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INFLUENCE OF ENZYMATIC PHOSPHOLIPID CLEAVAGE ON THE PERMEABILITY OF THE ERYTHROCYTE MEMBRANE

I. TRANSPORT OF NON-ELECTROLYTES VIA THE LIPID DOMAIN

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Summary

In order to further elucidate the influence of membrane lipids on transport via the lipid domain of the erythrocyte membrane, simple non-electrolyte diffusion was investigated by tracer flux measurements in whole cells after cleavage of up to 65% of phosphatidylcholine or sphingomyelin by phospholipase A_2 from *Naja naja*, or by sphingomyelinase.

A new type of labelled model non-electrolyte was used in this study, readily available by reacting a non-labelled thiol with a labelled alkylating SH-reagent.

In spite of the marked enzymatic alterations of the membrane, which lead to the occurrence of large quantities of lysophosphatidylcholine and long chain fatty acids, or of ceramide, the permeability of the lipid domain remained unaffected.

This finding is very surprising, since the physical properties of the lipid phase (microviscosity, structure of the membrane interface) are likely to be perturbed in the enzyme-treated membranes.

Sphingomyelinase-treated cells undergo stomatocytic shape changes followed by deep invaginations of the membrane and finally endocytosis, while phospholipase A_2-treated cells essentially maintain their normal shape.

Introduction

Solutes can pass cell membranes via the lipid bilayer phase or via intrinsic proteins. Transport of non-electrolytes through lipid bilayers has been shown,

Abbreviations: TG-MalNEt, 2-(3′-thioglyceryl)-*N*-ethylmaleimide; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

in liposomes and biological membranes, to be strongly dependent on the degree of unsaturation and the chain length of the fatty acid constituents [1,2]. Moreover, the presence of cholesterol in artificial and natural bilayers retards diffusion [3,4]. On the basis of spectroscopic evidence, these effects have been related to changes of the microviscosity of the apolar membrane interior [5] and consecutive changes of the intramembrane diffusion coefficients [1,5]. Alternatively, alterations of the interfacial region between the hydrophobic paraffin chains and the zwitterionic head groups might be responsible for the permeability effects of changes in lipid composition, although only little direct information is available on this subject [6–9].

A possible way to elucidate the relevance of the phospholipids for 'translipid' transport rates is to cleave them in situ by phospholipases attacking the interfacial region. Two such enzymes, phospholipase A_2 (from *Naja naja* and from bee venom) and sphingomyelinase (from *Staphylococcus aureus*) have been shown to hydrolyse substantial amounts of phosphatidylcholine or sphingomyelin in intact erythrocytes [10], without causing hemolysis. The present study deals with the effect of such cleavages on the transfer of 2-(3'-thioglyceryl)-*N*-ethylmaleimide (TG-MalNEt), a newly synthesized model nonelectrolyte, and of glycerol, through the lipid domain of the erythrocyte membrane.

Materials and Methods

Materials. Phospholipase A_2 from *Naja naja* venom (Sigma) and sphingomyelinase from *S. aureus* were purified as described in a previous paper [11]. [1-^{14}C]Glycerol was obtained from Amersham Buchler, Braunschweig. 2-(3'-Thioglyceryl)-*N*-ethyl[2,3-^{14}C]maleimide was freshly prepared every day from equimolar amounts of *N*-ethyl[2,3-^{14}C]maleimide (Amersham, 4.6 Ci/mol) and 3-thioglycerol (Fluka) as follows: 0.6 μmol of *N*-ethyl[2,3-^{14}C]maleimide were dissolved in 0.3 ml of a 5 mM solution of thioglycerol (1.5 μmol) in medium A (see Methods) and incubated for 15 min at 25°C and pH 7.4. Subsequently 0.2 ml of a 4.5 mM solution of non-radioactive *N*-ethylmaleimide (0.9 μmol) in medium A were added and the solution incubated for another 15 min. After this procedure, unreacted *N*-ethylmaleimide could not be detected in the solution by titration with soluble thiols and no radioactivity was retained by the cells during the efflux of the derivatized *N*-ethyl[2,3-^{14}C]maleimide, precluding the binding of unreacted reagent to the membrane or to the content of the cells. Human blood was obtained from the local blood bank, bovine blood from the local slaughter house. Standard chemicals used were of the highest purity available. Non-radioactive TG-MalNEt was prepared freshly every day by mixing 1 vol. of a 100 mM solution of thioglycerol in medium A (see Methods) with an equimolar amount of solid *N*-ethylmaleimide and stirring the solution for 15 min at 25°C (pH 7.4). This stock solution was diluted with 50 vols. of medium A prior to further use.

