Transbilayer distribution of lipids in a population of sarcoplasmic-reticulum vesicles sealed with their cytoplasmic side outwards

Katherine A. MCGILL,* Jonathan P. BENNETT,† Gerald A. SMITH, Robert W. PLUMB and
Graham B. WARREN‡

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

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A population of sarcoplasmic-reticulum vesicles, all of which were sealed with their cytoplasmic side outwards, was obtained by density-gradient centrifugation after loading the vesicles with calcium oxalate. The calcium oxalate could subsequently be removed from the vesicles by the reverse action of the calcium-transport system. Measurements of the catalysed exchange of the phosphatidylcholine in the sarcoplasmic-reticulum cytoplasmic monolayer with an exogenous phosphatidylcholine pool suggested that phosphatidylcholine is symmetrically distributed across the sarcoplasmic-reticulum membrane. A similar result was obtained for phosphatidylethanolamine when sarcoplasmic-reticulum lipids were labelled with trinitrobenzenesulphonic acid. Further catalysed lipid-exchange reactions showed that the transverse movement of phosphatidylcholine across the membrane was exceedingly slow ($t_{\frac{1}{2}} > 15$ days).

The asymmetric distribution of lipids in the human erythrocyte membrane was first reported by Bretscher (1972) and it is now well established that quantitative lipid asymmetry is a feature of many plasma membranes (for review see Rothman & Lenard, 1977). It is less clear whether intracellular membranes also display transverse lipid asymmetry. Bretscher (1973) predicted that intracellular membranes would be found to possess an asymmetry that was apparently the reverse of that in the plasma membrane, but conforming to the rule that the cytoplasmic-facing monolayer would have a similar composition in each membrane, thus allowing conservation of transverse lipid asymmetry during the fusion of plasma and intracellular membranes. However, at present there is no general agreement as to the pattern of lipid asymmetry in intracellular membranes. For example, several studies have been carried out on rat liver microsomal fraction ('microsomes') with markedly conflicting results

(Higgins & Dawson, 1977; Nilsson & Dallner, 1977; Sundler *et al.*, 1977). None of these workers fulfilled the essential experimental criteria listed by Rothman & Lenard (1977) to establish lipid asymmetry. The membranes under study should be present as a pure preparation (no contaminating lipid from other membranes), and all the membranes should form closed vesicles with the same sidedness. It must be shown that the labelling reagent, under the conditions of the experiment, neither penetrates the membrane nor leads to lysis.

In the present paper we describe a study of the transmembrane distribution of lipids across an intracellular membrane, sarcoplasmic reticulum, in which we fulfil those criteria. Although sarcoplasmic reticulum can be isolated in a relatively pure state (Meissner & Fleischer, 1971) these preparations are known to be heterogeneous (Hasselbach & Makinose, 1963; Deamer & Baskin, 1969). Previous studies of lipid asymmetry in sarcoplasmic-reticulum were not carried out on homogeneous preparations, and the possibility that a proportion of the sarcoplasmic-reticulum vesicles might be unsealed or even have inside-out orientation was not taken into account.

To obtain right-side-out sealed sarcoplasmic-reticulum vesicles for a study of lipid asymmetry we have used the rationale that only such vesicles will be functional in the ATP-dependent accumulation of

* Present address: Unité 109 de Neurobiologie, Centre Paul Broca de l'INSERM, 2^{ter} rue d'Alésia, 75014 Paris, France.

† Present address: Department of Experimental Pathology, School of Medicine, University College, London W.C.1, U.K.

‡ Present address: European Molecular Biology Laboratory, Postfach 10.2209, D-6900 Heidelberg, Federal Republic of Germany.

Ca^{2+} . In the presence of potassium oxalate, the accumulated Ca^{2+} within right-side-out sealed sarcoplasmic-reticulum vesicles precipitates as calcium oxalate. The dense calcium oxalate-containing vesicles were then purified from leaky vesicles and inside-out vesicles by sucrose-density-gradient centrifugation. In these right-side-out sealed sarcoplasmic-reticulum vesicles, both phosphatidylcholine and phosphatidylethanolamine were found to be approximately symmetrically distributed between the two leaflets of the lipid bilayer.

