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A sphingomyelinase-resistant pool of sphingomyelin in the nuclear membrane of hen erythrocytes

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Experiments in which hen erythrocytes were exposed to the action of exogenous sphingomyelinase (*Staphylococcus aureus*) or to their endogenous plasma membrane sphingomyelinase showed that about 15% of the total sphingomyelin was resistant to breakdown either in intact or lysed cells. This resistant pool of sphingomyelin seems likely to reside in the nuclear membranes of the cells, so that essentially all the plasma membrane sphingomyelin can be broken down by exogenous sphingomyelinase acting on intact cells, suggesting that plasma membrane sphingomyelin is exclusively localised in the outer lipid leaflet. Paradoxically, introduction of Ca^{2+} into the intact cells using A23187 causes the breakdown of up to 30% of total cell sphingomyelin inside the cells but without apparently affecting the putative nuclear pool of sphingomyelin and this suggests that Ca^{2+} may alter the original disposition of sphingomyelin in the membrane so that originally outer leaflet sphingomyelin becomes accessible to the endogenous sphingomyelinase inside the cells. No differences were seen in the fatty acid compositions of sphingomyelin degradable by exogenous sphingomyelinase, sphingomyelin degradable in the presence of A23187/Ca^{2+} or the enzyme-resistant pool of sphingomyelin.

Introduction

There is considerable evidence that the membranes of various cellular compartments possess characteristic lipid compositions and one of the best examples of this is the concentration of sphingolipids and particularly sphingomyelin, in plasma membranes [1]. Furthermore sphingomyelin in human erythrocyte plasma membranes seems to have a marked asymmetry in its transmembrane distribution with 80–85% of the total residing in the outer lipid leaflet [2]. Recent work by Boegheim and his colleagues [3] shows that the sphingomyelin on the outer leaflet (as judged by its accessibility to exogenous sphingomyelinase) has a distinctly different fatty acid composition from the residual sphingomyelin (presumed to be on the cytoplasmic leaflet), which is relatively rich in shorter chain (C_{16}, saturated; C_{18}, unsaturated) fatty acids. Such a difference in fatty acid compositions is not seen when comparing outer-leaflet with inner-leaflet phosphatidylcholine [4] but Boegheim et al. [2] argued that because 'flip-flop' of sphingomyelin was very much slower than that of phosphatidylcholine, an initial asymmetry in sphingomyelin fatty acid composition was conserved on a time scale comparable to the life span of individual cells.

A more complex situation is represented by the

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chicken erythrocyte, which because it is nucleated, possesses nuclear membranes in addition to a plasma membrane. However the chicken red cell plasma membrane appears to be almost identical in phospholipid composition to its human equivalent and seems to contain much of the sphingomyelin of the intact cell [5]. A considerable proportion (> 60%) of this sphingomyelin can be broken down by an endogenous sphingomyelinase which is latent in intact cells but which is activated when the cells are lysed [6–8]. A smaller amount (≈ 30%) can also be degraded when the endogenous enzyme is activated non-lytically by treating the cells with Ca^{2+} and ionophore A23187. Since under these conditions phosphorylcholine is released inside the cells [5], it appears that the pool of sphingomyelin which was susceptible to breakdown following Ca^{2+}/ionophore treatment resides on the inner face of the plasma membrane, and thus, on the basis of findings of Boegheim et al. [3] might have a characteristic fatty acid composition which was different from the overall composition of the sphingomyelin of the cell.

We therefore set out to characterise the fatty acid composition of sphingomyelin and ceramide after treatment of intact cells with (a) *S. aureus* sphingomyelinase to break down the outer-leaflet pool of sphingomyelin and (b) A23187/Ca^{2+} to break down the presumed inner-leaflet pool of sphingomyelin.

