NaCl while stirring at 0 °C. After 5 min, the mixtures were brought to 37 °C and stirred for 1 h. The resealed cells were washed four times with 160 mM NaCl (centrifuge 10 min, 12 000 × *g*, 0 °C) to remove external phospholipase. Two control experiments were run: one experiment without addition of phospholipase, and another experiment in which the enzyme was added after resealing for 1 h at 37 °C.

Incubation of resealed cells with trapped phospholipase A_2

The hydrolytic action of pancreatic phospholipase A_2 from the inside of the resealed cells produces lysis at a certain stage of the incubation. Therefore, 1 ml of the packed resealed ghosts was suspended in 5 ml 150 mM NaCl, 5 mM $CaCl_2$ at 37 °C while stirring, in order to start the enzymatic reaction from the inside. After different time intervals, 200 µl of the suspension were transferred into 5 ml 160 mM NaCl (or 5 ml water to detect 100 % lysis) and directly centrifuged at 12 000 × *g* for 5 min at 0 °C. The supernatants were monitored without further dilution at 418 nm, using 160 mM NaCl as a blank.

For determination of phospholipid hydrolysis, 4 ml of the packed resealed cell suspension was mixed with 20 ml 150 mM NaCl, 5 mM $CaCl_2$, and incubated at 37 °C while stirring. At different time periods, 6-ml samples were taken and transferred into test tubes containing 20 mg of EDTA in order to stop the enzymic reaction. To check for absence of enzymatic breakdown during the resealing and subsequent incubation procedure when Ca^{2+} was omitted, 2 ml of the packed, resealed ghost suspension was mixed with 10 ml 160 mM NaCl (without Ca^{2+}) and a 6-ml sample (corresponding to the longest time period) was taken and transferred to a test tube containing 20 mg of EDTA. The ghost samples were extracted according to Reed et al. [25] and the phospholipid degradation was determined as described above under Phospholipid analysis.

RESULTS AND DISCUSSION

Phospholipases

In contrast to previous findings [1], partial purification of sphingomyelinase C from *S. aureus* by Sephadex G-100 gel filtration appeared to be no longer satisfactory because, for unknown reasons, no preparations devoid of haemolytic activity

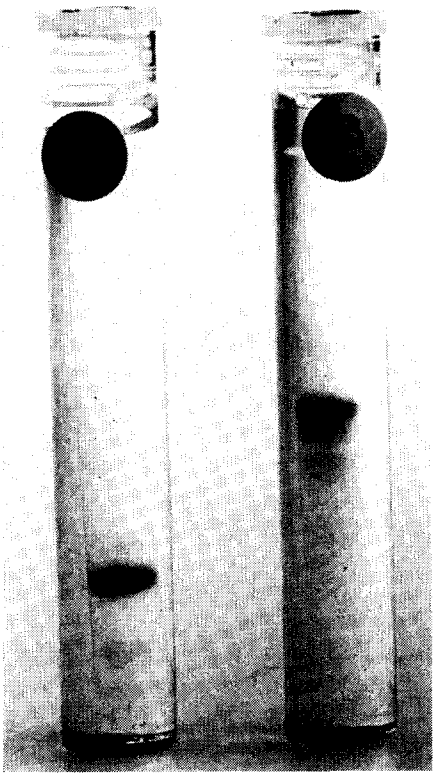

Fig. 1. Behaviour of purified *S. aureus* sphingomyelinase C (tube A) and purified *C. welchii* phospholipase C (tube B) on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, after reduction of the protein samples prior to electrophoresis. Electrophoresis of approx. 10 μg of protein was carried out in 5.6 % polyacrylamide gels containing 1 % sodium dodecyl sulfate as described by Fairbanks et al. [22]. Protein bands were visualized by staining with Coomassie blue.

