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# Endovesiculation of human erythrocytes exposed to sphingomyelinase C: a possible explanation for the enzyme-resistant pool of sphingomyelin

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**When human erythrocytes are treated with *Staphylococcus aureus* sphingomyelinase C at 37 °C they become susceptible to cold lysis and appear to endovesiculate. Endovesiculation has been confirmed by showing that in parallel with sphingomyelin breakdown, the cells accumulate [$^3$H]inulin or [$^{14}$C]sucrose (without losing intracellular $K^+$) and also experience a loss of cell-surface acetylcholinesterase activity into a latent intracellular pool which can be revealed by treatment with detergent. On the basis of these observations it can be calculated that endovesicles account for about 2–4% of cell volume and about 25% of total cell surface. Pretreatment of cells with bee venom phospholipase $A_2$ completely blocked sphingomyelinase-induced endovesiculation but this effect was related to a concomitant decrease in sphingomyelin breakdown which was reduced by about 90%. These results indicate that the pool of sphingomyelin which is not susceptible to attack by sphingomyelinase C (about 15% of total sphingomyelin) may be resistant because of membrane internalisation and not because it originally resides in the inner leaflet of the plasma membrane.**

## Introduction

Our knowledge of the asymmetric transbilayer distribution of phospholipids in the plasma membrane of the human erythrocyte is based on an extensive work using a variety of chemical and enzymatic membrane probes [1,2]. As is well known, this work generally concludes that the anionic phospholipids (phosphatidylethanolamine and phosphatidylserine) usually occupy the inner lipid leaflet of the membrane whereas choline phospholipids (phosphatidylcholine and sphingomyelin) are concentrated in the outer leaflet. Experiments using exogenous bacterial sphingomyelinase C have played an important part in establishing not only the transbilayer disposition of sphingomyelin but also of phosphatidylethanolamine, since the action of sphingomyelinase C appears to increase the accessibility of this phospholipid to other enzymes (e.g. phospholipase $A_2$) [3–6]. This and more recent work has indicated that since a maximum of 85% of total sphingomyelin can be degraded by *Staphylococcus aureus* sphingomyelinase C acting on intact red cells, about 15% must therefore be present on the inner lipid leaflet [7]. Although this hypothesis has been supported by the observation that the enzyme-resistant pool of sphingomyelin has an unusual (less saturated) fatty acid composition which is more typical of inner leaflet phospholipids than

Abbreviation: Mops, 4-morpholinepropanesulphonic acid.

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of 'normal' sphingomyelin [7], a recent report from our laboratory concerning the action of sphingomyelinase C on chicken erythrocytes casts doubt upon the above interpretation [8]. Furthermore, we were aware of past data which suggested that treatment of mammalian red cells with phospholipase C-type enzymes caused large but non-lytic alterations in cell morphology perhaps leading to loss of surface membrane into internal vesicles [3,9–13] and also to a dramatically increased susceptibility to cold lysis [9,13], which might therefore preclude a simple interpretation of the results of phospholipase action. We therefore decided to look more closely at the effect of sphingomyelinase C on human cells in an effort to establish the precise effects of this enzyme.

## Materials and Methods

*Staphylococcus aureus* sphingomyelinase C was obtained from Sigma Chemical Co. as a solution in 50% glycerol, 0.25 M phosphate buffer (pH 7.5). This preparation had a protein concentration of 0.24 mg/ml with an enzyme activity of 160 I.U./mg of protein (1 I.U. defined as 1 $\mu$mol of substrate degraded per min at 37°C). For some experiments this material was purified as described by Zwaal et al. [5] when the activity was 1500 I.U./mg of protein and a single band (mol. wt. 25000) was seen on SDS gel electrophoresis. Bee venom phospholipase $A_2$ (760 I.U./mg protein) was also obtained from Sigma Chemical Co. Purified *Bacillus cereus* phospholipase C (2 mg/ml) was a gift from Dr. Clive Little (University of Tromso, Norway) and possessed an activity of about 500 I.U./mg of protein when assayed on phosphatidylcholine.

