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THE ASYMMETRIC DISTRIBUTION OF PHOSPHOLIPIDS IN THE HUMAN RED CELL MEMBRANE

A COMBINED STUDY USING PHOSPHOLIPASES AND FREEZE-ETCH ELECTRON MICROSCOPY

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SUMMARY

1. Phospholipase A_2 (phosphatide acylhydrolase, EC 3.1.1.4) from *Naja naja* hydrolyses 68% of the lecithin of the intact human erythrocyte, without changing the freeze–fracture faces of the membrane. Phospholipase A_2 (*Naja naja*) treatment of ghosts produces complete breakdown of the glycerophospholipids and induces aggregation of particles on the freeze–fracture faces of the membrane.

2. Phospholipase C (phosphatidylcholine choline phosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* does not attack intact cells and no change in freeze–etch morphology is observed. The glycerophospholipids of ghosts are almost completely degraded by this enzyme, which causes a reduction in tangentially-splitted membranes and a formation of large diglyceride droplets, which are also visible by phase-contrast microscopy.

3. Sphingomyelinase (sphingomyelin choline phosphohydrolase) from *Staphylococcus aureus*, hydrolyses 80–85% of the sphingomyelin of the intact human red cell, and produces aggregation of the particles and the formation of small spheres (75 Å and 200 Å in diameter) on the outer fracture face with corresponding pits on the inner fracture face. Treatment of ghosts with this enzyme causes a complete degradation of the sphingomyelin and produces, in addition to aggregation of particles, the formation of droplets (1000–3000 Å in diameter) which are adherent to the membrane and are not visible by phase-contrast microscopy.

4. When the cells are treated successively with phospholipase A_2 (*Naja naja*) and sphingomyelinase (*Staphylococcus aureus*) no lysis occurs although the osmotic fragility is markedly increased. By this treatment, up to 48% of the total phospholipids are degraded. It is concluded that this phospholipid fraction (which contains the majority of the choline-containing phospholipids and some phosphatidylethanolamine) forms the outer monolayer of the membrane.

INTRODUCTION

The selective degradative action of lipases on lipid molecules can be used to investigate the molecular structure of the biological membrane. For this purpose

the action of phospholipase A_2 (phosphatide acylhydrolase, EC 3.1.1.4) and phospholipase C (phosphatidylcholine choline phosphohydrolase EC 3.1.4.3) on erythrocyte membranes has been extensively investigated (see for recent review ref. 1). Recently sphingomyelinase action on erythrocytes has also been studied[1,2]. These studies demonstrate clearly that the effect of the enzymes on the intact cells is different from the effect on isolated ghosts.

Pure pancreatic phospholipase A_2 does not hydrolyse any phospholipid in the membrane of the intact cell, whereas the main glycerophospholipid classes are completely degraded in ghosts[1,3]. However, phospholipases A_2 from sea snake venom[4], *Naja naja*[5] and bee venom[1] have been shown to produce phospholipid breakdown in the membrane of the human erythrocyte without causing lysis. Phospholipase C from *Clostridium welchii* produces degradation of 70% of the phospholipids of the ghosts, with the formation of the so-called "black dots"[6], visualized by phase-contrast microscopy. Pure phospholipase C from *Bacillus cereus*, though not able to degrade phospholipids in intact cells, causes complete degradation of the main phospholipids in ghosts with the exception of sphingomyelin, resulting in the release of 70% of the total lipid phosphorus[3]. Also in this case "black dots" are formed, which have been identified with the diglycerides produced by the action of *B. cereus* phospholipase C. Sphingomyelinase (sphingomyelin choline phosphohydrolase) from *Staphylococcus aureus* hydrolyses 80–85% of the sphingomyelin in intact human erythrocytes[2].

Phospholipases have been shown to be useful tools in the determination of asymmetrical distributions of phospholipids in erythrocyte membranes[1]. Such an asymmetry has been proposed by Bretscher[7,8], based on labelling of erythrocytes and ghosts with the relatively non-permeant reagent formylmethionylsulphone methyl phosphate. The present study deals with the determination of the asymmetrical distribution of phospholipids in the human erythrocyte membrane, as can be deduced from differences in lytic and non-lytic action of phospholipase A_2 (*N. naja*), phospholipase C (*B. cereus*) and sphingomyelinase (*S. aureus*). Influences of these phospholipases on the morphology of intact red cell membranes and ghosts are visualized by freeze–etch electron microscopy.

MATERIALS AND METHODS

Phospholipases

Phospholipase A_2 from *N. naja* venom was purified as described by Cremona and Kearney[9], with the exception that gel filtration on Sephadex G-75 was replaced by gel filtration on Sephadex G-100. Pure phospholipase C from *B. cereus* was prepared by the method of Zwaal *et al.*[10]. Sphingomyelinase from *S. aureus* was purified as described by Colley *et al.*[2].

Treatment of erythrocytes with phospholipases

Freshly collected human red cells (from acid–citrate–dextrose-treated blood; cells packed for 10 min at $3000 \times g$) were washed 4 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 were mixed with 5 ml of the isotonic saline solution, followed by addition of 0.25 ml of packed cells. The amounts of enzyme added were routinely: 10 I.U. of phospholipase A_2, 10 I.U. of phospholipase C and 5 I.U. of

sphingomyelinase. Incubations with *N. naja* phospholipase A_2 contained 10 mM $CaCl_2$. Unless otherwise stated, the mixtures were incubated for 1 h at 37 °C with gentle stirring, followed by centrifugation in a centrifuge. The supernatants, having a pH of 7.3–7.4, were collected and percentage haemolysis was determined as described previously[3]. Aliquots of the cells were taken for freeze–etch studies and the remaining cells were analysed for phospholipids.