were obtained. Therefore, further purification of this enzyme was undertaken, taking advantage of the observations of other investigators [18–20] that sphingomyelinase C appears to be isoelectric around pH 9.1. After filtration through DEAE-Sephadex A-25 at pH 9.0 and through CM-Sephadex C-25 at pH 9.25, the final product appeared as a major protein band after electrophoresis in sodium dodecyl sulfate polyacrylamide gels (Fig. 1). Two to three faint protein bands were also visible, indicating the presence of trace amounts of impurities. The specific activity on sphingomyelin (from sheep red cell ghosts) was calculated to be 1900 I.U./mg, assuming $E^{1\%}_{280nm} = 16$. This is considerably higher than purified preparations of staphylococcal β-haemolysin (sphingomyelinase C) recently obtained by Wadström and Möllby [18, 19] (spec. act. approx. 2 I.U./mg), and by Bernheimer et al. [20] (spec. act. approx. 140 I.U/mg). The difference in specific activity might be explained by the fact that our purification is carried out in the presence of 50 vol.% of glycerol, which is known to stabilize to a great extent enzymatic activities. The final product is completely stable when stored in 50 vol.% glycerol at -20 °C and is devoid of haemolytic activity.

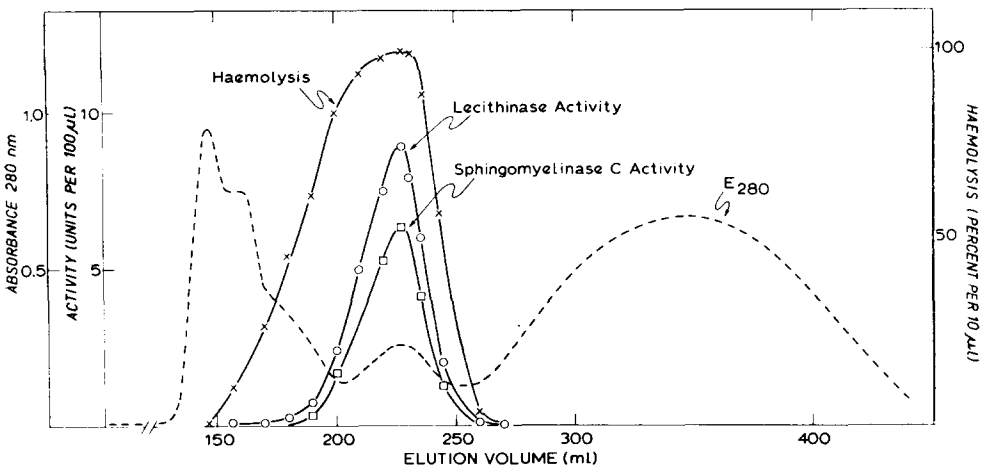

Fig. 2. Sephadex G-100 gel filtration of culture filtrate from *C. welchii*. Details described in the text. Elution void volume, 147 ml. Activity is expressed as international enzyme units per 100 µl of eluted fraction. Percentage haemolysis was determined as described earlier [24] with 10 µl of the eluted fractions.

Of all the phospholipases tested, only phospholipase C from *C. welchii* remained haemolytic during purification. Due to a favourable elution pattern of the lyophilized culture filtrate on Sephadex G-100 (Fig. 2), a second gel filtration on Sephadex G-100 resulted in a preparation which exhibited one main protein band and a few small impurities after sodium dodecyl sulfate-polyacrylamide electrophoresis (Fig. 1). The specific activity of the final preparation appeared to be 2000 I.U./mg for egg lecithin and 1400 I.U./mg for sphingomyelin, assuming $E^{1\%}_{280nm} = 16$. This preparation is comparable to the highly pure product recently isolated by Takahashi et al. [28], having a specific activity of 1620 I.U./mg protein, determined with lecithin as substrate but based on a different assay system. Other investigators [29–32] have reported much lower specific activities for their purified preparations, ranging from 72 to 370 I.U./mg protein. It is emphasized again that carrying out the whole purification procedure in the presence of 50 vol.% of glycerol, as described in this paper, has a marked preserving effect on enzymatic activities of phospholipases. In contrast to the observations of Pastan et al. [33], but in agreement with the results of Möllby and Wadström [31] and Takahashi et al. [28], no separation between lecithin- and sphingomyelin-hydrolyzing activities could be achieved (Fig. 2). The haemolytic activity of the eluate coincided very well with the enzyme activity, although some haemolytic activity might also be present in the preceding protein fraction eluted at 150–175 ml. Heat treatment of the enzyme at 56 °C in the presence of Ca^{2+} did not abolish the haemolytic activity while preserving the hydrolytic activity as described by Sabban et al. [34], but resulted in a gross inactivation of enzymatic activity similar to that described by Takahashi et al. [28].