Materials and Methods

10-ml samples of blood from the wing vein of Rhode Island Red hens were taken in a syringe containing 0.5 ml of 100 mM EGTA and the cells were washed four times in 0.15 M NaCl at 4°C by centrifugation at 1000 × *g* for 5 min. Packed red cells were withdrawn from below the buffy-coat layer using a Pasteur pipette and were diluted to 20% haematocrit in 20 mM morpholinepropanesulphonic acid (Mops)-NaOH buffer (pH 7.1)/0.15 M NaCl. Cells (1 ml aliquots) were incubated at 37°C either alone or in the presence of (a) 0.4 I.U. of *S. aureus* sphingomyelinase (Sigma Chemical Co.), (b) 0.05% Triton X-100, (c) 5 μM A23187 (Calbiochem) + 1 mM $CaCl_2$, or with various combinations of the above. Samples of packed cells (0.2 ml) were also lysed and incubated in 0.8 ml of 20 mM Mops-NaOH (pH 7.1). After appropriate incubation times, 3.75 ml of 2 : 1 methanol/chloroform was added and lipids were extracted as described previously [5]. Phospholipids were separated by TLC under N_2 on silica gel H plates using a solvent consisting of chloroform/methanol/acetic acid/water (75 : 45 : 12 : 6, v/v) [9] and containing 1 mg/ml butylated hydroxytoluene as antioxidant. Spots were identified by comparison with phospholipid standards (Sigma Chemical Co.) after staining with iodine (prior to phosphorus analysis) or with Rhodamine 6G (0.1% in acetone) prior to fatty acid analysis. In some cases where it was important to resolve sphingomyelin and lysophosphatidylcholine, samples were run in two dimensions using chloroform/methanol/water (65 : 25 : 4, v/v) as the first solvent. Neutral lipids were separated on silica gel H plates run in a solvent consisting of benzene/diethyl ether/acetic acid (50 : 40 : 0.2, v/v) and ceramide was identified by comparison with an authentic standard (Sigma Chemical Co).

For fatty acid analysis, spots excised from TLC plates were treated with 15% BF_3/methanol (Pierce) for 1 h at 100°C under N_2 as described by Morrison and Smith [10]. Fatty acid methyl esters were separated and identified by comparison with authentic standards by GLC (Pye 204) on 1.5-m columns of (a) 5% diethyleneglycolsuccinate (DEGS) on 80/100 mesh Supelcoport (Supelco) run isothermally at 190°C and (b) 1.5% Dexsil (Supelco) on 80/100 mesh Supelcoport using a gradient from 200 to 280°C at 8 Cdeg/min. Quantification of peaks was achieved using a Shimadzu RC1B computing integrator.

Where necessary, confirmation of the identity of sphingomyelin was achieved (a) by subjecting the presumed sphingomyelin to mild alkaline hydrolysis [11] and demonstrating that the lipid rechromatographed in its original position; (b) by showing that following treatment with BF_3/methanol, the lipid remaining in the aqueous layer was sphingosine. This was confirmed by extracting the lipid into diethyl ether after addition of NaOH and showing that the silylated lipid chromatographed in same position on Dexsil as did authentic sphingosine (between 20 : 0 and 22 : 1 fatty acid methyl esters); (c) by demonstrating that the isolated lipid chromatographed as cera-

mide after treatment with *S. aureus* sphingomyelinase or *Clostridium welchii* phospholipase C (Sigma Chemical Co).

Attempts were made to assay phospholipase A activity in the *S. aureus* sphingomyelinase preparation by incubation of up to 2 units of enzyme for 2 h at 37°C with 200–500 nmol of pure phosphatidylcholine (Sigma) sonicated in 0.5 ml of 20 mM Mops-NaOH buffer containing 0.15 M NaCl.

Cells treated with A23187/Ca^{2+} were sedimented at 1000 × *g* for 5 min and the distribution of phosphorylcholine in cells and supernatant solution was measured [5]. Determination of phosphorylcholine was carried out as described previously [5] but using a modified TLC solvent system (methanol/1 mM EDTA, 75 : 25, v/v) in which phosphorylcholine moved with an R_F of 0.12 and all other cellular water-soluble phosphates (nucleotides, sugar phosphates, etc.) moved close to the front. The identity of phosphorylcholine isolated in this way from cell extracts was confirmed by treating the material with alkaline phosphatase (Sigma Chemical Co.) and measuring the choline released by the procedure of Appleton et al. [12].