Fresh blood was obtained from volunteers and red cells were isolated by centrifugal washing as described previously [14]. To minimise contamination with leukocytes, red cell samples were withdrawn from below the buffy coat layer after each centrifugation. Cells were finally resuspended at 20% haematocrit in 20 mM Mops NaOH buffer containing 150 mM NaCl with the addition of 1 mM $MgCl_2$ and 10 mM glucose (Mops-saline).

5 ml of the cell suspension was treated with 50 mU of sphingomyelinase C at 37°C for 20 min. During this period, cell morphology was monitored using light microscopy, and at the end of the incubation a sample was taken into cacodylate-buffered 1% glutaraldehyde for subsequent electron microscopy [14]. To measure enzyme-induced cold lysis, 10-$\mu$l aliquots of treated cells were added to 1 ml of Mops-saline buffer containing 1 mM EDTA instead of $MgCl_2$ (to inhibit the sphingomyelinase C), incubated for 5 min at various temperatures from 37°C to 0°C and sedimented at $10000 \times g$ for 1 min in an MSE Microcentaur centrifuge.

Cell lysis was measured as the relative absorption at 540 nm of the original supernatant solution after incubation compared with the absorption of an equivalent number of cells lysed in 10 ml of distilled water. $K^+$ loss from the cells during incubation was similarly determined on the supernatant solutions and compared with lysed cells using a $K^+$-sensitive electrode (Philips plc Cambridge, U.K.).

To measured endocytosis of extracellular medium accompanying breakdown of sphingomyelin we employed a procedure which resembled that described by Ben-Bassat et al. [15] but with a fluid phase radioactive label rather than the membrane-bound label used by these authors. 10 $\mu$Ci of $^3H$ inulin (Amersham International plc, Amersham, U.K.) was added to 8 ml of cell suspension which was then divided into two aliquots. After incubation for 5 min at 37°C; 25 mU of sphingomyelinase C solution was added to one of the aliquots and an equivalent amount of glycerol was added to the other as a control. Incubation was continued for up to 2 h and at intervals, 0.5 ml samples from both control and enzyme-treated incubations were added to conical glass centrifuge tubes containing 10 ml of warm (37°C) Mops-saline buffer in which $MgCl_2$ was replaced by 1 mM EDTA, and the samples were centrifuged at $500 \times g$ for 5 min. The supernatant solution was retained to measure any cell lysis or loss of cell $K^+$ which occurred and the cells were washed three times in the same medium, taking care to maintain the temperature at 37°C. Finally the cells were resuspended in a total volume of 1 ml and vortexed with 3.75 ml of 2:1 methanol/chloroform. After 30 min 1.9 ml each of chloroform and water were added, the samples were again vortexed and then they were centrifuged as

before to achieve phase separation. Radioactivity associated with the cells was measured by adding 1.0 ml of the upper (aqueous) phase to 10 ml of PCS scintillation fluid (Amersham International plc) and counting in a Searle liquid scintillation counter. The lower (chloroform) phase was dried and sphingomyelin was analysed as described previously using thin-layer chromatography [7]. Other phospholipids including lysophosphatidylcholine were determined in the same way.

Similar experiments were carried out using phospholipase C from *B. cereus* (10 I.U./ml) instead of the sphingomyelinase C and using bee venom phospholipase $A_2$ prior to treatment with sphingomyelinase C. For the latter experiments, 10 ml of cells at 20% haematocrit were preincubated for 1 h at 37°C with 58 I.U./ml of phospholipase $A_2$ in Mops-saline containing 0.5 mM $CaCl_2$. After washing once with Mops-saline containing 1 mM EGTA and then twice with Mops-saline containing 1 mM $MgCl_2$ the cells were incubated with sphingomyelinase C as above. Control cells were treated identically except that no phospholipase $A_2$ was added. In each case measurements were made of phospholipid breakdown, lysis and uptake of [$^3$H]inulin, although in some experiments [$^{14}$C]sucrose or [$^3$H]inositol were employed instead of [$^3$H]inulin.