Experimental

Materials

Pure synthetic 1,2-dioleoyl-*sn*-glycero-3-phosphocholine was prepared by the method of Robles & Van den Berg (1969). 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine was prepared by the method of Smith *et al.* (1978). Radiolabelled dioleoyl phosphatidylcholine tritiated in the choline head group was prepared by the method of Smith *et al.* (1978). Phosphatidylserine was obtained from Lipid Products, South Nutfield, Surrey, U.K. Tri[1- ^{14}C]oleoylglycerol was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Isolation of right-side-out sealed sarcoplasmic-reticulum vesicles

Rabbit sarcoplasmic reticulum was isolated by the method of Robinson *et al.* (1972) as modified by Warren *et al.* (1974). Sarcoplasmic-reticulum protein (30mg) was added to 30ml of a solution containing 50mM-MgATP $^{2-}$, 15mM-potassium oxalate and 10mM-histidine adjusted to pH 7.3 with HCl at room temperature. This mixture was gently stirred for 30min during which time 5.0ml of 40mM- CaCl_2 /80mM-MgATP $^{2-}$, adjusted to pH 6.7 with KOH, was added at a rate of 0.2ml/min. The suspension was then layered on gradients comprising 5.0ml of 40% (w/v) sucrose, 5.0ml of 60% (w/v) sucrose and 0.2ml of 80% (w/v) sucrose all in a solution containing 5.0mM-MgATP and 50mM-histidine/HCl, pH 7.3, and centrifuged at 63 000g for 60min at 20°C.

The band at the 60%/80% sucrose interface was resuspended in 68ml of a solution containing 20mM-glucose, 10mM-MgSO $_4$, 50mM-EGTA, 1.0mM-MgATP $^{2-}$, 3.6 units of hexokinase/ml, 0.125M-sucrose, 0.50M-KCl, 50mM-K $_2\text{HPO}_4$, adjusted to pH 8.0 with 50mM-KH $_2\text{PO}_4$, and incubated for 3h at 10°C. The sarcoplasmic reticulum was then sedimented by centrifugation at 70 000g for 60min at 40°C on 0.2ml of an 80% (w/v) sucrose solution. The pellet was resuspended in a total volume of 68ml of a solution containing 0.25M-sucrose, 1.0M-KCl, 50mM-K $_2\text{HPO}_4$ /KH $_2\text{PO}_4$, pH 8.0, and the centrifugation step was repeated.

The pelleted material was resuspended and layered on 10ml of a solution containing 60% (w/v) sucrose, 50mM-histidine/HCl, pH 7.3, and centrifuged at 63 000g for 60min at 4°C. The pellet was discarded and the supernatant which contained the sarcoplasmic reticulum, was dialysed into the required buffer solution.

Characterization of right-side-out sealed sarcoplasmic-reticulum vesicles

Samples for electron microscopy were dialysed in a buffer containing 10mM-K $_2\text{HPO}_4$ /KH $_2\text{PO}_4$, pH 8.0. After deposition on to carbon-blocked grids, samples were negatively stained with an aq. 2% (w/v) solution of phosphotungstic acid, pH 6.7, and examined at 40 000 times magnification in an AEI EM 801 electron microscope.

Polyacrylamide-gel electrophoresis was carried out by the method of Shapiro *et al.* (1967) adapted to give a final concentration of 5% acrylamide and 0.1% sodium dodecyl sulphate in the gels and 0.1% sodium dodecyl sulphate in the electrophoresis buffer. Samples were applied in a solution containing 10mM-Na $_2\text{HPO}_4$ adjusted to pH 7.2 with 10mM-NaH $_2\text{PO}_4$, 3% sodium dodecyl sulphate, 140mM-mercaptoethanol and 10% (v/v) glycerol. Electrophoresis was carried out with a constant current of 6mA/tube. Gels were fixed and then stained with Coomassie Blue.

Analysis of lipid head-group composition was by the method of Rouser *et al.* (1970); the lipid fatty-acid content was analysed by g.l.c. Protein was assayed by the biuret method (Warren *et al.*, 1974). Lipid phosphorus was measured by the method of Bartlett (1959).