Phospholipase action on intact cells

The action of the various phospholipases towards intact human red cells and ghosts is summarized in Table I. With the exception of *C. welchii* phospholipase C, none of the purified phospholipases produced haemolysis. In agreement with previous

TABLE 1

ACTION OF PHOSPHOLIPASES ON INTACT HUMAN ERYTHROCYTES AND GHOSTS

		Intact cells						Ghosts				
		Haemolysis	Percent degradation of phospholipids					Percent degradation of phospholipids				
			Total phospholipid	Sphingomyelin	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylserine	Total phospholipid	Sphingomyelin	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylserine
Phospholipase A_2	Pancreas	−	0	0	0	0	0	70–74	0	100	100	100
	Cr. adamanteus	−	0	0	0	0	0	70–74	0	100	100	100
	N. naja	−	20	0	68	0	0	70–74	0	100	100	100
	Bee venom	−	19	0	55	9	0	70–74	0	100	100	100
Phospholipase C	*B. cereus*	−	0	0	0	0	0	68–74	0	100	100	90–100
	C. welchii	+	Production of ghosts (see right)					75–85	100	100	80–100	0
Sphingomyelinase C	*S. aureus*	−	20	85	0	0	0	25	100	0	0	0
Phospholipase D	Cabbage	−	0	0	0	0	0	65–75	0	100	80–100	70–100
Phospholipase A_2 + sphingomyelinase C	*N. naja** + *S. aureus**	−	48	82	76	20	0	100	100	100	100	100
Phospholipase C + sphingomyelinase C	*B. cereus*** + *S. aureus***	+	Production of ghosts (see right)					98–100	100	100	100	90–100

* Taken from Verkleij et al. [2].

** Taken from Colley et al. [11].

observations [1–3], sphingomyelinase C and the phospholipases A_2 from *N. naja* and bee venom produced a considerable non-haemolytic degradation of sphingomyelin and lecithin, respectively, whereas pure pancreatic phospholipase A_2 and *B. cereus* phospholipase C appeared to be ineffective in this respect. In addition, both *Cr. adamanteus* phospholipase A_2 and cabbage phospholipase D failed to produce any detectable breakdown of phospholipids in intact human red cells, in spite of the fact that these two preparations are still relatively impure after the purification procedures described above. Since both preparations are inactive towards intact red cells, further purification has not been carried out. It should be mentioned that the individual action of all the phospholipases on non-sealed ghosts produced a complete breakdown of those phospholipids which are proper substrates for the enzymes involved (Table I). It has been shown in previous studies [1, 2] that the subsequent action of *N. naja* phospholipase A_2 and sphingomyelinase C produces a non-haemolytic breakdown of all the phospholipids in the outer-membrane layer, comprising the majority of the choline-containing phospholipids and one-fifth of the phosphatidylethanolamine. On the other hand, the combined action of sphingomyelinase C and *B. cereus* phospholipase C is haemolytic for human red cells, although neither of these enzymes produces lysis independently. Apparently, the production of ceramides, lyso-compounds and fatty acids in the outer-membrane layer does not result in haemolysis, although the osmotic resistance of the cell is strongly impaired. In contrast, the simultaneous formation of both ceramides and diacylglycerols in the outer-membrane layer appears to be a haemolytic process [3].