The putative loss of cell surface by endocytosis was measured by assaying the membrane marker enzyme acetylcholinesterase [16,17]. 2 ml of a 10% cell suspension in Mops-saline containing 1 mM $MgCl_2$ and 10 mM glucose was divided into two equal portions and incubated at 37°C. To one aliquot was added 25 mU of *S. aureus* sphingomyelinase C and to the other, an equivalent amount of 50% glycerol as a control. After 20, 40 and 60 min, duplicate 1 μl samples of control and enzyme-treated incubations were added to 0.5 ml of the assay medium (10 μl of 75 mM acetylthiocholine iodide, 10 μl of 10 mM dithiobisnitrobenzoic acid in 100 mM phosphate buffer (pH 8.0 at 37°C)) in four cells of an LKB Ultrospec 4050 spectrophotometer, and increase of absorbance at 412 nm was followed for 10 min. At this time, 5 μl of 10% Triton X-100 was added to each sample to lyse the cells and reveal any latent acetylcholinesterase activity, which was monitored as before for 10 min. Similar measurements of acetylcholinesterase activity with and without addition of sphingomyelinase C, were made on preparations of well-washed red cell ghosts prepared as described previously [14].

## Results

Confirming past indications [9,13] that breakdown of sphingomyelin on the surface of erythrocytes resulted in an increased susceptibility to cold lysis, Fig. 1 shows that below 20°C human cells treated with sphingomyelinase C undergo massive lysis. At 30°C and above, however, the treated cells remained intact for several hours, in accordance with many previous reports [3–7]. These results made us aware of the importance of maintaining the temperature of the cells at 37°C in subsequent experiments where it was vital to minimise cell lysis.

Treatment of human erythrocytes with sphingomyelinase C also produced large changes in cell morphology as seen in the light microscope: the

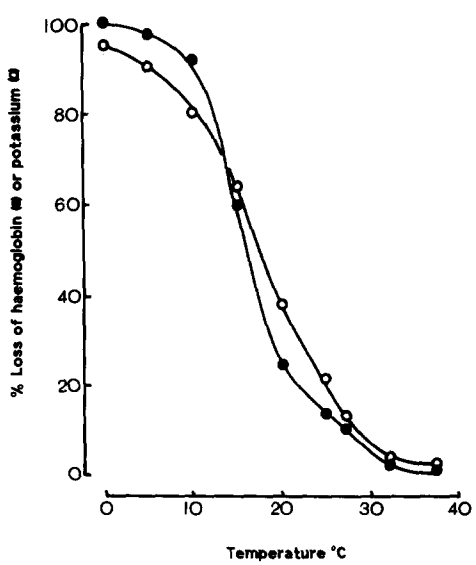


Fig. 1. Temperature-dependent lysis of human erythrocytes treated with *S. aureus* sphingomyelinase C. Cells were pretreated with sphingomyelinase C, incubated for 5 min at various temperatures and then centrifuged, as described under Methods. Lysis was determined as release of haemoglobin (●) and $K^+$ (○). The experiment shown is one of four which gave essentially identical results.

original discocytes rapidly became stomatocytic and then progressed to a spherical shape with apparent intracellular inclusions. The suggestion that endovesiculation was occurring was supported by electron microscopy which revealed a large number of small vesicular structures apparently inside the cells (Fig. 2). Although some of these structures, particularly the larger ones, could be due to sections taken across simple invaginations of the cell surface, it would be difficult to explain all the small vesicular profiles in this way.

Two other experiments supported the idea that sphingomyelinase C treatment caused endovesiculation. Firstly, an impermeant marker of the extracellular space ([$^3$H]inulin) accumulated in the cells in parallel with sphingomyelin breakdown and in the absence of significant cell lysis or loss of intracellular $K^+$ (Fig. 3). This marker was not taken up by control cells nor by cells which were pretreated with sphingomyelinase C before addition of inulin. Based on the amount of radioactivity accumulated during treatment with sphingomyelinase C, it was calculated that endovesicles could account for about 2–4% of total cell volume. Similar data was obtained uing [$^{14}$C]sucrose and with [$^3$H]inositol (results not shown) although in these cases there was a small progressive uptake of the radioactive markers into control cells.