Treatment of ghosts with phospholipases

Human erythrocyte ghosts were isolated according to Dodge *et al.*[11] and finally washed twice with 0.05 M Tris buffer (pH 7.4; 10 mM $CaCl_2$, 0.25 mM $MgCl_2$).

Suspensions of 10 mg ghosts in 5 ml of the same buffer were incubated with stirring for 60 min at 37 °C with 10 I.U. of phospholipase A_2, 10 I.U. of phospholipase C or 5 I.U. of sphingomyelinase. The ghosts were packed at $27000 \times g$ for 20 min and the supernatants, having a pH of 7.3–7.4, were removed by suction. Aliquots of the ghosts were taken for freeze-etch studies and the remaining ghosts were analysed for phospholipids.

Phospholipid analyses

Enzymatic activity was inhibited by addition of a mixture of *o*-phenanthroline and EDTA (final concentration: 4 mM of each) prior to lipid extraction of the cells or ghosts by the method of Reed *et al.*[12]. The extracts were taken to dryness under reduced pressure and the residue was dissolved in 150 μl of chloroform–methanol (1:1, v/v). The phospholipids were separated by two-dimensional thin-layer chromatography using the procedure of Broekhuyse[13], and determined as phosphorus after destruction with 70% $HClO_4$ at 190 °C by a modification[14] of the procedure of Fiske and SubbaRow. Percentage degradation of glycerophospholipids after phospholipase A_2 or C attack was determined using sphingomyelin (which is not degraded) as an internal standard. For determination of sphingomyelin degradation by sphingomyelinase, one of the glycerophospholipids (usually lecithin) was used as an internal standard.

In the case of phospholipid breakdown by the combined action of phospholipase A_2 and sphingomyelinase none of the phospholipids can be used as an internal standard, since in principle each of them can be degraded. Therefore, lipids extracted from incubated cells and corresponding blanks were quantitatively applied to the thin-layer plates, allowing direct comparison of the quantity of each of the individual phospholipid class before and after treatment of the cells. The two-dimensional thin-layer chromatography technique of Broekhuyse[13] easily allows determination of lysoglycerophospholipids. Since the lyso-compounds produced by phospholipase A_2 action towards erythrocytes remain in the membrane[1], percentage conversion of glycerophospholipids by this enzyme can also be determined by directly comparing the remaining phospholipid with the corresponding lyso-derivative formed. Within the limits of accuracy, both methods gave the same results.

Freeze–etching electron microscopy

Samples of intact cells and ghosts were quenched from 37 °C in a mixture of liquid and solid nitrogen and prepared further in a Denton freeze–etch apparatus as described before[15]. Micrographs were made with a Siemens Elmiskop 1.A.

TABLE I

PERCENTAGE HAEMOLYSIS PRODUCED BY COMBINED ACTION OF PHOSPHOLIPASES IN HUMAN ERYTHROCYTES

All incubations were carried out for 3 h. Enzymes were added to the incubation mixtures after time intervals indicated. Values are mean of four experiments. Although other enzyme combinations are possible they are intentionally omitted, since they provide no additional information relevant to this paper.

Addition of enzyme at			*% haemolysis after 180 min*
t = 0 min	*t = 60 min*	*t = 120 min*	
–	–	–	2
Phospholipase A_2 (*N. naja*)	–	–	1
Phospholipase C (*B. cereus*)	–	–	1
Sphingomyelinase (*S. aureus*)	–	–	2
–	Sphingomyelinase (*S. aureus*)	Phospholipase C (*B. cereus*)	100
Phospholipase A_2 (*N. naja*)	Sphingomyelinase (*S. aureus*)	Phospholipase C (*B. cereus*)	5
–	–	Phospholipase C (*B. cereus*) + sphingomyelinase (*S. aureus*)	100
–	Phospholipase A_2 (*N. naja*)	Sphingomyelinase (*S. aureus*)	3
–	Sphingomyelinase (*S. aureus*)	Phospholipase A_2 (*N. naja*)	30–40
–	–	Phospholipase A_2 (*N. naja*) + sphingomyelinase (*S. aureus*)	11
Phospholipase A_2 (*N. naja*)	–	Phospholipase C (*B. cereus*)	1
–	–	Phospholipase A_2 (*N. naja*) + phospholipase C (*B. cereus*)	1

RESULTS

Haemolysis by phospholipases

When human erythrocytes are incubated separately with *N. naja* phospholipase A_2, *B. cereus* phospholipase C or *S. aureus* sphingomyelinase, no haemolysis above blank is observed (Table I). The percentage haemolysis produced by the combined action of the enzymes appears to be strongly dependent upon the sequence of addition of enzymes to the incubation mixtures. Cells treated with sphingomyelinase are completely lysed by addition of phospholipase C, which is consistent with previous observations[2]. However, when the cells are first incubated with phospholipase A_2 and then treated with sphingomyelinase and phospholipase C, no significant haemolysis is produced. Although phospholipase A_2-treated cells are not lysed upon addition of sphingomyelinase, the reverse order of addition of these enzymes produces 30–40% haemolysis. When both these enzymes are added simultaneously some haemolysis is observed. The combined action of phospholipases A_2 and C appears not to be lytic.

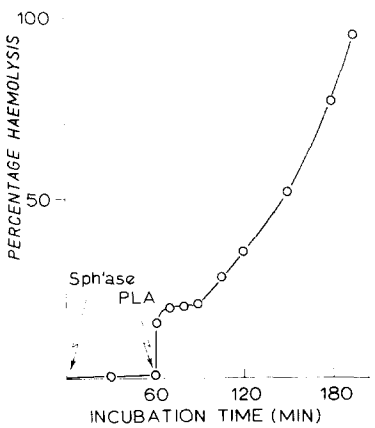


Fig. 1. Percentage haemolysis produced by the successive action of sphingomyelinase (Sph'ase) and phospholipase A_2 (PLA) towards human erythrocytes.