In this respect, the haemolytic action of *C. welchii* phospholipase C may be interesting, since this enzyme has been shown to attack preferentially both choline-containing phospholipids (lecithin and sphingomyelin) [28–32], thus exhibiting a similar action as a mixture of *B. cereus* phospholipase C and *S. aureus* sphingomyelinase C. At this stage of the investigation, however, the possibility could not be completely ruled out that haemolysis was produced by a minor contaminant in the enzyme preparation. As shown in Fig. 3., *C. welchii* phospholipase C produced complete haemolysis of human erythrocytes within 5 min incubation time. However, when the

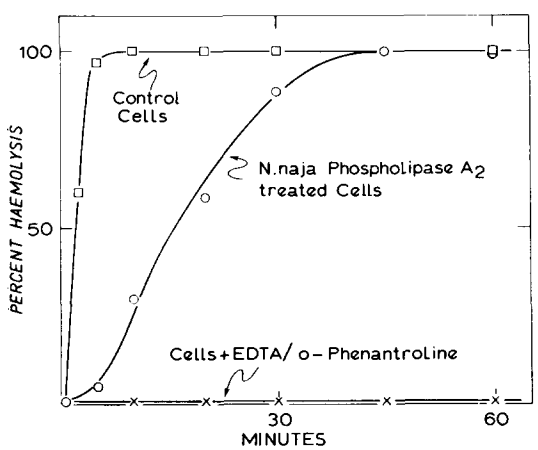

Fig. 3. Haemolytic activity of purified *C. welchii* phospholipase C under different conditions. For details see the text.

enzymatic activity was completely blocked by addition of a mixture of EDTA and *o*-phenanthroline, no haemolysis occurred and also no phospholipid hydrolysis could be established in these cells. This may be taken as evidence that the enzyme itself, rather than a possible contaminant, exhibits a haemolytic action towards human red cells. This is further supported by the observation that red cells treated first with *N. naja* phospholipase A_2, followed by treatment with *C. welchii* phospholipase C, are lysed at a much slower rate than control cells with this phospholipase C alone, in spite of the strongly increased osmotic fragility of the phospholipase A_2-treated cells. One would expect that osmotically fragile cells are lysed at a faster rate in case haemolysis is caused by a contaminating haemolytic toxin in the *C. welchii* phospholipase C preparation. The most obvious explanation is that with control cells, *C. welchii* phospholipase C is able to degrade both lecithin and sphingomyelin (which produces lysis similar to the combined action of *B. cereus* phospholipase C and *S. aureus* sphingomyelinase), while with phospholipase A_2-treated cells, *C. welchii* phospholipase C can only attack sphingomyelin and the lysolecithin molecules, produced in the outer-membrane layer by the pretreatment with phospholipase A_2. The conversion of lysolecithin by *C. welchii* phospholipase C proceeds at a much slower rate than the breakdown of lecithin [32], resulting in a much lower rate of the haemolysis process.

We have suggested before [4] that the phospholipids are directly available on the outside of the intact red cell membrane, but that the ability of the different phospholipases to exert their action depends strongly on the compression state of the lipids in the native membrane. This suggestion is further supported by studies dealing with the activity of phospholipases towards mono-molecular films of phospholipids at various initial surface pressures. Details of these investigations are described in the following paper [12]. It is most striking that those phospholipases which fail to exert their action on intact cells, are also unable to produce lipid breakdown when injected under a monolayer of choline-containing phospholipids spread at an air water interface at an initial surface pressure above 31 dynes/cm. On the other hand, those phospholipases which are able to attack the intact erythrocyte membrane, also produce breakdown of monolayers of choline-containing phospholipids with an initial surface pressure above 31 dynes/cm. Furthermore, *B. cereus* phospholipase C is able to exert its action to a mixed monolayer of lecithin and sphingomyelin with an initial surface pressure above 31 dynes/cm, provided that sphingomyelin is degraded first or simultaneously by *S. aureus* sphingomyelinase C. Therefore, it is likely that with intact cells, breakdown of sphingomyelin enables *B. cereus* phospholipase C to hydrolyse the glycerophospholipids at the outside of the membrane (mainly lecithin), resulting in lysis of the cells. Since *C. welchii* phospholipase C is able (i) to hydrolyse both lecithin and sphingomyelin, and (ii) to attack mixed monolayers of these two choline-containing phospholipids at initial surface pressures above 31 dynes/cm, it is concluded that the intrinsic properties of this enzyme are responsible for producing haemolysis of human erythrocytes. At present, no other pure phospholipase has been shown to have haemolytic properties.