Secondly, treatment with sphingomyelinase C decreased by about 25% the amount of acetylcholinesterase activity measurable in intact cells and this missing activity was revealed when the cells were subsequently exposed to a lytic detergent, Triton X-100 (Table I). Since acetylcholinesterase is a marker for the external surface of human erythrocytes, these observations are consistent with a sphingomyelinase C-dependent internalisation of about a quarter of total cell surface. Treatment of washed red cell ghosts with sphingomyelinase C did not change their acetylcholinesterase activity (Table I), suggesting either that no endovesiculation occurred in the ghosts or that the membranes were permeable to the reagents.

No changes in phospholipids other than sphingomyelin were seen in these experiments including lysophosphatidylcholine (about 1% of total

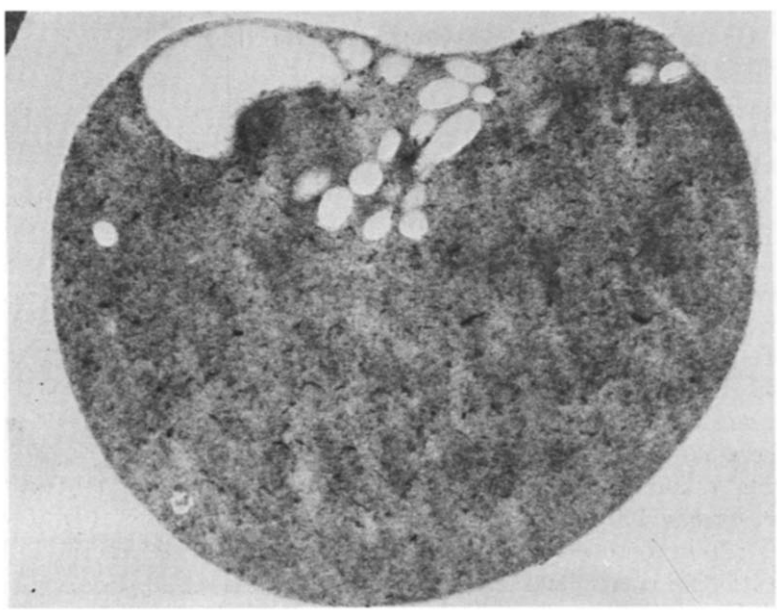

Fig. 2. Electron micrograph of a human erythrocyte after treatment with *S. aureus* sphingomyelinase C. After treatment of cells with sphingomyelinase C for 15 min, samples were fixed in 1% glutaraldehyde, 0.1 M cacodylate buffer pH 7.4 and sections were prepared for electron microscopy as described previously [34]. Virtually all the cells in the preparation had a morphology similar to the cell illustrated. Magnification ×20000.

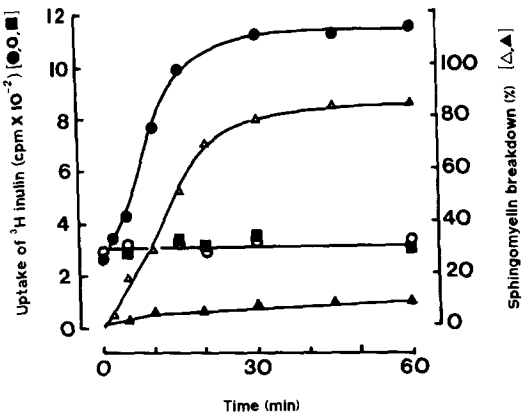


Fig. 3. Time course of uptake of [$^3$H]inulin and breakdown of sphingomyelin in erythrocytes treated with *S. aureus* sphingomyelinase C with and without prior exposure to phospholipase $A_2$. Cells were incubated with sphingomyelinase C in the presence of [$^3$H]inulin and at various times the enzyme activity was halted by addition of EDTA. Another sample of cells was treated with phospholipase $A_2$ (see Methods) prior to exposure to sphingomyelinase C. The cells were sedimented and uptake of the radioactive markers (●, +sphingomyelinase; ■, +phospholipase $A_2$ +sphingomyelinase; ○, control) was measured and compared with the amount of sphingomyelin degradation (△, +sphingomyelinase; ▲, +phospholipase $A_2$ +sphingomyelinase) (see Methods). Similar results were seen in four further experiments.

phospholipid) and lysophosphatidylethanolamine (about 0.5%). Cell lysis as measured by haemoglobin release never exceeded 1% and release of cell $K^+$ was always less than 2% of the total in the cells.