The variation in the degree of haemolysis which is observed when sphingomyelinase-treated cells are incubated with phospholipase A_2 (Table I), required some further investigation. A time-curve of the haemolysis produced by such an incubation system is shown in Fig. 1. As expected[2], sphingomyelinase alone does not produce haemolysis. Addition of phospholipase A_2 after 1 h produces a sharp increase in haemolysis which tends to level off at 20%*. However, after 90 min of total incubation time, haemolysis proceeds again and goes to completion. It should be emphasized that although phospholipase A_2 initially produces 20% lysis, no formation of ghosts can be observed by phase-contrast microscopy and the cells are still in a biconcave shape. On the other hand, ghosts are produced when haemolysis proceeds again after 90 min.

* Similar results are obtained when pure pancreatic phospholipase A_2 is used.

Degradation of phospholipids by phospholipases in intact cells and ghosts

The observation that no lysis is produced by the separate action of the enzymes does not necessarily mean that no lipid degradation has taken place. Although phospholipase C is not able to attack the phospholipids in the intact cell, both phospholipase A_2 and sphingomyelinase accomplish a non-lytic degradation of phospholipids in intact human erythrocytes (Table II). Phospholipase A_2 only attacks lecithin, converting 68% of the original lecithin into lysolecithin. This represents a breakdown of 20% of the total phospholipid fraction. Treatment of intact cells with sphingomyelinase results in the degradation of 85% of the sphingomyelin, representing another 20% of the membrane phospholipid. In contrast to the incubations with intact cells, incubations of ghosts with these phospholipases result in a complete degradation of those phospholipids which are suitable substrates for the enzymes (Table III).

Since phospholipase A_2-treated erythrocytes are not lysed by subsequent incubation with sphingomyelinase (Table I), it was of interest to determine the non-lytic phospholipid degradation by this enzyme combination. While phospholipase

TABLE II

NON-LYTIC DEGRADATION OF PHOSPHOLIPIDS IN INTACT HUMAN ERYTHROCYTES BY PHOSPHOLIPASES

Values are mean of eight experiments.

	% *degradation of phospholipids*				
	Total phospholipid	*Sphingomyelin*	*Phosphatidylcholine*	*Phosphatidylethanolamine*	*Phosphatidylserine*
Phospholipase C (*B. cereus*)	0	0	0	0	0
Phospholipase A_2 (*N. naja*)	20	0	68	0	0
Sphingomyelinase (*S. aureus*)	20	85	0	0	0

TABLE III

DEGRADATION OF PHOSPHOLIPIDS IN HUMAN RED CELL GHOSTS BY PHOSPHOLIPASES

Values are mean of eight experiments.

	% *degradation of phospholipids*				
	Total phospholipid	*Sphingomyelin*	*Phosphatidylcholine*	*Phosphatidylethanolamine*	*Phosphatidylserine*
Phospholipase C (*B. cereus*)	68–74	0	100	100	90–100
Phospholipase A_2 (*N. naja*)	70–74	0	100	100	100
Sphingomyelinase (*S. aureus*)	25	100	0	0	0
Sphingomyelinase + phospholipase A_2	95–100	100	100	100	100