Phospholipase A_2 action within resealed ghosts

Further evidence for the existance of a non-random phospholipid distribution can be obtained by directly attacking the inside of the red cell membrane, after

trapping a phospholipase without its cofactor inside resealed ghosts. Starting the enzymic reaction by addition of cofactor will lead to phospholipid hydrolysis which is restricted to those phospholipid classes which are present at the membrane interior, provided that no lysis of the resealed cells occurs. For this purpose it is essential that the enzyme is trapped within the greatest possible number of resealed cells, and that no hydrolysis of phospholipids occurs during the resealing process.

Although pancreatic phospholipase A_2 fails to attack the phospholipids in intact cells, this enzyme appeared to be able to attack the phospholipids at the inside of the membrane after trapping within resealed ghosts in the presence of EDTA. This resealing process is not accompanied by phospholipid breakdown by the pancreatic phospholipase A_2. Activation of the enzyme by addition of Ca^{2+} produced substantial breakdown of glycerophospholipids before the cells started to lyse (Fig. 4). At this point, approx. 25 % of the lecithin, 50 % of the phosphatidylethanolamine, and 65 % of the phosphatidylserine were found to be degraded. This comprises 30–35 % of the total phospholipid fraction of the membrane. It is remarkable that before the onset of lysis the degradation of phosphatidylcholine tended to cease, whereas hydrolysis of the other two glycerophospholipids appeared to be in full swing. As expected, breakdown of all the glycerophospholipids went to completion during lysis of the resealed cells. In a control experiment it was established that pancreatic phospholipase A_2 is unable to produce phospholipid breakdown when added to ghosts after the resealing procedure.

It has first been recognized by Hoffman [35, 36] that a ghost suspension cannot be considered as a homogeneous population. A certain fraction of the ghost population is capable of resealing, whereas the rest of the ghosts remain leaky. Bodemann and Passow [37] and Schwoch and Passow [38] have distinguished between three types of ghosts, one of these (called Type II) representing a ghost population which reseals after reversion of the haemolysis procedure by addition of alkali ions. It

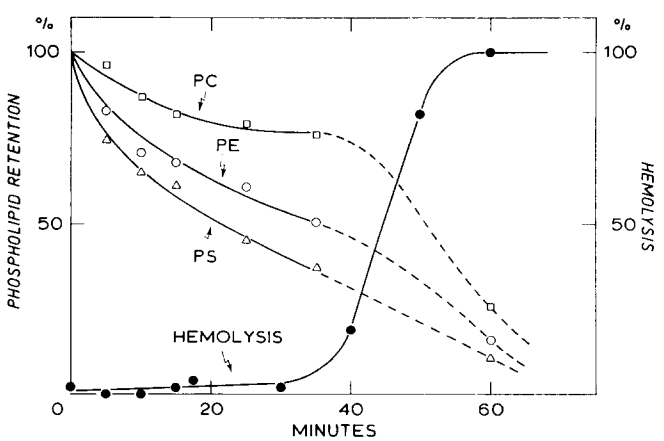

Fig. 4. Percentage retention of glycerophospholipids after trapping of pancreatic phospholipase A_2 (without Ca^{2+}) inside resealed human erythrocyte ghosts followed by addition of Ca^{2+} to start enzymic breakdown at $t = 0$.
Abbreviations: PC, lecithin; PE, phosphatidylethanolamine; PS, phosphatidylserine.

needs no argument that for studying phospholipase action at the inside of resealed ghosts, the fraction of Type II ghosts should be as high as possible. This has been realized by lysing the red cells at 0 °C, pH 6, followed by reversing tonicity and subsequent resealing at 37 °C. The observation that, after Ca^{2+} addition, lecithin degradation by trapped pancreatic phospholipase A_2 tends to level off around 25 % breakdown (corresponding to 75 % retention in Fig. 4), does suggest that this fraction is present at the inner side of the human erythrocyte membrane. This fraction may correpond to that lecithin fraction which cannot be hydrolysed by *N. naja* phospholipase A_2 treatment of intact cells (see also ref. 2). On the other hand, the non-lytic degradation of phosphatidylethanolamine and phosphatidylserine from the inside is not completely complementary to the observation with intact erythrocytes, described earlier [2]. This is mainly due to the fact that the resealed cells apparently start to lyse when 30–35 % of the total phospholipids of the membrane have been converted into their respective lyso-derivatives*. The relatively rapid attack of both phosphatidylethanolamine and phosphatidylserine does suggest, however, that a major fraction of these two phospholipids is present at the membrane interior, although from this study no quantitative estimations as to what extent can be made.