Methods. Erythrocytes were washed three times in 154 mM NaCl. 1 vol. of cells was suspended in 10 vols. of a medium containing, besides TG-MalNEt or glycerol at concentrations given in the legends, the following constituents (mM): KCl (100), NaCl (50), sucrose (44), $MgCl_2$ (0.25), Hepes (10) (medi-

um A). $CaCl_2$ was added at 0.25 or 10 mM depending on the type of phospholipase to be used.

Cells were incubated in these solutions for equilibration with non-radioactive test substance for at least 60 min (37°C, pH 7.4). Cleavage of phospholipids was achieved during this period by addition of either phospholipase A_2 from *Naja naja* (requiring 10 mM Ca^{2+}) or of sphingomyelinase (0.25 mM Ca^{2+}). The extent of phospholipid hydrolysis was varied via the concentration of the enzymes (1—10 I.U./ml of erythrocytes) and via the duration of exposure (0—120 min). Cells incubated in the absence of enzymes served as controls.

After the incubations the cells were washed three times with medium A (pH 7.4) and the extent of phospholipid cleavage determined as described previously [11]. An aliquot of the cells was resuspended in medium A at a hematocrit of 50% and equilibrated with labelled TG-MalNEt or glycerol (0.1 μCi/ml suspension) at 25°C. Tracer efflux into tracer-free but otherwise identical media (hematocrit 5%) was measured as described elsewhere [12,13].

The extent of hemolysis during the efflux period was below 5% in all cases, thus not affecting the validity of the rate coefficient of tracer appearance in the medium as a measure of transmembrane flux under the standardized conditions used in our experiments. The water content of control and phospholipase-treated cells was determined by measurements of dry weight, changes of the cell surface/volume ratios by measurements of osmotic fragility according to standard procedures.

Results

Transfer characteristics of thioglycerylmaleimide

The assessment of the permeability of the lipid phase of the erythrocyte membrane requires a permeant which does not penetreate by other pathways, such as hydrophilic pores or carriers. A number of non-electrolytes, in particular certain amides, probably fulfill this prerequisite [14,15]. Their penetration rates, however, are too high to be resolved by conventional tracer flux measurements. In bovine erythrocytes, the problem can be solved by using glycerol, which in this species passes the membrane only via the lipid phase according to a number of criteria [16]. In human erythrocytes, however, glycerol penetrates

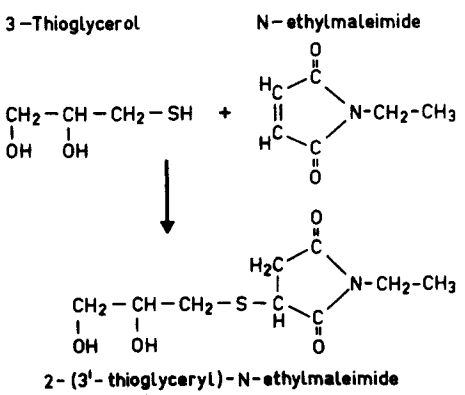

Fig. 1. Scheme of the synthesis of 2-(3'-thioglyceryl)-*N*-ethylmaleimide.

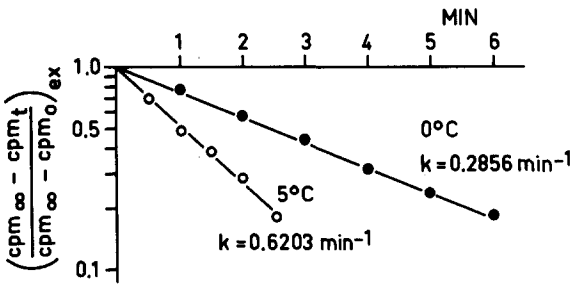

Fig. 2. Kinetics of the efflux of 2-(3′-thioglyceryl)-*N*-[2,3-^{14}C]ethylmaleimide from preloaded human erythrocytes. Measurements as described in the text. Rate coefficients are obtained from the slope of linear regressions calculated for the data points. pH 7.35, hematocrit 5%. cpm 0, cpm *t* and cpm ∞ refer to extracellular radioactivities at time zero, after various time intervals and after attainment of tracer equilibrium. Data from one representative experiment.