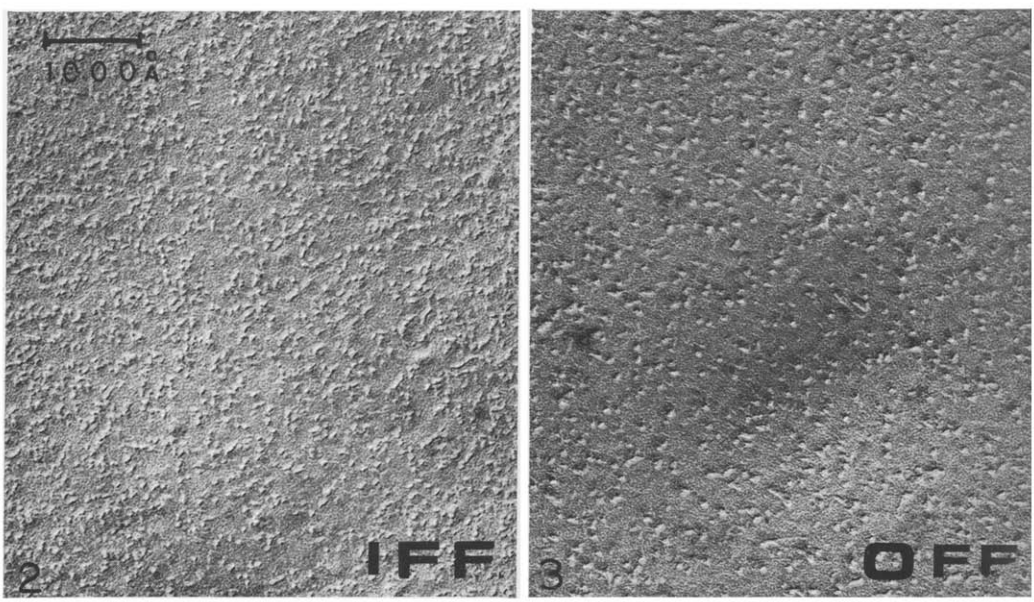
TABLE IV

NON-LYTIC DEGRADATION OF PHOSPHOLIPIDS BY PHOSPHOLIPASES IN HUMAN ERYTHROCYTES

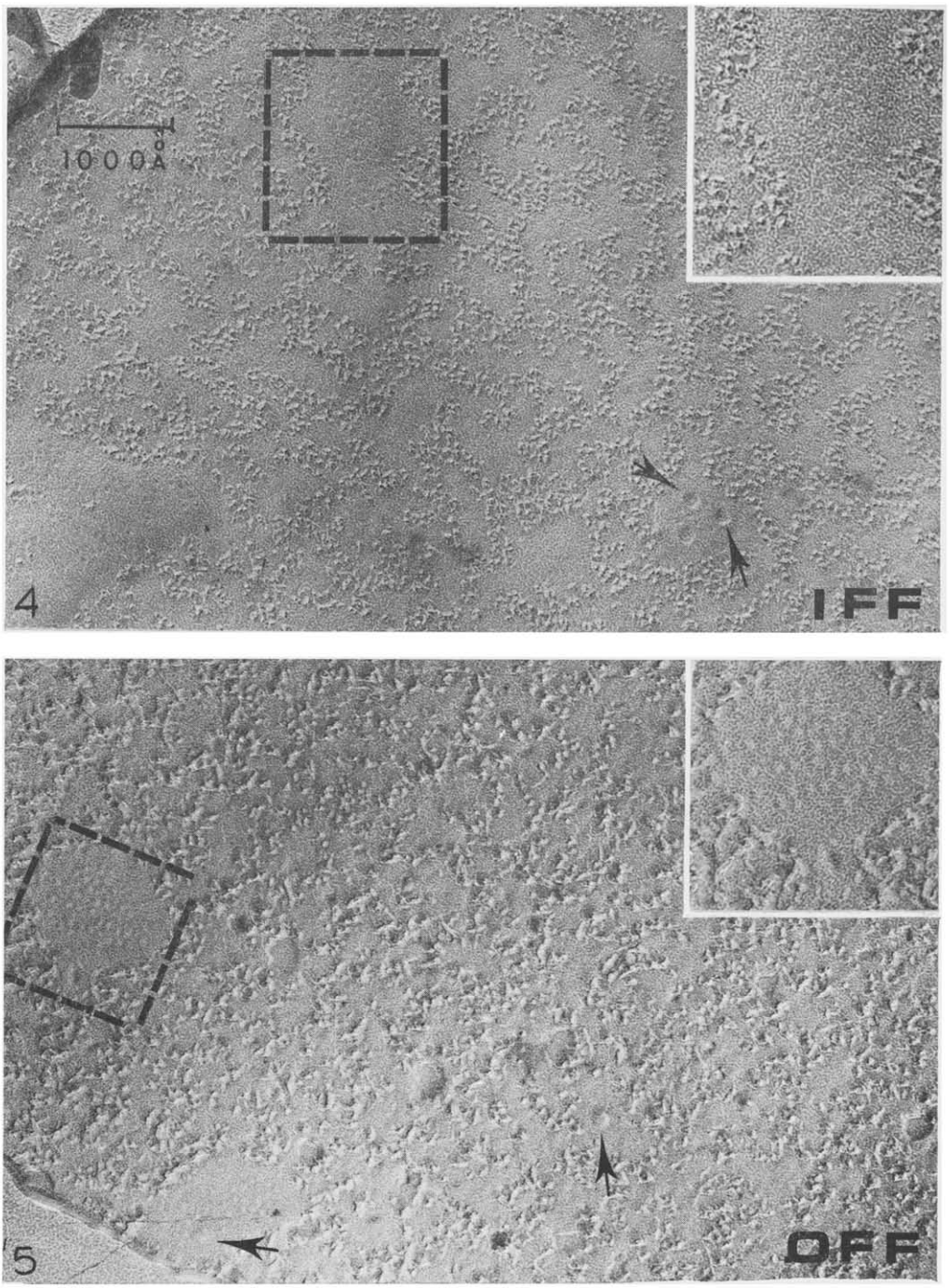
Incubations were started in the presence of phospholipase A_2 (*N. naja*), followed by addition of sphingomyelinase (*S. aureus*) after 1 h. Values are mean of three experiments.

Incubation time (min)	*Enzyme*		*% degradation*				
	Phospholipase A_2 (N. naja)	*Sphingomyelinase (S. aureus)*	*Total phospholipid*	*Sphingomyelin*	*Phosphatidylcholine*	*Phosphatidylethanol amine*	*Phosphatidylserine*
0	↓	↓	0	0	0	0	0
60			20	0	68	0	0
120			48	82	76	20	0