In order to assess the distribution of different phospholipids between the plasma membrane and nucleus of hen erythrocytes, estimates of the cross-contamination between the two fractions prepared as described by Beam et al. [13] were made assuming that spectrin was present solely in the plasma membrane and histones were present exclusively in the nuclei. Quantification of these proteins on SDS gels using a microdensitometer (Bio-Rad) in conjunction with a Shimadzu RC1B computing integrator, allowed a graphical comparison to be made between phospholipid composition and the relative amounts of plasma membrane and nuclear material in each preparation. Breakdown of sphingomyelin which occurred during incubation of the two separate fractions in Mops-saline buffer with or without addition of *S. aureus* sphingomyelinase, was measured as described above for cells.

Results

Treatment of intact chicken erythrocytes with *S. aureus* sphingomyelinase gave very similar results to those obtained with human erythrocytes [2,3]: about 85% of the total sphingomyelin was degraded after 60 min incubation (Table I, Fig. 1) and this amount did not increase further after 6 h incubation (Table I). Treated cells changed from their normal ellipsoidal shape to a buckled crescent-shaped form but did not become stomatocytic or invaginated. Addition of the enzyme to lysed cells or to cells treated with 0.05% Triton X-100 (which released > 95% of total haemoglobin) gave considerably faster breakdown (Fig. 1) but after 60 or 120 min the total breakdown was scarcely any different from that seen with intact cells. Treatment with Triton alone activated the endogenous sphingomyelinase, giving much faster breakdown of sphingomyelin than in cells lysed without Triton (Fig. 1), but still leaving a resistant pool of phospholipid comigrating with sphingo-

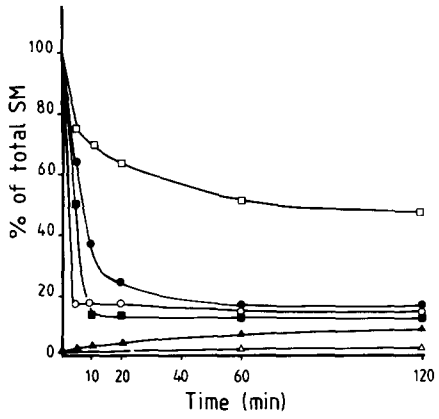

Fig. 1. Time-course of breakdown of hen erythrocyte sphingomyelin in cells exposed to exogenous and/or endogenous sphingomyelinase. Cells were incubated at 37°C with (a) 0.4 units of *S. aureus* sphingomyelinase in 0.15 M NaCl/20 mM Mops-NaOH buffer (pH 7.1) (●, ▲); (b) 0.05% Triton X-100 in 0.15 M NaCl/20 mM Mops-NaOH buffer (■, △); (c) 20 mM Mops-NaOH buffer (pH 7.1) (□); (d) 20 mM Mops-NaOH buffer (pH 7.1) + 0.4 units of *S. aureus* sphingomyelinase (○). Cells (0.2 ml packed volume in 1 ml buffer) were incubated as described under Methods. Results shown are means of duplicate determinations from a single experiment which was one of four similar experiments giving essentially the same results. Production of lysophosphatidylethanolamine is not shown (for the sake of clarity) but was very similar in magnitude and time-course to production of lysophosphatidylcholine. The amount of lysophosphatidylcholine (triangular symbols) is expressed as a percentage of the total sphingomyelin (circles and square symbols) in the cells which itself represented about 17% of total phospholipid.

TABLE I

SPHINGOMYELIN CONTENT OF HEN ERYTHROCYTES EXPOSED TO EXOGENOUS AND/OR ENDOGENOUS SPHINGOMYELINASES

Cells were incubated with exogenous *S. aureus* sphingomyelinase or with Triton or A23187/Ca^{2+} to activate the endogenous sphingomyelinase as described under Methods. Results are expressed as % of original sphingomyelin (SM) phosphorus remaining and represent means of duplicate samples derived from a single experiment. Two further similar experiments gave substantially the same results.