Due to its absolute requirement for Ca^{2+} [39], pancreatic phospholipase A_2 can be completely inhibited by EDTA. This is an essential premise for experiments with resealed ghosts, when no breakdown of phospholipids can be allowed to occur during the resealing procedure. Moreover, in spite of the presence of a membrane-bound Ca^{2+}-ATPase, addition of Ca^{2+} to the resealed ghost suspension apparently results in a sufficient Ca^{2+} concentration inside the ghosts so as to activate pancreatic phospholipase A_2. So far, other phospholipases have been found to be less satisfactory in trapping experiments. For example, the ghosts are difficult to seal in the presence of *N. naja* phospholipase A_2; they often remain leaky and some phospholipid degradation may occur during the resealing process even in the presence of high EDTA concentrations.

It is remarkable that, although pancreatic phospholipase A_2 is unable to attack the membrane outside intact erythrocytes and resealed ghosts, it is capable of attacking the membrane inside resealed cells. Similar observations have been reported for *B. cereus* phospholipase C [40]. The finding [12] that both pancreatic phospholipase A_2 and *B. cereus* phospholipase C are able to attack monolayers of the negatively charged phospholipids at much higher initial surface pressures than monolayers composed of lecithin, may provide an explanation of the phenomenon that these two enzymes can act on the membrane interior and not on the exterior.

CONCLUDING REMARKS

The degradative action of phospholipases on intact red cells results in a dramatic increase in osmotic fragility, but does not always lead to haemolysis of the cell. Apparently, all the glycerophospholipids of the outer-membrane layer can be converted in lyso-derivatives and fatty acids without producing haemolysis. On the other

* It should be noted that non-haemolytic attack of intact human erythrocytes by phospholipase A_2 (*N. naja* or bee venom) does not convert more than 28 % of the glycerophospholipids in their respective lyso-derivatives.

hand, the conversion of the glycerophospholipids into lyso-compounds and fatty acids at the membrane inside, appears to result in haemolysis after breakdown of a considerable fraction of the glycerophospholipids at this side of the membrane. This difference in response between the two sides of the membrane may be due to the relative abundancy of sphingomyelin (which is not attacked by phospholipase A_2) in the outer-membrane layer, whereas this phospholipid is nearly absent at the membrane inside. Even when the sphingomyelin molecules in the outer-membrane layer are converted in ceramides by sphingomyelinase C after the degradative action of phospholipase A_2, the permeability barrier for haemoglobin is retained. For unknown reasons, the reverse order of attack by these two enzymes appears to be a haemolytic process [2]. Haemolysis also occurs when the split products, produced by phospholipase A_2 action on intact cells, are removed by addition of serum albumin [3, 10]. Moreover, the simultaneous formation of ceramides and diacylglycerols in the outer-membrane layer impairs the membrane structure to such an extent that haemolysis seems to be inevitable. This is probably due to a phase-separating aggregation of diacylglycerols and ceramides into discrete pools, similar to the formation of "black dots" in ghosts during treatment with phospholipase C [1, 3, 41], leading to a destruction of the permeability barrier of the membrane.

A second aspect of phospholipase treatment of intact red cells concerns the observation that some phospholipases are intrinsically able to exert their action, whereas other phospholipases fail to have access to the membrane phospholipids. In this respect, it is concluded that the accessibility of red cell membrane phospholipids in situ to phospholipases is governed by at least three underlying principles: (i) substrate specificity of the phospholipases, (ii) sidedness of phospholipids when only one side of the membrane is exposed to the phospholipase action and, (iii) compression state of the lipids in the membrane. The influence of the last-mentioned effect on phospholipase action is studied in more detail in the following paper [12], using phospholipid monolayers at various initial surface pressures as a model system.

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