In order to determine if the morphological changes seen with sphingomyelinase C treatment could also be induced by a phospholipase C which attacked glycerolipids but not sphingomyelin, cells were exposed to purified *B. cereus* phospholipase C. Even when large amounts of this enzyme were used (10 I.U./ml) little breakdown of phosphatidylcholine was measured (5–10% after 1 h) although this was accompanied by the conversion of a small number (< 5%) of cells into stomatocytes or spherocytes. However, there was no measurable increase in uptake of [$^3$H]inulin compared with untreated controls. Attempts to increase further the number of stomatocytes by addition of more enzyme or by extending the incubation period failed due to the onset of unacceptable levels of cell lysis. Cells treated with phospholipase C did not undergo significant lysis in the cold.

TABLE I

THE EFFECT OF *S. AUREUS* SPHINGOMYELINASE C ON THE ACCESSIBILITY OF ERYTHROCYTE ACETYLCHOLINESTERASE

Acetylcholinesterase activity was measured as described under Methods in (a) untreated control cells, (b) cells pretreated with sphingomyelinase C, (c) control cells in the presence of 1% Triton X-100, (d) sphingomyelinase C-treated cells in the presence of Triton, (e) washed ghosts made from the same number of cells and (f) ghosts treated with sphingomyelinase C. Results are expressed as means ± S.D. from five separate experiments, taking the activity of untreated cells at 100%. *P* values were calculated using Student's *t*-test.
n.s., not significantly different from untreated cells.

| | Activity |
|---|---|
| Untreated cells | 100 |
| Cells treated with sphingomyelin C | 76 ± 4 ($P < 0.001$) |
| Control cells + Triton X-100 | 96 ± 3 (n.s.) |
| Cells treated with sphingomyelinase C + Triton | 98 ± 4 (n.s.) |
| Untreated ghosts | 97 ± 5 (n.s.) |
| Ghosts treated with sphingomyelinase | 99 ± 3 (n.s.) |

Pretreatment of cells with phospholipase $A_2$ removed about 60% of the phosphatidylcholine within 1 h and rendered the cells echinocytic in agreement with previous reports [3–5,11]. It also largely abolished the endovesiculation produced by sphingomyelinase C as measured by uptake of [$^3$H]inulin (Fig. 3). In the light microscope the cells appeared discocytic or slightly stomatocytic but showed no sign of endocytosis. Furthermore the rate of sphingomyelin breakdown was decreased by about 90% after prior treatment with phospholipase $A_2$ and even after 1 h the cells exposed to both enzymes still retained 90% of their original sphingomyelin. As reported by others [3], treatment of the cells with sphingomyelinase C before addition of phospholipase $A_2$ caused almost complete lysis.

All of the above experiments gave substantially the same results with purified sphingomyelinase C substituted for the semi-purified commercial enzyme.

## Discussion

The results of these experiments are interesting from several different aspects. To begin with, we

have confirmed previous indications [9,13] that sphingomyelinase C treatment causes erythrocytes to become very susceptible to cold lysis (Fig. 1) and this suggests that conversion of sphingomyelin to ceramide produces a change in membrane structure which drastically alters the sensitivity of the membrane to reduced temperature. Although we have no clear explanation for this effect, we think it is important to note the evidence for a strong and specific interaction between sphingomyelin and cholesterol in membranes [18–20] and the consequence that breakdown of sphingomyelin by sphingomyelinase C may leave cholesterol less strongly associated with phospholipids. Since cholesterol fluidises a mixture of phospholipids below its normal phase transition [21,22], removal of cholesterol from its association with phospholipids is likely to promote gelation of the fatty acid chains at subnormal temperatures and this could be the critical event which initiates cold lysis.