	Incubation time	% SM remaining
Control (no addition)	–	100
Incubated control	1 h	101
Intact cells + A23187/Ca^{2+}	20 min	73
Intact cells + *S. aureus* sphingomyelinase	1 h	16
	6 h	14
Cells lysed in 0.05% Triton	1 h	16
	6 h	9
0.1% Triton	1 h	5
0.2% Triton	1 h	3
A23187/Ca^{2+}-treated cells lysed in 0.05% Triton	1 h	15
A23187/Ca^{2+}-treated cells + *S. aureus* sphingomyelinase	1 h	14
Cells lysed in 0.05% Triton + *S. aureus* sphingomyelinase	1 h	11
	6 h	4

myelin on the TLC plate. This residual phospholipid appeared to be sphingomyelin since (a) it was chromatographically unchanged by mild alkaline hydrolysis but destroyed by 2 M HCl at 100°C; (b) it gradually disappeared after prolonged incubation with 0.05% Triton or with higher concentrations of this detergent (Table I); (c) a quantity of sphingosine equivalent to the phosphorus measured was recovered from the aqueous fraction after BF_3 or HCl methanolysis; (d) treatment of pure phospholipid extracted from cells with either *C. welchii* phospholipase C or *S. aureus* sphingomyelinase led to complete disappearance of the phospholipid migrating in the region of sphingomyelin.

Initial studies using one-dimensional TLC, on the fatty acid composition of the sphingomyelin which was resistant to *S. aureus* sphingomyelinase gave results rather similar to those obtained by Boegheim et al. [3]: unusually large quantities of short chain unsaturated fatty acids (especially 18:1 and 18:2) were found (Table IIB(e)), in contrast to the results for total sphingomyelin and ceramide where it was difficult to measure any of these two fatty acids (Table II). In contrast, the resistant sphingomyelin after activation of the endogenous sphingomyelinase by lysis or treatment with Triton, had a fatty acid composition quite

TABLE II

FATTY ACID COMPOSITION OF SPHINGOMYELIN AND CERAMIDE FROM HEN ERYTHROCYTES EXPOSED TO SPHINGOMYELINASES

Sphingomyelin and ceramide were analysed as described under Methods from cells incubated in 0.15 M NaCl/20 mM Mops-NaOH (pH 7.1) (unless otherwise stated) treated as follows: (a) untreated; (b) A23187/Ca^{2+} 20 min; (c) lysed in 20 mM Mops-NaOH buffer (1 h); (d) lysed in the presence of 0.05% Triton; (e) intact cells incubated for 1 h with *S. aureus* sphingomyelinase; (f) as (e) but SM separated by two-dimensional TLC. 22:*x* refers to components eluting from the column between 22:0 and 24:0/24:1.

Fatty acid	A. Ceramide				
	(a)	(b)	(c)	(d)	(e)
16:0	14.3±2.5	15.0±1.3	15.8± 2.3	14.3±2.1	15.1±2.6
18:0	2.9±0.6	2.2±0.4	2.1± 0.8	1.3±0.5	1.8±0.7
18:1	–	1.1±0.9	0.8± 0.6	1.0±0.6	1.4±0.8
18:2	–	–	–	–	–
22:0	17.3±1.9	14.5±1.7	15.6± 2.1	16.7±1.4	16.4±2.2
22:*x*	–	2.8±1.5	3.2± 1.7	2.3±1.1	2.7±0.5
24:0	19.4±2.2	18.6±2.4	20.2± 1.5	20.1±0.9	22.1±2.9
24:1	36.8±1.7	38.8±1.7	36.9± 2.5	38.9±1.8	39.8±3.1
24:*x*	5.0±1.3	6.4±2.1	7.1± 2.4	8.5±2.1	6.5±2.7
% total	6 ±2	28 ±5	65 ±10	87 ±3	85 ±2