predominantly by mediated transfer [16]. Tracer measurements of the permeability of the lipid phase have been attempted in these cells by studying glycerol transfer in the presence of inhibitors of the mediated transfer system [16]. In addition, non-ionic diffusion of acetate was used for this purpose [16]. In order to circumvent the difficulties presented by these approaches, we prepared a model non-electrolyte, 2-(3′-thioglyceryl)-*N*-ethylmaleimide, by

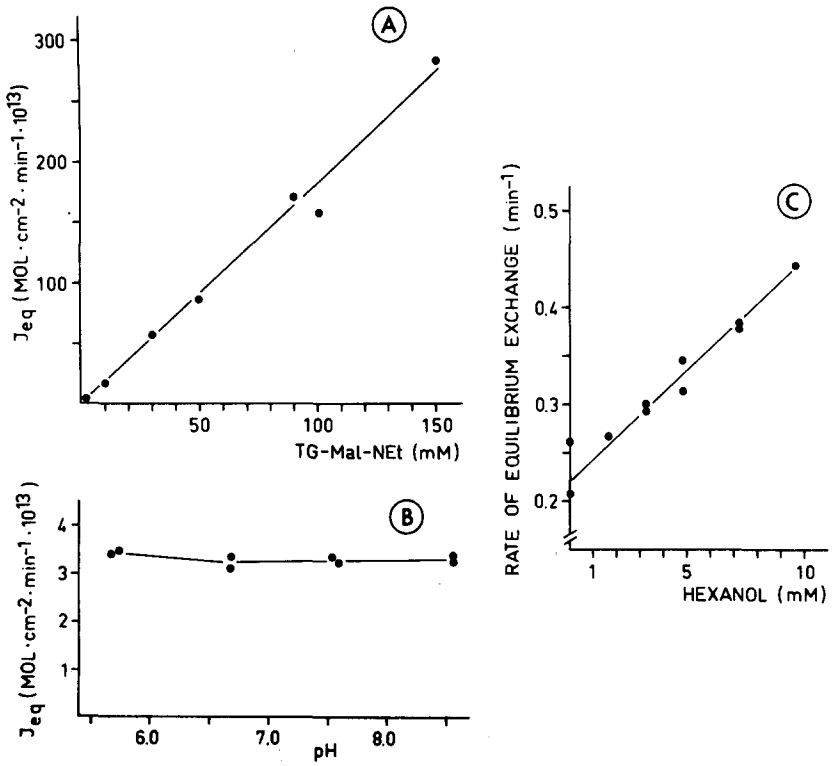

Fig. 3. Characteristics of the transfer of TG-MalNEt across the human erythrocyte membrane. (a) Concentration dependence of equilibrium fluxes, calculated according to Ref. 12, pH 7.35, 0°C. (b) pH dependence of equilibrium fluxes, 0°C, 2 mM TG-MalNEt. (c) Influence of hexanol, present during the efflux measurement, 0°C, 2 mM TG-MalNEt, pH 7.35, hematocrit 5%.

reacting a thiol, 3-thioglycerol, with *N*-ethylmaleimide, an alkylating SH-reagent (Fig. 1). The products of such alkylations are known to be stable. Back-exchange of labelled TG-MalNEt from preloaded cells into media containing only non-labelled TG-MalNEt could be measured at 0 and 5°C by conventional techniques (Fig. 2). The mean rate coefficient k, at 0°C, 0.2585 ± 0.0250 min^{-1} corresponds to a half-time of 2.7 min. Elevation of the temperature to 5°C increases k to 0.6387 ± 0.0184, which is equivalent to a Q_{10} value of about 6.

The efflux follows first-order kinetics and leads to complete equilibration of the tracer between the cellular and extracellular compartment. The flux increases linearly with concentration up to 150 mM (Fig. 3a), indicative of a simple diffusion process. It is independent of pH (Fig. 3b) and becomes enhanced in the presence of increasing concentrations of hexanol (Fig. 3c). The same effect occurs in the presence of phloretin (0.25 mM), which increases the rate coefficient (at 0°C) from 0.2425 min^{-1} to 0.4096 min^{-1}. These enhancements indicate transfer via the lipid phase of the membrane (see Discussion).