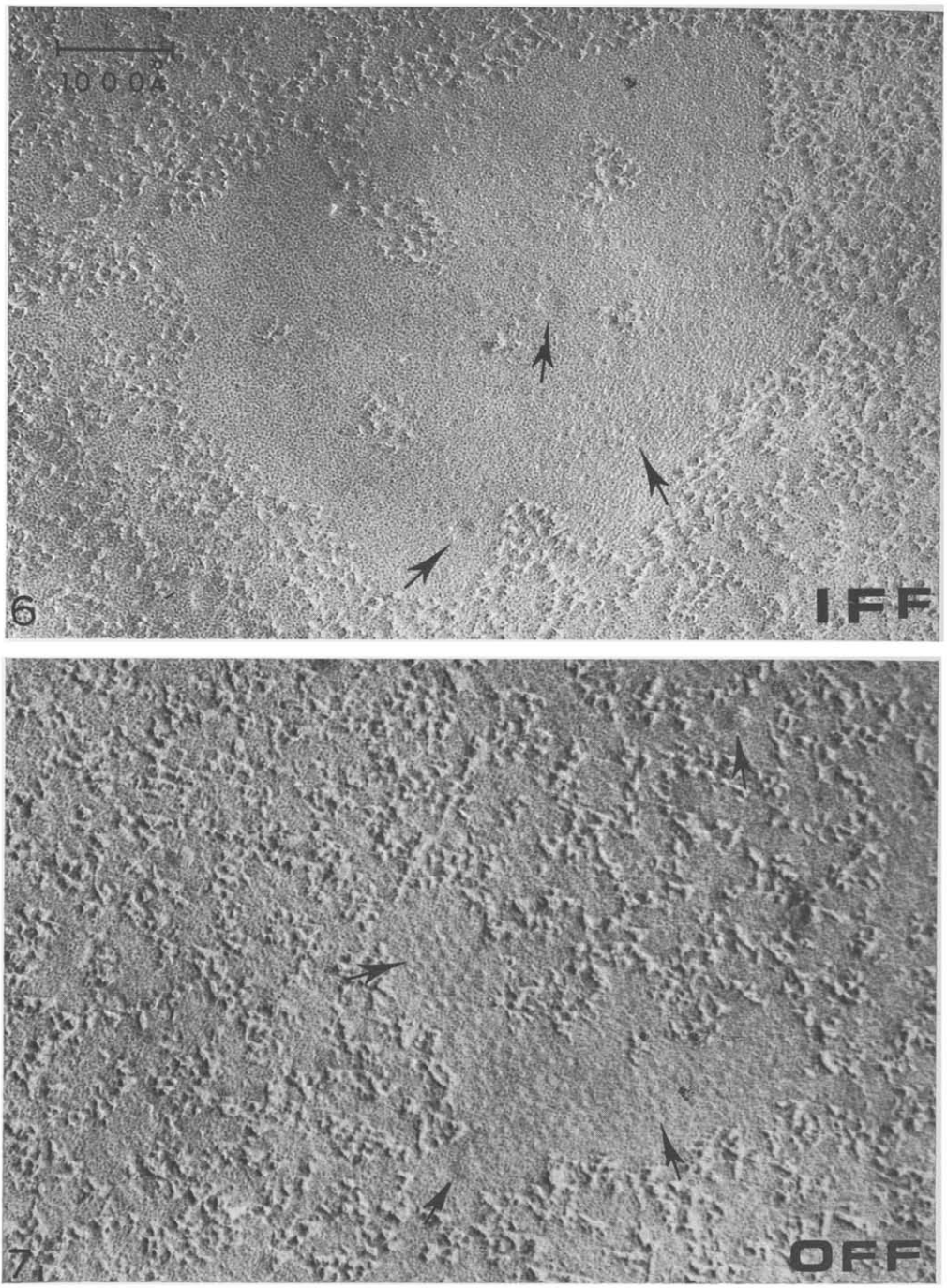
A_2 alone exclusively attacks phosphatidylcholine in intact cells, addition of sphingomyelinase not only results in the hydrolysis of 82% of the sphingomyelin, but also enables phospholipase A_2 to attack 20% of the phosphatidylethanolamine and another 8% of the phosphatidylcholine (Table IV). Prolonged incubation times fail to produce more phospholipid breakdown. This successive action of the two enzymes, though not producing lysis of the cells, degrades up to 48% of the membrane phospholipids. It should be noted that no phosphatidylserine is degraded, although this phospholipid is more rapidly hydrolysed than the other phosphoglycerides when ghosts are subjected to phospholipase A_2 treatment. It is emphasized that further treatment of the cells with phospholipase C does not result in a further degradation of the phospholipids.



Figs 2 and 3. Inner fracture face (IFF) and outer fracture face (OFF) of the intact erythrocyte membrane. ×120000.



Figs 4 and 5. Inner fracture face (IFF) and outer fracture face (OFF) of the intact red cell membrane after treatment with sphingomyelinase. Notice the characteristic areas with pits (75 Å in diameter) on the inner fracture face (inset Fig. 5) and the corresponding spheres on the outer fracture face (inset Fig. 6). Arrows indicate pits and spheres (200 Å in diameter) on inner fracture face and outer fracture face, respectively. ×160000. Insets ×240000.



Figs 6 and 7. Inner fracture face (IFF) and outer fracture face (OFF) of the intact red cell membrane treated successively with phospholipase A_2, sphingomyelinase and phospholipase C. Arrows indicate pits and spheres, both of 75 Å and 200 Å in diameter. × 160000.