Cholesterol can apparently segregate out of the bilayer as a result of phospholipase C activity, since there is some evidence that treatment of red cell ghosts with a phospholipase C (whether or not it attacks sphingomyelin) causes accumulation of hydrophobic lipids including cholesterol, diacylglycerol and ceramide into lipid droplets separate from the phospholipid bilayer [4,9,23–25]. Breakdown of sphingomyelin alone appears to produce large (> 1000 Å) droplets (presumably containing ceramide) in ghosts and there is some evidence that even in intact cells, sphingomyelinase C can cause clustering of intramembranous particles and possibly formation of small lipidic droplets (< 200 Å) [3,9].

Both removal of phospholipid headgroups and any subsequent segregation of hydrophobic lipids into droplets could cause a decrease in membrane surface area which might contribute to the spherocytosis observed in cells treated with phospholipase C or sphingomyelinase C. However, the major determinant of spherocytosis seems likely to be the sphingomyelinase C-dependent endovesiculation process which on the basis of acetylcholinesterase assays appears to internalise up to 25% of total membrane. The endovesicles only account for 2–4% of cell volume based on uptake of insulin and sucrose and it can be calculated from these values that the mean diameter of the vesicles is about 10% that of the intact cell, a value which is consistent with the results of electron microscopy (Fig. 2) and with previous information [3,9,12]. The larger profile in Fig. 2 could represent a section through an invagination rather than a sealed vesicle. Although previous investigators have shown convincingly that red cell ghosts also vesiculate when treated with sphingomyelinase C [9], we were unable to see any corresponding change in the acetylcholinesterase activity of ghosts treated with this enzyme (Table I). These results could be due to an enhanced permeability of ghosts to the reagents used.

There are other possible interpretations of our findings which do not involve the idea of endovesiculation. For instance, it could be suggested that breakdown of sphingomyelin causes an increase in membrane permeability to inulin which might explain the enhanced uptake in enzyme-treated cells. However, this could not explain the observation that uptake of inulin was only increased when this radioactive marker was added prior to sphingomyelinase C and not when it was added after the sphingomyelinase C-dependent morphological changes had reached completion. Alternatively, it is possible that acetylcholinesterase, which seems to show preferential binding to sphingomyelin liposomes [22], is inhibited by degradation of this phospholipid rather than being internalised as a result of endovesiculation, although it is not obvious on this hypothesis why acetylcholinesterase activity should be recovered on treatment of the cell with Triton X-100. In addition, since there was no inhibition of acetylcholinesterase when washed ghosts were treated with sphingomyelinase C, sphingomyelin did not appear to be necessary for activity and ceramide was not inhibitory. Taking the results of radioactive marker uptake and acetylcholinesterase experiments together with the morphological observations our data is best explained by the endovesiculation model.

The mechanism of endocytosis induced by sphingomyelinase C is at present unknown. However, our observations do imply that either removal of the phosphorylcholine headgroup from sphingomyelin or the concurrent generation of ceramide are the cause of the membrane fusion

events which appear to be necessary for the formation of endovesicles. The initial direction of membrane bending can be predicted from the bilayer couple hypothesis of Sheetz and Singer [26] either on the basis that the cone-shaped ceramide molecule occupies a smaller area than the original sphingomyelin [27] or on the assumption that like diacylglycerol, ceramide can rapidly cross the membrane bilayer, thus causing a differential expansion of the inner lipid leaflet [28]. The immediate cause of the fusion event leading to endovesiculation remains obscure but it may be related to the clustering of intramembranous particles and apparent formation of lipidic droplets seen after freeze-fracture of sphingomyelinase C-treated cells. It is at present not clear whether breakdown of glycerolipids to give diacylglycerol can have similar morphological effects to sphingomyelinase C, since the phospholipase C from *Clostridium perfringens* which easily degrades glycerolipids on intact erythrocytes also attacks sphingomyelin, whereas the phospholipase C from *B. cereus* which does not attack sphingomyelin breaks down glycerolipids in intact cells only slowly, if at all [4,25]. However, our evidence suggests that breakdown of glycerolipids by the *B. cereus* enzyme does produce some minor morphological changes analogous to those obtained with sphingomyelinase C but does not cause significant increases in uptake of [$^3$H]inulin or in susceptibility to cold lysis.