Influence of phospholipid cleavage on non-electrolyte transfer via the lipid phase of the erythrocyte membrane

Treatment of human erythrocytes with phospholipase A_2 or sphingomyelinase leads to a cleavage of up to 65% of the phosphatidylcholine or the sphingomyelin, without affecting the aminophospholipids. Cleavage of phosphatidylcholine produces a minor and variable decrease of the rates of efflux of TG-MalNEt. As a maximum, a 20% reduction was observed in some experiments at 60% cleavage (Fig. 4a). Treatment with sphingomyelinase has no effect on the exchange of TG-MalNEt, measured at 0°C (Fig. 4b). The same is true for glycerol self-exchange in bovine erythrocytes, measured at 20°C (Fig. 4c),

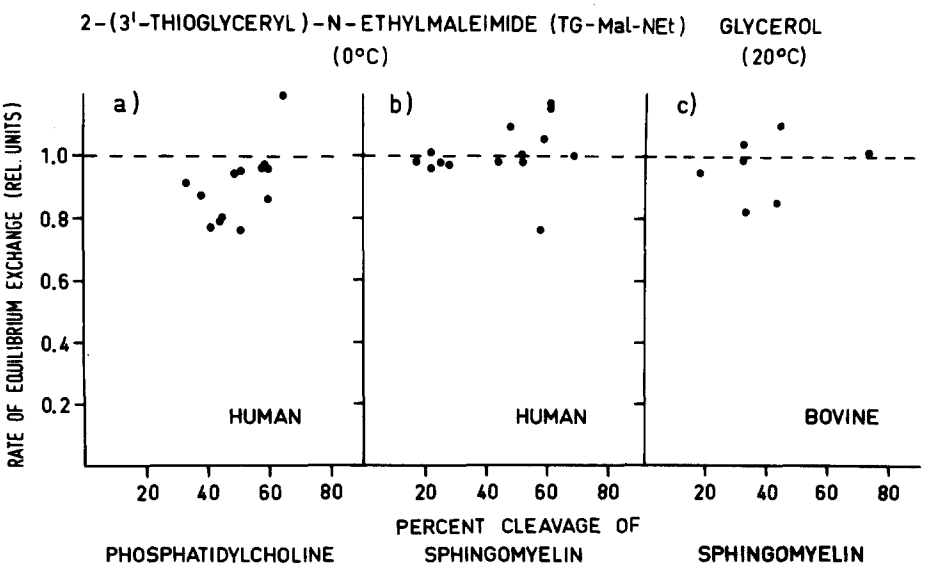

Fig. 4. Rates of equilibrium exchange of TG-MalNEt and glycerol in human and bovine erythrocytes treated with phospholipase A_2 (*Naja naja*) and sphingomyelinase. Treatment with phospholipases and efflux measurements (pH 7.35, hematocrit 5%) as described under Materials and Methods. Values in arbitrary units, relative to the controls.

indicating that the lack of effects has nothing to do with the nature of the non-electrolytes, with the animal species, or with temperature.

Influence of phospholipid cleavage on osmotic fragility and cell shape

The surface area of a lipid layer depends on the nature of its constituents. Modification of membrane phospholipids by hydrolytic cleavage might therefore be expected to alter the surface area of the erythrocyte. This question was investigated by measuring the osmotic fragility of the cells, which depends on the membrane surface area for a given cell volume. The studies were supplemented by measurements of the cellular water content and by morphological studies. Cleavage of phosphatidylcholine by phospholipase A_2 from *Naja naja* slightly enhances osmotic fragility (Fig. 5a) which can probably be accounted for by a swelling of the cells, as indicated by a small increase of the water content from 64.3 to 66.0%. Surface area thus seems to remain constant.

Cell morphology is not altered by treatment with phospholipase A_2 from *Naja naja*, except for the occurrence of a small number of echinocytes. These shape changes, however, are most likely due to the additional presence of 10 mM Ca^{2+}, since treatment with phospholipase A_2 from bee venom (cf. subsequent paper), which only requires 0.25 mM Ca^{2+}, has no effect on cell shape.