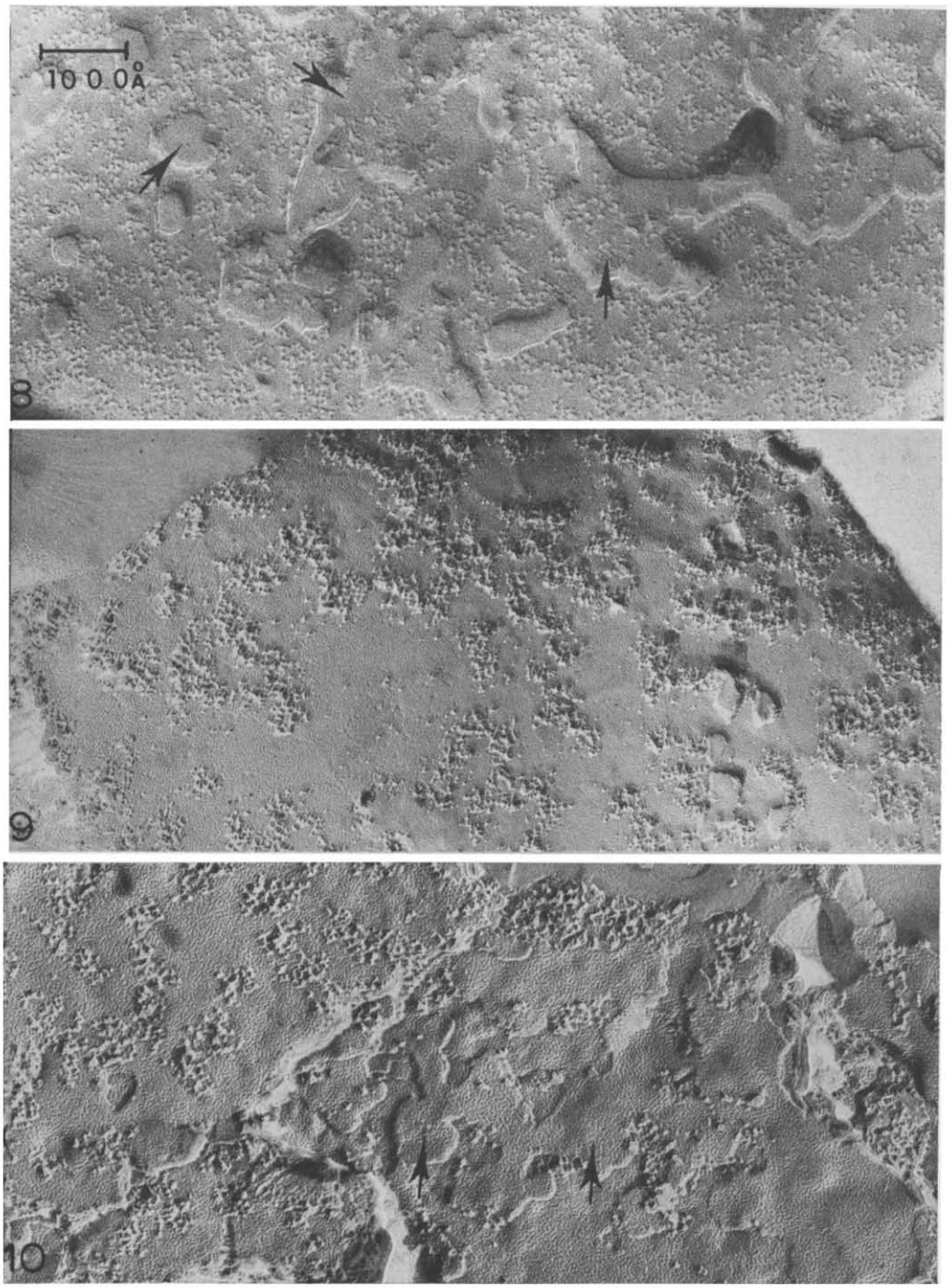


Fig. 8. Control ghosts with typical plaques (see arrows). × 120000.

Fig. 9. Ghosts treated with phospholipase A_2. × 120000.

Fig. 10. Ghosts treated with phospholipase A_2 showing typical plaques (see arrows). × 120000.