similar to that of total sphingomyelin or ceramide (before or after enzyme treatment). Closer investigation revealed that the bacterial enzyme appeared to be associated with phospholipase A activity since small spots corresponding in mobility to lysophosphatidylethanolamine (ninhydrin positive) and lysophosphatidylcholine were present after incubation of cells with *S. aureus* sphingomyelinase (Fig. 1) but not after activation of the endogenous enzyme. Moreover, the spot presumed to be lysophosphatidylcholine was destroyed by alkaline hydrolysis (unlike residual sphingomyelin) and contained large amounts of 18 : 1 and 18 : 2 fatty acids (Table IIC). When a better separation of the residual sphingomyelin from lysophosphatidylcholine was obtained by TLC in two dimensions it was clear that the fatty acid composition of the residual sphingomyelin was very similar to that of total sphingomyelin or ceramide (Table IIB(f)), thus confirming that the original observation of 18 : 1 and 18 : 2 fatty acids in the resistant sphingomyelin was probably due to contamination with lysophosphatidylcholine presumably arising as a result of phospholipase A activity. However, attempts to demonstrate phospholipase A activity in the *S. aureus* enzyme preparation proved negative using sonicated phosphatidylcholine as substrate: activity was less than 1 nmol per h per unit of sphingomyelinase activity.

When hen erythrocytes were treated with A23187/Ca^{2+} about 30% of the total sphingomyelin was degraded to ceramide as described previously [5]. This ceramide and the residual sphingomyelin had rather similar fatty acid compositions to each other and to the same lipids either in untreated cells or in cells treated with Triton or *S. aureus* sphingomyelinase (Table II). We confirmed our original observation [5] that an amount of phosphorylcholine equivalent to the sphingomyelin broken down was generated inside the cells, so that under these conditions it appeared that about 30% of the cellular sphingomyelin was available to the endogenous sphingomyelinase within the cells. Addition of 0.05% Triton alone to cells pretreated with A23187/Ca^{2+} still did not lead to the breakdown of the resistant pool of sphingomyelin (Table I) which accounted for about 15% of the total sphingomyelin. Neither did addition of *S. aureus* sphingomyelinase to A23187-treated cells cause a significant reduction in the amount of the resistant sphingomyelin (Table I).

The precise subcellular localisation of the sphingomyelin in the hen erythrocyte is more difficult to determine than in the human red cell

24 : *x* is a single component eluting just after 24 : 1 on DEGS and just after 24 : 0 on Dexsil. It could be 1-hydroxy 22 : 0 based on the data of O'Brien and Rouser [24]. The proportion of each fatty acid is expressed as percentage of total recovered in each case. Ceramide totals are expressed as a percentage of total SM fatty acid. SM and lysoPC totals are expressed as a percentage of total SM phosphorus.

B. Sphingomyelin						C. LysoPC
(a)	(b)	(c)	(d)	(e)	(f)	(e)
12.3 ± 2.4	12.7 ± 1.2	15.6 ± 2.7	13.5 ± 1.1	22.5 ± 3.6	16.2 ± 2.3	20.3
2.6 ± 0.8	3.2 ± 1.1	2.9 ± 1.3	3.1 ± 1.7	4.1 ± 2.3	5.1 ± 2.5	6.3
0.2 ± 0.3	1.0 ± 0.8	1.2 ± 0.6	1.3 ± 0.7	6.5 ± 2.5	0.8 ± 0.4	30.2
< 0.1	< 0.2	< 0.5	< 0.5	13.5 ± 3.2	< 0.5	43.9
13.9 ± 0.9	14.3 ± 2.1	15.8 ± 2.9	16.4 ± 2.5	10.2 ± 2.1	14.1 ± 0.7	–
2.8 ± 1.7	1.9 ± 0.8	1.7 ± 0.8	2.1 ± 0.6	3.1 ± 1.7	2.2 ± 0.5	–
19.1 ± 1.3	18.3 ± 2.2	21.1 ± 2.6	22.2 ± 3.1	11.5 ± 1.7	17.9 ± 1.3	–
39.8 ± 2.3	39.7 ± 3.6	35.6 ± 4.1	34.7 ± 3.9	24.5 ± 2.0	38.2 ± 2.7	–
7.6 ± 1.2	8.2 ± 2.7	6.6 ± 2.1	5.3 ± 1.7	2.1 ± 1.7	6.2 ± 2.1	–
(100)	27 ± 6	59 ± 10	15 ± 3	16 ± 4	12 ± 2	8 ± 3