Treatment with sphingomyelinase also produces minor increases of osmotic fragility as long as the cleavage is low. More extensive cleavage, however, enhances fragility markedly (Fig. 5b). This effect is due to a pronounced change of the cell shape: cells are converted via stomatocytic [17] forms into invaginated spheres (Fig. 6). These invaginations are finally lost to some extent into the cell interior by endocytotic vesiculation (Fig. 7) as was already

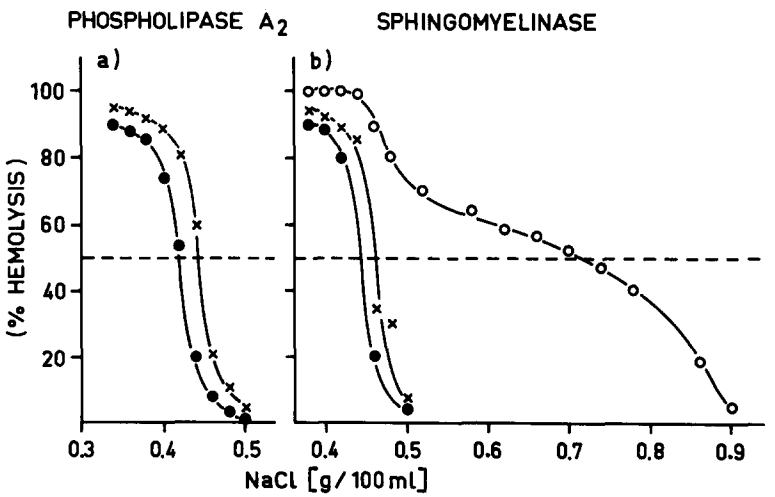

Fig. 5. Influence of phospholipid cleavage on the osmotic fragility of human erythrocytes. (a) Phospholipase A_2: cells, pretreated with enzyme (*Naja naja*, 5 I.U./ml cells) in 140 mM NaCl, 10 mM $CaCl_2$, 10 mM glycylglycine, 0.25 mM $MgCl_2$, were washed three times in 150 mM NaCl, 0.25 mM $CaCl_2$ and suspended in 1.5 vols. of that medium. 0.05 ml of this suspension were mixed with 5 ml NaCl solution of decreasing osmolarity. Swelling and lysis at room temperature were stopped after 10 min exposure by addition of 0.3 ml 10% NaCl. The suspension was centrifuged and hemoglobin determined in the supernatant by photometry. •, control; X, 64% cleavage of phosphatidylcholine. (b) Sphingomyelinase: same procedure, except that enzyme treatment was carried out in 150 mM NaCl, 0.25 mM $CaCl_2$. •, control; X, 18%, o, 41% cleavage of sphingomyelin.

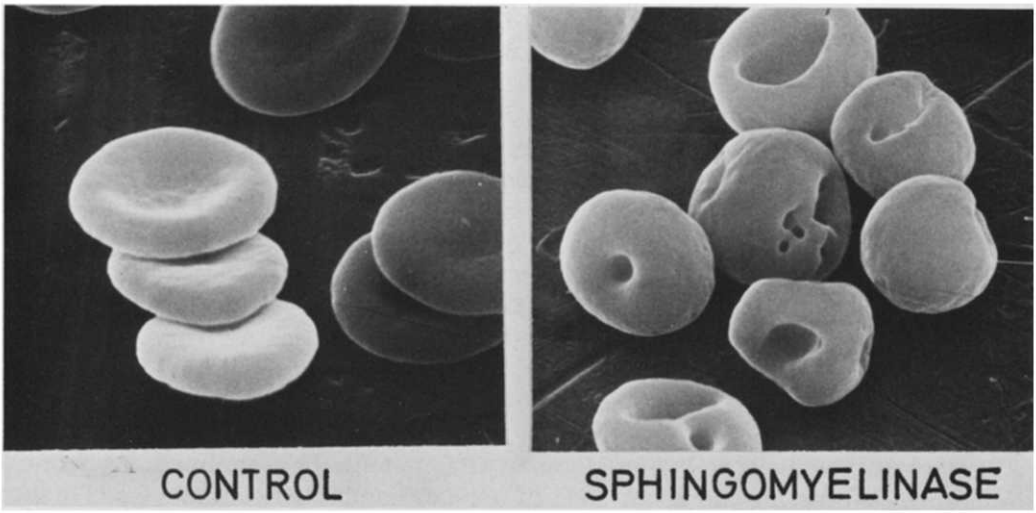

Fig. 6. Scanning electron microscopy of sphingomyelinase-treated erythrocytes. Sphingomyelin cleavage 45%. Control and enzyme-treated cells were washed three times in medium A and prepared for microscopy according to Ref. 42. Electron microscopy: Leitz-AMR 1000 A. Magnification X10 000.