Since sphingomyelinase C treatment causes internalisation of up to 25% of the plasma membrane it seems quite possible that the reason for the existence of the sphingomyelinase C-resistant pool of sphingomyelin is not because this pool represents sphingomyelin on the inner leaflet of the plasma membrane [7] but because some of the sphingomyelin is internalised in endovesicles and thus becomes inaccessible to exogenous enzyme. Even though some sphingomyelinase C would presumably be endocytosed it can be calculated that this would account for only about 0.5% of the total enzyme added so that attack on internalised sphingomyelin should be correspondingly two orders of magnitude slower than attack on external sphingomyelin. The apparent discrepancy between the values for membrane internalisation (25%) and residual sphingomyelin (15%) could be explained by a combination of enzyme attack immediately prior to internalisation together with a very slow breakdown after endovesicle formation.

Recent work [7] has indicated that the residual sphingomyelin in human cells treated with sphingomyelinase C is enriched in unsaturated fatty acids and we have not been able to account for this observation in terms of contamination with lysophosphatidylcholine as suggested by our experiments with chicken erythrocytes [8]. It is possible that sphingomyelin containing unsaturated fatty acids is either less rapidly attacked by sphingomyelinase C or is selectively incorporated into endocytic vesicles following attack by this enzyme.

It is not clear why pretreatment with phospholipase $A_2$ should produce a complete inhibibition of sphingomyelinase-induced endovesiculation as measured by inulin uptake (Fig. 3) but this effect must certainly be related to the fact that sphingomyelin breakdown was far slower in cells exposed to phospholipase $A_2$ before sphingomyelinase C. A similar result was obtained by Schrier et al. [29] but the effect was less marked than in our experiments, perhaps because these workers used much larger amounts of sphingomyelinase C. Insensitivity to sphingomyelinase and the associated inhibition of endovesiculation may both be due to a change in the physical characteristics of the membrane resulting from the production of lysophosphatidylcholine and fatty acid from phosphatidylcholine following the action of phospholipase $A_2$. Whatever the nature of this resistance to sphingomyelinase C after phospholipase $A_2$ treatment, it seems clear that it cannot be explained in terms of endocytosis.

Our results do not seem to be consistent with those of Verkleij et al. [3] who found that sphingomyelin breakdown in cells treated with phospholipase $A_2$ and sphingomyelinase C was only a little less than in cells treated with sphingomyelinase C alone. However, these workers used *Naja naja* phospholipase $A_2$ in the presence of 10 mM $CaCl_2$ and did not remove the phospholipase $A_2$ or $CaCl_2$ before addition of sphingomyelinase C so that their results are therefore difficult to compare directly with our findings. Recent data [30] have suggested that phospholipase $A_2$ may

under certain conditions cause increased transbilayer reorientation of some phospholipids in an already destabilised membrane. Possibly, in circumstances where phospholipase $A_2$ is active in the presence of sphingomyelinase C and $Ca^{2+}$ [3] such a destabilisation does occur and gives misleading results for phospholipid asymmetry.

Finally, in view of the confirmation in these experiments that sphingomyelinase C action can cause endocytosis in erythrocytes it is worth considering the possibility that endogenous sphingomyelinase C may be associated with some other kinds of cellular events which involve fusion of membranes. In this context it is interesting to note that the acrosome reaction, a membrane fusion event normally accompanying fertilisation, may involve breakdown of sphingomyelin [31] and also that activation of the endogenous sphingomyelinase C of chicken erythrocytes by treatment of the cells with A23187/$Ca^{2+}$ appears to be associated with membrane fusion events [32,33]. It has been suggested previously that formation of diacylglycerol from phospholipids may be involved in membrane fusion [28]; possibly the analogous production of ceramide from sphingomyelin could have a similar role.

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