Isolation of the phospholipid-exchange protein from beef heart

This was prepared essentially by the method of Johnson & Zilversmit (1975) with the omission of the Sephadex G-75 step. After ion-exchange chromatography on CM-52, the material was applied to a column of DE-52 cellulose equilibrated in 10mM-Tris/acetic acid, pH 6.0. The column was washed with 5 bed vol. of 10mM-Tris/acetic acid, pH 6.0, and then the phospholipid exchange activity was eluted as a sharp band with 150mM-Tris/acetic acid, pH 6.0. The active fractions were frozen in liquid N $_2$ and stored at -20°C.

The specific activities of our preparations were typically 250 units/mg of protein, using the assay of Johnson & Zilversmit (1975). These preparations showed two bands on sodium dodecyl sulphate/polyacrylamide gels. They did not contain any measurable proteolytic or phospholipase activity and did not catalyse the exchange of exogenous phospholipid with any sarcoplasmic-reticulum class other than phosphatidylcholine.

Preparation of unilamellar dioleoyl phosphatidylcholine vesicles

The method of Huang (1969) was used to prepare unilamellar [^3H]dioleoyl phosphatidylcholine (4×10^4 c.p.m./mg) vesicles that contained a trace of tri[1- ^{14}C]oleoylglycerol (2×10^4 c.p.m./mg of [^3H]dioleoyl phosphatidylcholine) to act as a fusion marker. Of the dioleoyl phosphatidylcholine in these vesicles 65% was found to be available for exchange with an excess of mitochondrial lipids when this was measured by the method of Johnson *et al.* (1975).

Measurement of the sarcoplasmic-reticulum exchangeable phosphatidylcholine pool by catalysed exchange with [^3H]dioleoyl phosphatidylcholine

Right-side-out sealed-sarcoplasmic-reticulum-vesicles (6.0 mg of protein) was incubated at 37°C with 3.75 mg of unilamellar [^3H]dioleoyl phosphatidylcholine/tri[1- ^{14}C]oleoylglycerol vesicles and the phosphatidylcholine-exchange protein in 1.5 ml of 0.25 M-sucrose / 2.0 mM-EDTA / 50 mM-Tris / HCl, pH 7.4. At the end of the incubation, samples were layered on discontinuous sucrose gradients at 37°C comprising 2.5 ml of 15% (w/v) sucrose and 0.2 ml of 50% (w/v) sucrose in 1.0 M-KCl / 50 mM- K_2HPO_4 / KH_2PO_4 , pH 8.0. They were centrifuged at 200 000 g for 90 min at 35°C . The total time of incubation was taken as the time from the start of the incubation to the start of the centrifugation. The band of material collecting at the 15%/50% interface was resuspended in 4 ml of a solution containing 1.0 M-KCl, 50 mM- K_2HPO_4 / KH_2PO_4 , pH 8.0, and centrifuged at 150 000 g for 1 h at 35°C again on a 0.2 ml pad of 50% sucrose. This washing step was repeated once more on the pelleted material, which was then resuspended in a final volume of 2.0 ml of 0.25 M-sucrose / 1.0 mM-EDTA / 50 mM-Tris / HCl, pH 7.4. Protein yields of approx. 85% were routinely obtained.

After the above washing procedure, less than 5% of the [^3H]dioleoyl phosphatidylcholine transferred to right-side-out sealed sarcoplasmic-reticulum vesicles was due to non-specific association of lipid vesicles with right-side-out sealed sarcoplasmic-reticulum vesicles, as determined from the presence of tri[1- ^{14}C]oleoylglycerol (which is not transferred by the phospholipid-exchange protein). A correction was made for this non-specific lipid association in calculating the exchangeable phosphatidylcholine.

The amount of exchanged lipid was calculated by both radioactive counting and g.l.c. analysis. The size of the exchangeable dioleoyl phosphatidylcholine ^3H pool was calculated and from this the proportion of the phosphatidylcholine in the sarcoplasmic-reticulum membrane that participated in the exchange reaction was estimated.

Measurement of the rate of transbilayer movement of phosphatidylcholine in right-side-out sealed sarcoplasmic-reticulum vesicles

Five tubes were incubated at 37°C for up to 96 h. Each contained 2.0 mg of right-side-out-sealed-sarcoplasmic-reticulum-vesicle protein, 1.25 mg of [^3H]dioleoyl phosphatidylcholine/tri[1- ^{14}C]oleoylglycerol vesicles and 350 units of exchange protein in 3.0 ml of 0.25 M-sucrose / 1.0 mM-EDTA / 50 mM-Tris / HCl, pH 7.4, plus 0.2% (w/v) NaN_3 . At 24 h intervals, one sample was withdrawn and subjected to the standard washing procedure. The amount of exchange was determined as described above.