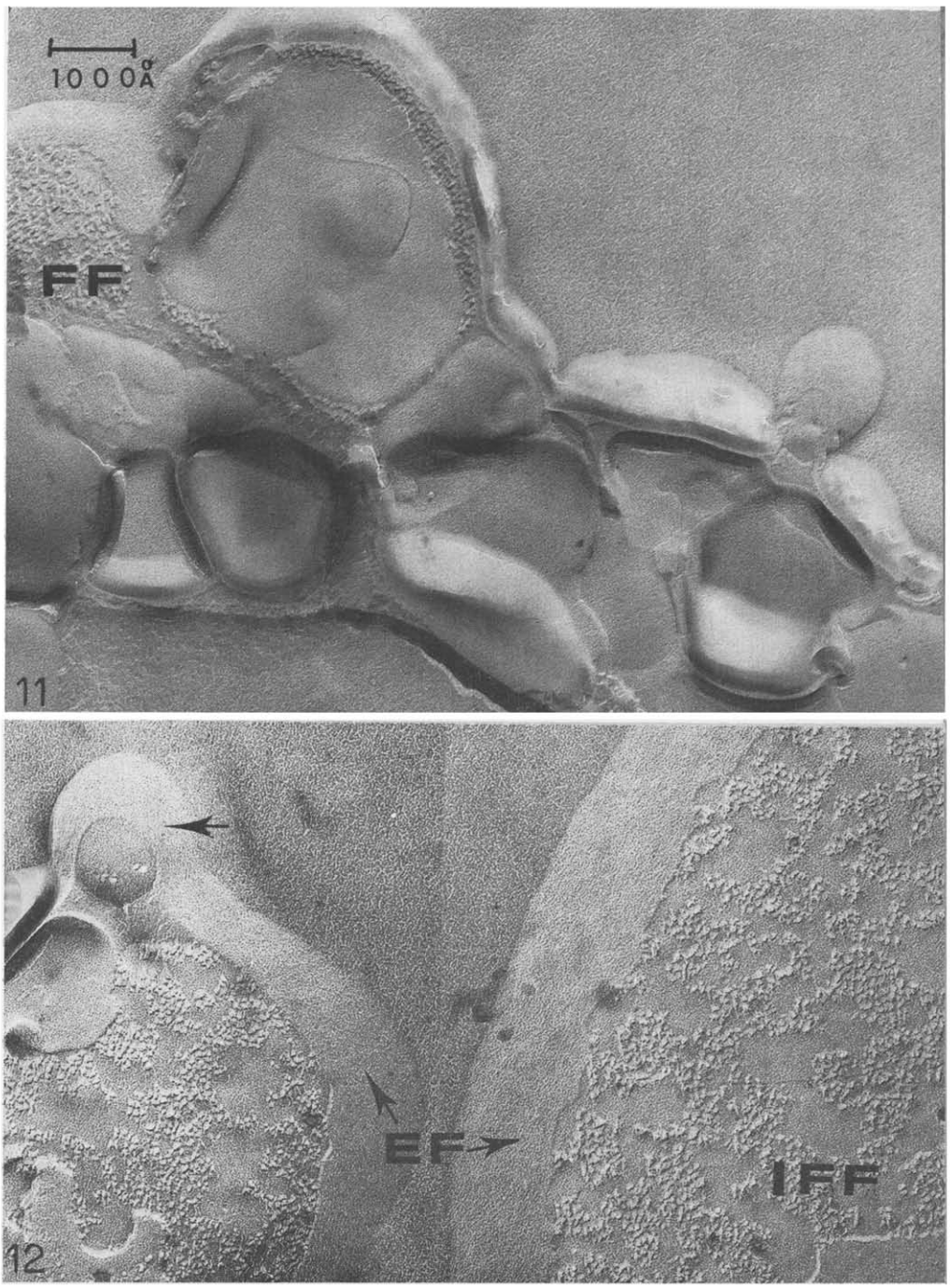


Fig. 11. Ghosts treated with phospholipase C (etched at −100 °C during 1 min). × 120000. FF, fracture face.

Fig. 12. Ghosts treated with sphingomyelinase (etched at −100 °C during 1 min). × 120000. EF, etched face.

Freeze–etch studies of phospholipase-treated human erythrocytes and ghosts

Intact erythrocytes. Figs 2 and 3 exhibit the normal fracture faces of the membrane of the human erythrocyte. A homogeneous distribution of particles on the inner and outer fracture faces is observed. More particles are visible on the inner fracture face than on the outer fracture face. Incubation of intact cells with phospholipase A_2 does not alter the fracture faces although 68% of phosphatidylcholine has been degraded. Phospholipase C is not able to attack phospholipids in intact cells and also no change in freeze-etch morphology is observed. Sphingomyelinase causes 80–85% degradation of sphingomyelin, which alters the fracture faces markedly (Figs 4 and 5). In contrast to control cells the particles of the inner fracture face and outer fracture face are not distributed randomly but are grouped in clusters. In addition to this phenomenon, one observes areas with pits only on the inner fracture face and corresponding spheres only on the outer fracture face. These areas represent 10–20% of the total fracture face. The diameter of these pits and spheres is approx. 75 Å. Also larger pits are observed on the inner fracture face which may correspond to the larger spheres on the outer fracture face. The diameter of both these pits and spheres is approx. 200 Å. Similar fracture faces are observed after incubation of intact red cells with successively phospholipase A_2 and sphingomyelinase, in which case 48% of the membrane phospholipids are degraded. However, it appears that the areas with pits on inner fracture face corresponding with the spheres on outer fracture face are migrated together (Figs 6 and 7). Even if the cells treated with the two enzymes are subsequently incubated with phospholipase C, no further alteration of freeze–etch morphology is observed.

Erythrocyte ghosts. The freeze–fracture faces of the ghosts are similar to those of the intact erythrocytes as has also been observed by others[16–18]. However, one also observes arrays with rather regular plaques, made up of adherent membranes as has been reported earlier[19] (Fig. 8). Phospholipase A_2 produces complete breakdown of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine. Freeze–etching shows irregularly distributed particles clusters and large smooth fracture faces (Figs 9 and 10).

Phospholipase C breaks phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine into diglycerides and their respective polar headgroups. In freeze–etch replicas we could find only a few tangentially fractured membranes (Fig. 11). Small fractured areas with particles (70–120 Å in diameter) are present and no smooth fracture faces are observed within these areas. Typical droplets adherent to these areas are observed. The diameter of these droplets is between 1000 and 10000 Å. Sphingomyelinase is able to degrade all the sphingomyelin of the ghost membrane. In this case freeze-etch morphology is different from that of intact erythrocyte membranes treated with this enzyme (Fig. 12). Instead of the characteristic pits on the inner fracture face and spheres on the outer fracture face one observes small droplets of 2000–3000 Å adherent to the membrane (see arrow). In addition to this phenomenon the particles are strongly clustered.

DISCUSSION

Phospholipase A_2 from *N. naja* and sphingomyelinase from *S. aureus*, unlike phospholipase C from *B. cereus*, are able to produce non-lytic degradation of phos-

pholipids in intact human erythrocytes. In intact cells, up to 68% of the lecithin can be hydrolysed by phospholipase A_2 and 80–85% of the sphingomyelin can be degraded by sphingomyelinase. This is in agreement with previous observations from this laboratory[1,2]. In addition, when intact erythrocytes are treated successively with phospholipase A_2 and sphingomyelinase almost half of the membrane phospholipid can be degraded, comprising mainly the choline-containing phospholipids and some phosphatidylethanolamine. On the other hand, treatment of ghosts with this enzyme combination results in a complete degradation of the major phospholipid classes.

It has been argued[1] that enzymatic degradation of phospholipids in intact cells provides direct information about the lipids on the outer surface of the cell membrane. Enzymatic degradation in non-sealed ghosts will in addition provide information about the lipids on the interior of the membrane, since the enzymes can then act towards both sides of the membrane. This supposition becomes even more likely when half of the phospholipids were found to be attacked in intact cells whereas essentially all the phospholipids appeared to be hydrolysed in ghosts. On this basis, the phospholipid distribution between inner and outer layer of the human erythrocyte membrane can be deduced. The outer layer consists mainly of the two choline-containing phospholipids, *e.g.* lecithin and sphingomyelin, while in addition one-fifth of the phosphatidylethanolamine is present (Fig. 13). The inner layer is mainly composed of phosphatidylethanolamine and phosphatidylserine, while a minor fraction of the choline-containing phospholipids may also be present. Recently, Bretscher[7,8] and also Gordeski and Marinetti[20] proposed a similar arrangement of the phospholipids in the erythrocyte membrane. In these studies the supposition was mainly based on indirect evidence obtained by labelling the cell with relatively non-permeant reagents.