since there is the possibility in the avian cells that some of the total sphingomyelin is present in the nuclear membrane. An attempt was made to assess the amount of nuclear membrane sphingomyelin by comparison of the phospholipid content of nuclei with that of plasma membranes, adjusting the results to allow for cross-contamination between the two fractions (see Methods). This led to the conclusion (Fig. 2) that although 'pure' nuclei contained only a small proportion of the total sphingomyelin (equivalent to about 2–3% of their total phospholipid) this was nevertheless sufficient to account for the pool of sphingomyelin which was resistant to enzyme attack. This was confirmed by experiments with isolated nuclear and plasma membrane fractions which showed that the nuclear sphingomyelin was relatively stable to incubation with or without sphingomyelinase whereas the plasma membrane sphingomyelin was almost completely degraded (Table III).

Fig. 2 also confirmed that triphosphoinositide is exclusively localised in the plasma membrane but that the nuclear membrane does contain some

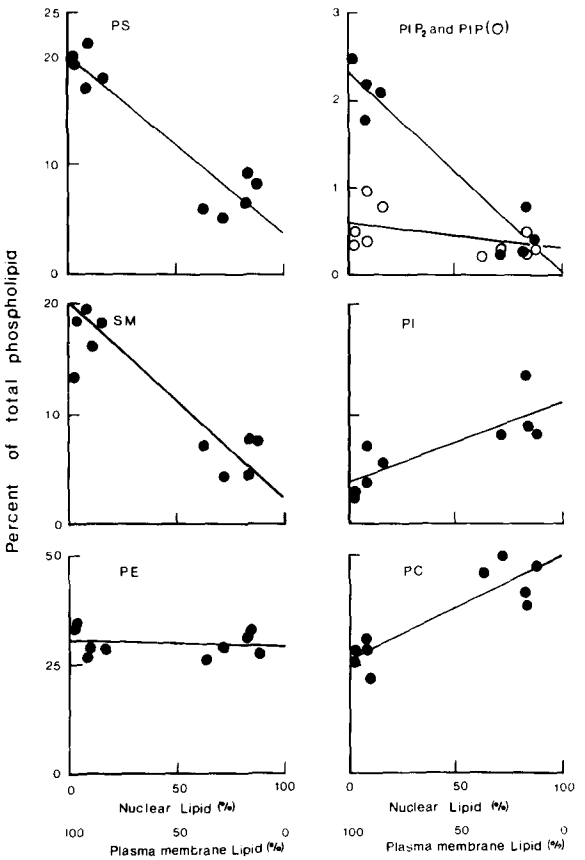

Fig. 2. Phospholipid content of plasma membrane and nuclear preparations from hen erythrocytes. Analyses of phospholipid classes were carried out as described previously [5] on samples of plasma membrane and nuclei from various experiments using different chickens. In each case, the relative amounts of nuclear contamination in the plasma membrane and plasma membrane contamination of the nuclei was assessed by gel electrophoresis of each sample (see Methods). A plot of phospholipid content versus relative quantities of plasma membrane/nuclei allowed an estimate to be made of the amounts of each phospholipid in the 'pure' fractions. Abbreviations: PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-diphosphate; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin.

TABLE III

BREAKDOWN OF SPHINGOMYELIN IN ISOLATED PLASMA MEMBRANES AND NUCLEI INCUBATED WITH OR WITHOUT *S. AUREUS* SPHINGOMYELINASE

Plasma membranes and nuclei were prepared as described by Beam et al. [13] and nuclei were purified by a further sonication and washing. Triplicate samples of each fraction (equivalent to about 500 nmol of total phospholipid) were incubated in 20 mM Mops-NaOH buffer/0.15 M NaCl with or without addition of 0.4 units of *S. aureus* sphingomyelinase. Sphingomyelin was determined as described under Methods. The reported values represent the means ± S.D. of three separate experiments.