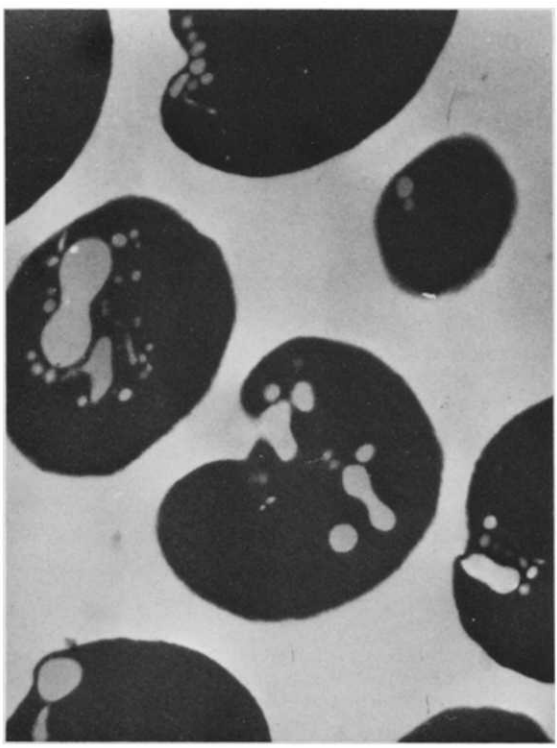

Fig. 7. Transmission electron microscopy of human erythrocytes after cleavage of 45% of the sphingomyelin. Enzyme-treated cells were washed three times in enzyme-free suspension medium, prepared for electron microscopy according to Ref. 43 and embedded in araldite. Electron microscopy: Philips 300. Final magnification X6450.

suspected by earlier investigators [19,20]. Similar shape changes have previously been demonstrated with cationic drugs, e.g. chlorpromazine and tetracaine [18]. A common mechanism may underlie endocytosis provoked by these two types of membrane perturbation. The shape effects of sphingomyelinase treatment could result from the removal of phosphorylcholine head groups from the outer lipid layer, if one assumes that this modification reduces the area of the outer layer relative to that of the inner layer. According to the 'bilayer couple hypothesis' of erythrocyte shape changes [21] this should induce stomatocytosis *. The final endocytotic vesiculation, which requires membrane fusion, is probably facilitated by aggregation of the intercalated protein particles [10], which produces regions of pure lipids, assumed to enhance fusion [22]. An increase of membrane fluidity, likely to result from sphingomyelinase treatment (cf. Refs. 23 and 24) may constitute an additional stimulus for fusion.

Discussion

The enzymatic cleavage, in situ, of phospholipids in the erythrocyte membrane, without concomitant lysis of the cell, has proven a valuable tool for the elucidation of its membrane structure [10]. Present concepts of an asymmetric phospholipid distribution have largely been derived from studies with sphingomyelinase and phospholipase A_2. The results of these studies [10] indicate that the choline phospholipids, phosphatidylcholine and sphingomyelin, are predominantly located in the outer membrane layer, while the aminophospholipids reside in the inner layer. Enzymatic degradation of the two exofacial phospholipids leads to the formation of equivalent quantities of split products. Hydrolysis of phosphatidylcholine produces stoichiometric amounts of lysophosphatidylcholine and long chain fatty acids (Fig. 8), predominantly of the unsaturated type [25]. Both degradation products remain bound to the membrane under the conditions of our experiments [11,26]. Cleavage of sphingomyelin releases phosphorylcholine into the aqueous phase, while ceramide stays in the membrane.

The chemical alterations produced by both types of phospholipid cleavage would be expected to induce functional perturbations. Surprisingly, the barrier properties of the lipid domain of the erythrocyte membrane are not affected (Fig. 4). This conclusion rests of course on the validity of the evidence that the non-electrolytes tested in fact penetrate via the lipid phase. Besides the simple diffusion kinetics (Fig. 3 and Ref. 16), which exclude carrier-mediated transport, striking similarities between the transfer properties of artificial lipid membranes and the pathway for TG-MalNEt in the human, and glycerol in the bovine erythrocyte membrane support this assumption: In both systems the transfer exhibits a high activation energy (Fig. 2 and Ref. 16), is enhanced by

* While this paper was under review, Allen et al. [44] demonstrated a transmembrane reorientation, from the outer to the inner membrane layer, of diglycerides formed in erythrocytes treated with phospholipase C. They proposed a causal role of this 'flip' for the stomatocytogenic effect [19,20] of phospholipase C treatment, based on the bilayer coupled hypothesis. In view of the structural similarities between ceramide and diglycerides such a reorientation might also account for the stomatocytogenic action of sphingomyelinase treatment.