As a control, 1.5 mg portions of right-side-out-sealed-sarcoplasmic-reticulum-vesicle protein were incubated at 37°C in 0.5 ml of a buffer containing 0.25 M-sucrose, 1.0 mM-EDTA, 50 mM-Tris / HCl, pH 7.4, plus 0.2% (w/v) NaN_3 . Exchange protein and sonicated [^3H]dioleoyl phosphatidylcholine / tri[1- ^{14}C]oleoylglycerol vesicles were incubated separately at 37°C . At 24 h intervals for 4 days, 350 units of exchange protein (1.0 mg) and 0.94 mg of the [^3H]dioleoyl phosphatidylcholine vesicles were withdrawn and added to 4.0 mg of right-side-out sealed sarcoplasmic-reticulum vesicles. After allowing lipid exchange to reach equilibrium (4 h) the samples were subjected to the standard washing procedure and the exchangeable phosphatidylcholine pool was measured as described previously.

Trinitrobenzenesulphonate labelling of phosphatidylethanolamine and phosphatidylserine in right-side-out-sealed-sarcoplasmic-reticulum vesicles

This was carried out essentially by the method of Rothman & Kennedy (1977). Right-side-out-sealed-sarcoplasmic-reticulum-vesicle protein (11 mg in the case of phosphatidylethanolamine labelling and 40 mg of protein for phosphatidylserine labelling) was incubated with 3.0 mM-trinitrobenzenesulphonate in a final volume of 8.0 ml for phosphatidylethanolamine labelling and 48 ml for phosphatidylserine labelling in a solution of 50 mM- K_2HPO_4 / KH_2PO_4 , pH 8.0. Both components of the reaction mixture were equilibrated at the reaction temperature before mixing. In the case of phosphatidylethanolamine labelling 1.32 ml samples were subsequently withdrawn into microfuge tubes containing 0.2 ml samples of 30% (w/v) trichloroacetic acid on ice and 12 μl of bovine serum albumin (10 mg/ml) was added to each to quench the reaction. In the case of phosphatidylserine labelling 5.3 ml samples were withdrawn into 1.5 ml samples of 30% (w/v) trichloroacetic acid on ice and 100 μl of bovine serum albumin (10 mg/ml) was added.

The withdrawn samples were immediately vortexed and stored on ice for at least 15 min. They were then centrifuged at 15 000 g for 3 min, the

pellets were washed with water and centrifuged down again. Lipid was extracted from each sample by the method of Folch *et al.* (1957). The lipids were re-dissolved in 2.0 ml of chloroform/methanol/formic acid/water (13:5:1:1, by vol.) and applied to silica-gel-coated t.l.c. plates (Merck). The plates were developed in chloroform/methanol/water/conc. NH_3 (65:30:3:1, by vol.). The trinitrophenyl phosphatidylethanolamine and trinitrophenyl phosphatidylserine bands were scraped from the plates and the lipid derivatives were eluted in two 1.0 ml volumes of chloroform/methanol/water/formic acid (13:5:1:1, by vol.). The samples were centrifuged (15000 *g* for 10 min) to precipitate the silica gel and the absorbance of the supernatant at 337 nm was measured against a blank prepared by taking an equal volume of clean silica gel through the same elution procedure. The amount of trinitrophenyl phosphatidylethanolamine formed was read from a standard curve prepared by reacting to completion known amounts of synthetic dioleoyl phosphatidylethanolamine with trinitrobenzenesulphonate in chloroform/methanol/water (12:6:1, by vol.) eluting the trinitrophenyl phosphatidylethanolamine and reading the absorbance at 337 nm as described above.

Results

Isolation and characterization of right-side-out sealed sarcoplasmic-reticulum vesicles

As shown in Table 1, 63% of the sarcoplasmic reticulum was isolated from the 60%/80% sucrose interface when subjected to density-gradient centrifugation after loading with calcium oxalate and must

therefore be sealed and right-side out. In the absence of Ca^{2+} less than 5% of the sarcoplasmic reticulum was recovered from this interface.