	Nuclei	Plasma membrane
Control (unincubated)	3.5 ± 0.4 *	25.2 ± 2.6 *
20 min 37°C	3.4 ± 0.6	4.4 ± 0.8
20 min 37°C + sphingomyelinase	3.2 ± 0.3	0.9 ± 0.3

* These figures included ceramide, to account for sphingomyelinase broken down during the preparation of these fractions. This breakdown was generally about 20–25% of the total sphingomyelin in the plasma membrane fraction (see Ref. 5).

diphosphoinositide and phosphatidylserine. Phosphatidylinositol and phosphatidylcholine partition 2 : 1 in favour of the nuclear membrane whereas phosphatidylethanolamine appears to be equally distributed between the nuclear and plasma membranes.

Discussion

Initial studies of the breakdown of hen erythrocyte sphingomyelin by endogenous sphingomyelinase gave results rather similar to those obtained previously with human cells [2,3]. About 85% of the sphingomyelin was degraded without cell lysis and the residual sphingomyelin appeared to have a different fatty acid composition from the main pool of sphingomyelin, being unusually rich in short chain unsaturated fatty acids (Table IIB(e)). This unusual fatty acid composition however seemed to be an artifact arising from contamination with lysophosphatidylcholine which appeared during treatment of cells with *S. aureus* sphingomyelinase, since when two-dimensional TLC was employed to improve the separation between sphingomyelin and lysophosphatidylcholine, the fatty acid composition of the residual sphingomyelin was virtually identical to that of the pool of sphingomyelin which was susceptible to enzyme degradation. No 'unusual' fatty acid composition was observed for the residual sphingomyelin (separated by one-dimensional TLC) and no formation of lysolipid was observed when sphingomyelin was degraded by the endogenous sphingomyelinase, which therefore was not accompanied by any phospholipase A activity. These results suggest that there is no pool of sphingomyelin with a fatty acid composition significantly different from that of the bulk sphingomyelin in hen erythrocytes and contrast with the findings of Boegheim et al. [3] who used a similar *S. aureus* enzyme preparation in their investigations on human cells. Since we were unable to demonstrate any phospholipase A activity in the *S. aureus* sphingomyelinase preparation using purified phosphatidylcholine as substrate it is not clear whether the phospholipase A activity exhibited against intact cells is an intrinsic property of this enzyme preparation or whether breakdown of sphingomyelin somehow stimulates endogenous phospholipase A activity in the cells. Whatever the source of this activity it seems to have the characteristics of a phospholipase A_1 since the residual lysolipid is relatively rich in unsaturated fatty acids which are usually present on the 2-position of glycerophospholipids. Zwaal and his co-workers [2,19] did not observe any phospholipase A activity when using their purified *S. aureus* sphingomyelinase with human cells but since they measured decreases in phospholipid rather than increases in lysophospholipid it is unlikely that they would have detected the low levels of activity (breakdown of about 2% of total phosphatidylcholine) seen in the present work.

It has been assumed by previous workers that erythrocyte sphingomyelin which is not attacked by *S. aureus* sphingomyelinase under non-lytic conditions is probably on the inner leaflet of the plasma membrane [2,3]. However with hen erythrocytes even after lysis in low concentrations of Triton, the 12–15% of the total sphingomyelin which resists exogenous sphingomyelinase is still not susceptible to breakdown by either endogenous or exogenous enzyme (Table II, Fig. 1). It is only under more extreme conditions of incubation (higher Triton concentrations or longer incubation periods) that this pool seems to be degraded. The resistant pool of sphingomyelin could be on either leaflet of the plasma membrane but somehow sequestered, perhaps by interaction with protein so that it could not be degraded by either exogenous or endogenous enzyme activity. The 15% of total sphingomyelin represented by the resistant pool is equivalent to about 150 nmol/10^{10} cells or about 10^7 molecules per cell, a figure which is an order of magnitude higher than the number of copies per cell of band 3 protein [14] which is the major membrane polypeptide, so that it seems unlikely that there is enough protein to bind this amount of sphingomyelin. An alternative and perhaps more appealing hypothesis is that the resistant pool is in the nuclear membrane of the hen erythrocyte where it is not available to either exogenous sphingomyelinase or the endogenous enzyme which appears to be localised in the plasma membrane [6–8]. Estimation of nuclear sphingomyelin is difficult because the level is very low and because isolated nuclei are invariably contaminated with plasma membrane, but we have made an attempt to measure the nuclear sphingomyelin by determining the degree of contamination with plasma membrane using spectrin as a plasma membrane marker and then making an allowance for contaminating plasma membrane sphingomyelin in the nuclei (Fig. 2). These results show that 'pure' nuclei contain about 2–3% of