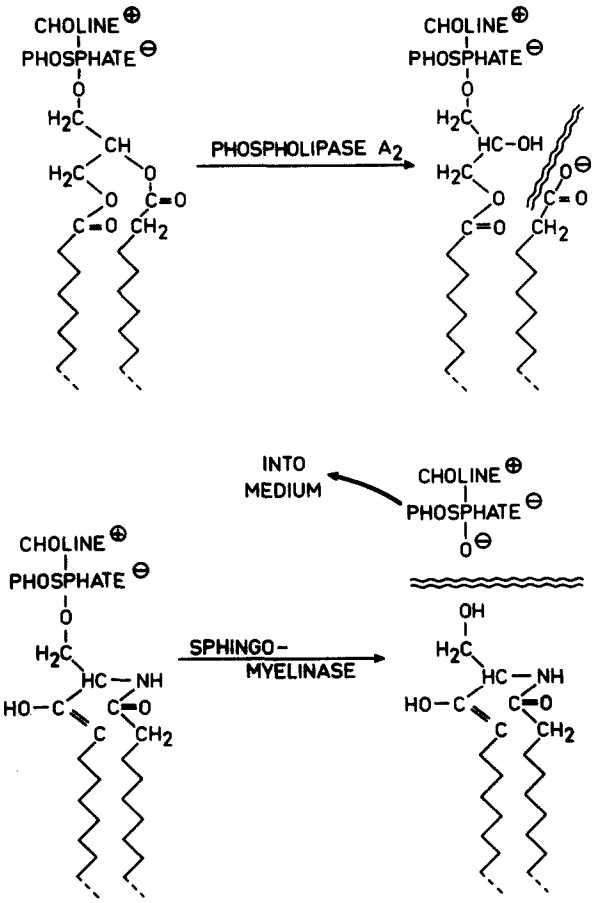

Fig. 8. Scheme of phospholipid cleavage.

cholesterol depletion [4,16], and increased by small aliphatic alcohols and phloretin (Fig. 3 and Ref. 16). In particular, the latter effect provides an important criterion that both test compounds penetrate via the lipid domain [16].

The principle of synthesizing labelled model non-electrolytes from a thiol and a labelled alkylating SH-reagent may open new possibilities for tracer studies on simple non-electrolyte transport, which have been hampered considerably in the past by the lack of suitable labelled test substances. Even families of homologues can be obtained by this simple technique, which makes available the vast amount of commercially available non-radioactive thiols for tracer transport studies. The permeability of TG-MalNEt at 0°C, $1.68 \cdot 10^{-7}$ cm/s, is about 20 times that calculated for the non-mediated transfer of glycerol and 1/4 of that obtained for ethylene glycol (Ref. 16, p. 42).

The lack of effect of phospholipid cleavage on the transfer of the two non-electrolytes is particularly unexpected in view of the pronounced effects of other modifications of the lipid phase of the erythrocyte, such as cholesterol depletion [4,16] or alcohol treatment [16]. The following considerations may resolve this apparent discrepancy for the case of phosphatidylcholine.

(1) Cholesterol depletion and alcohol treatment produce an increase of membrane fluidity [27,28], which can in principle account for the increases of non-electrolyte permeability observed under these conditions. Cleavage of phosphatidylcholine, on the other hand, seems to rigidify the lipid domain of the erythrocyte membrane [29]. Membrane rigidification, however, has been shown to have lesser effects on non-electrolyte permeability than membrane fluidization [30].

(2) Phospholipase A_2-treated erythrocytes can be compared with artificial lipid membranes containing lysophosphatidylcholine and phosphatidylcholine. In such systems, only fractional lysophosphatidylcholine concentrations exceeding 20 mol% markedly enhance non-electrolyte permeability [31]. After cleavage of 60% of its phosphatidylcholine, the erythrocyte membrane contains only about 10 mol% of lysophosphatidylcholine.