Phospholipase C can only produce lysis (and phospholipid breakdown) when the cells are treated first or simultaneously with sphingomyelinase. It has been sug-

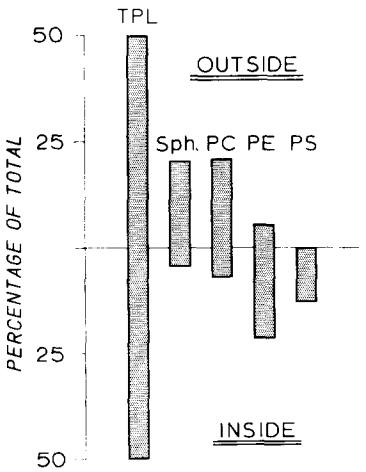


Fig. 13. Proposed distribution of phospholipids between inner and outer layer of the human erythrocyte membrane. Abbreviations: TLP, total phospholipid; Sph, sphingomyelin; PC, lecithin; PE, phosphatidylethanolamine; PS, phosphatidylserine.

gested[2] that degradation of sphingomyelin by sphingomyelinase exposes the glycerophospholipids to the action of phospholipase C leading to lysis of the cells. This is probably the result of the formation of diglycerides, which migrate into discrete pools (*e.g.* black dots)[2,6]. However, the combination of sphingomyelinase and phospholipase C does not produce haemolysis if the cells are pretreated with phospholipase A_2. In this case, the glycerophospholipids in the outer layer of the membrane are first converted into their lyso-derivatives. Although subsequent addition of sphingomyelinase hydrolyses sphingomyelin, the following treatment with phospholipase C does not produce lysis since the formed lyso-compounds in the outer layer of the membrane hardly serve as substrates for this enzyme. (The activity of phospholipase C towards lysolecithin is in the order of 10^4 times slower than the activity towards lecithin; B. Roelofsen and R. F. A. Zwaal, unpublished).

As has been suggested before, treatment of the cells with successively phospholipase A_2 and sphingomyelinase produces a non-lytic degradation of the phospholipids present on the exterior of the cell membrane. However, when these two enzymes are added in a reverse order, the cells start to lyse in a non-linear way. It is suggested that immediately after addition of phospholipase A_2 the cells become temporarily leaky for haemoglobin and seal again after a few minutes, since no ghosts are formed during this state. At present, it is unclear what causes this leaking and sealing phenomenon. During the initial leakage of haemoglobin the phospholipases can penetrate towards the cell interior from where they can degrade the inner layer of the membrane. This may explain why the sealed cells start to lyse again, accompanied with the production of ghosts.

Differences in action of phospholipase A_2, phospholipase C and sphingomyelinase towards intact cells and ghosts can be correlated with differences in freeze–etch morphology. Phospholipase A_2 does not change the fracture faces of the intact cell membrane although two-thirds of the lecithin has been degraded. Similar results are obtained with the protoplast of *B. cereus* after treatment with pancreatic phospholipase A_2, which results in an almost complete breakdown of the phospholipids[21]. Apparently the formation of lysolecithin in the intact red cell membrane has no implications for the freeze-fracture faces because the degradation products remain in the membrane and do not migrate into discrete pools[1]. Incubation of ghosts with phospholipase A_2, which results in a complete degradation of the glycerophospholipids, produces a complete reorganization of the membranes. Large smooth fracture faces and irregularly distributed particles-clusters are present on both fracture faces. Speth *et al*[19] showed that phospholipase A_2 (*N. naja*) treatment of ghosts results in a marked increase in the number of plaques made up of adherent membrane material, and no aggregation of particles could be observed. Our observations are at variance with these results in that the most striking effect of phospholipase A_2 treatment is the aggregation of particles accompanied with the formation of large smooth areas. Is should be noted that this is not due to pH alterations, which have been shown to induce similar particle aggregation[16].

Phospholipase C only acts on ghosts, which results in a marked change in morphology. With the exception of sphingomyelin, all the phospholipids are degraded in diglycerides which migrate into discrete pools[3]. This could explain the lower number of tangentially fractured membranes. The few fracture faces which are still present, are fully occupied with particles and no smooth areas are visible.

Two striking effects of sphingomyelinase on intact cells are observed: (i) the particles are aggregated on both fracture faces and (ii) separate areas with pits only on the inner fracture face and spheres only on the outer fracture face are present. We suggest that the spheres on the outer fracture face represent small droplets of ceramides, produced by the breakdown of sphingomyelin in the outer monolayer of the membrane. Sphingomyelinase does not produce spheres and pits on the fracture faces of ghosts, although in this case all the sphingomyelin has been degraded. However, droplets of 1000–3000 Å, which are not visible by phase-contrast microscopy, are adherent to the membrane. We suggest that these droplets contain the ceramides, produced by sphingomyelinase action towards ghosts.

Treatment of intact erythrocytes with successively phospholipase A_2 and sphingomyelinase shows similar fracture faces as with sphingomyelinase treatment alone. Apparently freeze–etching fails to demonstrate that the lysophospholipids and fatty acids produced by phospholipase A_2 action on intact cells are formed at the outer fracture face. It is likely that these reaction products do not cause a gross disorganization of the outer monolayer of the membrane, irrespective of whether ceramides or sphingomyelins are present.

It has been shown[1,2], however, that cells treated with either or both enzymes show increased osmotic fragility relative to control cells. Although phospholipase A_2 does not significantly alter the fracture faces of the intact cell membrane, the formation of lyso-compounds and fatty acids in the outer monolayer might very well alter its integrity and therefore the permeability properties of the membrane. In the case of sphingomyelinase, the increase in osmotic fragility might be explained by the formation of small ceramide droplets which will no longer contribute to the organization of the outer monolayer.

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