their phospholipid as sphingomyelin and thus account for 10–15% of total cell sphingomyelin, assuming approximately equal amounts of phospholipid in plasma membrane and nuclei [15]. Incubation of isolated nuclei with *S. aureus* sphingomyelinase caused little breakdown of sphingomyelin whereas degradation of plasma membrane sphingomyelin was almost complete under the same conditions (Table III) and this suggests that nuclear sphingomyelin may reside either in the inner nuclear membrane or in the inner leaflet of the outer nuclear membrane. These experiments confirmed that the amount of nuclear membrane sphingomyelin is about the same as the amount of enzyme-resistant sphingomyelin in cells.

If the enzyme-resistant pool of sphingomyelin is in the nuclei, then this means that essentially all of the enzyme-susceptible pool of sphingomyelin is on the outside face of the cell since it can be broken down by exogenous sphingomyelinase. This conclusion makes it very difficult to explain how A23187/Ca^{2+} can promote the breakdown of up to 30% of total cell sphingomyelin with phosphorylcholine appearing inside the cells and without apparently attacking the enzyme-resistant pool of sphingomyelin assumed to be present in the nuclei. If all the plasma membrane sphingomyelin is on the outside of the cell, how can up to 35% of it be broken down inside the cell?

Introduction of Ca^{2+} per se into human erythrocytes does not appear to produce any rapid changes in phospholipid asymmetry [16] but the situation is much more complicated in hen erythrocytes because here Ca^{2+} causes cytoskeletal beakdown and what appears to be fusion between nucleus and plasma membrane [5,17]. Conceivably, at the nexus between nuclear membrane and plasma membrane, sphingomyelin that was originally in the outer leaflet of the plasma membrane could become accessible to inner-leaflet sphingomyelinase. Alternatively, Ca^{2+}-induced separation of the plasma membrane bilayer from the cytoskeleton may allow transbilayer migration of sphingomyelin under the influence of sphingomyelinase present on the inner leaflet of the bilayer. This could be a similar situation to that described recently by Franck et al. [18] to explain apparently anomalous results of phospholipase A_2 action on human erythrocytes which had been exposed to diamide. Disorganisation of membrane structure by lysis which itself could alter interactions between bilayer and cytoskeleton, appears to be sufficient to activate the endogenous plasma membrane sphingomyelinase of hen erythrocytes [6–8] although the breakdown of sphingomyelin is very much faster in the presence of detergent (Fig. 1). There are other examples where membrane disorganisation increases the susceptibility of phospholipids to phospholipases [19–21].

The status of the original experiments of Boegheim et al. [3] in which human erythrocytes were treated with *S. aureus* sphingomyelinase is at present not clear. There is a possibility that the 'unusual' fatty acids and at least part of the phosphorus found in the enzyme-resistant sphingomyelin pool are due to contamination with lysophosphatidylcholine, although it seems unlikely that all the resistant pool of sphingomyelin could be explained in this way. However, there is an additional factor which could account for an apparently resistant pool of sphingomyelin in human erythrocytes. This stems from the observation that red cells treated with phospholipase C or sphingomyelinase C tend to invaginate and vesiculate internally without lysis [22,23] and this could lead to internalisation of some sphingomyelin which would thus not be available to an external enzyme. Although the morphology of hen erythrocytes is altered by treatment with these enzymes, it did not appear that they underwent endovesiculation perhaps because of constraints exercised by their complex cytoskeleton [5], and this could explain the more complete breakdown of plasma membrane sphingomyelin in hen cells treated with exogenous sphingomyelinase.

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