(3) Generation of lysophosphatidylcholine in situ leads to a concomitant formation of fatty acids in the erythrocyte membrane, which also contains about 45 mol% cholesterol. Artificial membrane systems containing one or both of these components in addition to lysophosphatidylcholine have been reported to be as stable as systems containing the respective diacyl phospholipid [32] and to retain their barrier properties for non-electrolytes even at high lysophosphatidylcholine concentrations [33].

The lack of effect of phosphatidylcholine cleavage is also of particular interest under the aspect of the 'hydrogen belt' concept [6] of non-electrolyte permeability: hydrogen bonds between ester carbonyl oxygens in phospholipids and hydroxyl or amide hydrogens in cholesterol, sphingolipids or water are claimed to constitute a rigid network in the region between the hydrophobic membrane interior and the polar head groups [6,34—37], which may be relevant for non-electrolyte permeability [6—9]. Although the cleavage of only one ester bond in phosphatidylcholine, as in our experiments, does not necessarily affect phospholipid-cholesterol interactions [9] the creation of additional negative charges in the membrane interface, due to the formation of fatty acids *, should perturb the hydrogen belt, particularly, since the fatty acid is able to change its position perpendicular to [38] and in the plane of [39] the membrane. Increases of non-electrolyte permeability have in fact been observed after incorporation of negatively charged constituents into artificial membrane systems [40].

The lack of effect of sphingomyelin hydrolysis on the permeability of the bilayer domain is presently unexplainable. In view of the increases of fluidity to be expected in membranes treated with sphingomyelinase, in analogy to findings with other phospholipases C [23,24], sphingomyelin cleavage should lead to an increase of permeability. Furthermore, cleavage of 60% of the sphingomyelin in human erythrocytes removes about 30% (50% in ox erythrocytes) of the exofacial phosphorylcholine groups and creates an equivalent amount of ceramide. The OH group on this residue could participate in hydrogen bonds [34] and thus influence the properties of the 'hydrogen belt'.

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* The extent to which these fatty acids are ionized is not known. Assuming that due to the presence of negative charges in the interface the pH is 2—3 units lower than bulk pH [41] 50—90% ionization are a reasonable estimate for acids with a pK of about 4.8.

The absence of permeability changes may therefore cast some doubts on concepts regarding this region as a barrier for non-electrolyte transfer.

In order to explain the lack of effect of both phospholipases, one might also adduce the exofacial localization of their attack, if one assumes the inner layer of the membrane to constitute the rate-limiting barrier for non-electrolyte efflux. In this case, one would not necessarily expect selective cleavage of the outer lipid layer to affect non-electrolyte permeability. This argument implies, that the degradatives remain confined to the outer layer. A number of observations, however, can be regarded as inferential evidence that transbilayer reorientation of all three degradatives is possible: (1) treatment of erythrocytes with long chain fatty acids increases membrane surface area [45], which is only possible if the compound is introduced into both halves of the bilayer. (2) Exogeneous lysophosphatidylcholine and fatty acids have been shown [46—49] to be converted into phosphatidylcholine at the inner surface of the erythrocyte membrane, which requires reorientation. (3) Sphingomyelinase treatment produces stomatocytes, which can be accounted for by a transmembrane reorientation of ceramide (cf. footnote p. 395 and Ref. 44). (4) A rapid transbilayer movement of lysophosphatidylcholine in artificial membranes has been observed in the presence of erythrocyte membrane proteins [50].

Moreover, an assignment of the non-electrolyte barrier to the inner layer would be difficult to reconcile with arguments supporting a limiting role of the outer layer, namely the species differences in non-electrolyte permeability which parallel differences in the phospholipid composition of the outer but not the inner layer [16], and the lack of effect on non-electrolyte permeability of a removal of the least tightly bound fraction of erythrocyte membrane cholesterol [16], a fraction, which is most probably localized in the inner layer [51]. On the basis of these observations a limiting role of the outer layer would seem probable, which, on the other hand, is difficult to reconcile with the data presented in this paper.

The lack of effect of two different types of phospholipid cleavage on the permeability of the lipid bilayer thus raises a number of questions concerning the significance of the interfacial and the hydrophobic region and of the two layers of the membrane lipid phase. These will have to be answered by studying in more detail the fate of the degradatives as well as the physical properties of the modified membranes.

Acknowledgements

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