To remove the precipitated calcium oxalate from within these vesicles we exploited the reversibility of the Ca^{2+} pump (see Hasselbach, 1978). Sarcoplasmic reticulum loaded with calcium oxalate was incubated in the presence of glucose, hexokinase and ATP (as an ADP-generating system) and EGTA. By using $^{45}\text{Ca}^{2+}$ it was found that 86% of accumulated Ca^{2+} could be removed from the vesicles in 3 h at 10°C. If this material was subsequently re-loaded with calcium oxalate and it was again subjected to density-gradient centrifugation all of it (>97%) was recovered from the 60%/80% sucrose interface. This material therefore consists of a functionally homogeneous preparation of right-side-out sealed sarcoplasmic-reticulum vesicles.

Analysis of protein content by gel electrophoresis (Fig. 1) shows that isolation of right-side-out sealed sarcoplasmic-reticulum vesicles leads to the purification of a polypeptide of apparent mol.wt. 115 000, which has been identified as the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase (the Ca^{2+} -pump protein). The lipid headgroup composition of right-side-out sealed sarcoplasmic-reticulum vesicles does not differ significantly from that of unfractionated sarcoplasmic reticulum (Table 2). The fatty acid composition was similar to that previously reported (Warren *et al.*, 1974) and did not differ significantly between

Table 1. *The recovery of Ca^{2+} -loaded vesicles from discontinuous sucrose gradients*

The preparation of right-side-out sealed sarcoplasmic-reticulum vesicles described in the Experimental section was carried out in the presence or absence of Ca^{2+} in the loading solution. After centrifugation on the discontinuous sucrose density gradients, the recovery of sarcoplasmic reticulum was determined by assaying for protein.

Position in gradient	Sarcoplasmic-reticulum vesicles recovered (%)	
	Ca^{2+} added to 'loading' solution	Ca^{2+} omitted
Supernatant/40% sucrose interface	25	84
40%/60% sucrose interface	10	10
60%/80% sucrose interface	63	< 5
Total	98	> 94

Fig. 1. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of (a) 100 µg of sarcoplasmic-reticulum protein and (b) 100 µg of right-side-out sealed sarcoplasmic-reticulum vesicular protein*
See the Experimental section for details.

Table 2. *The headgroup composition of sarcoplasmic-reticulum and right-side-out sealed sarcoplasmic-reticulum vesicles*

See the Experimental section for details. Results are means for three determinations.

Phospholipid class	Right-side-out sealed sarcoplasmic-reticulum vesicles content (%)	Sarcoplasmic reticulum content (%)
Phosphatidylcholine	72	73
Phosphatidylethanolamine	11.6	11.3
Phosphatidylinositol	7.6	7.4
Phosphatidylserine	4.2	3.9
Sphingomyelin	4.3	4.4
Cardiolipin	<1	<1
Neutral lipids	<2	<2
Phosphatidic acid	<1	<1

right-side-out sealed sarcoplasmic-reticulum vesicles and the batch of sarcoplasmic reticulum from which they were prepared. From electron micrographs (e.g. Plate 1a) the average size of right-side-out sealed sarcoplasmic-reticulum vesicles was calculated, and was found to be sufficiently large for us to ignore the contribution of curvature on the number of lipid molecules in each monolayer.

Transbilayer distribution of phosphatidylcholine measured by using a phospholipid-exchange protein

Fig. 2 shows the time course of catalysed exchange between right-side-out sealed sarcoplasmic-reticulum vesicles and filtered sonicated dioleoyl phosphatidylcholine vesicles in which 65% of the lipid was found to be available for exchange with an excess of mitochondrial lipid. Although the initial rate of exchange between right-side-out sealed sarcoplasmic-reticulum vesicles and the dioleoyl phosphatidylcholine vesicles depended on the amount of exchange protein present all of the time courses reach equilibrium at a level of exchange corresponding to an exchangeable sarcoplasmic-reticulum pool of size equal to 50% of the membrane phosphatidylcholine. That the vesicles remained intact throughout the exchange reaction was demonstrated by their capacity for ATP-dependent Ca^{2+} uptake.

The rate of transbilayer movement (flip-flop) of phosphatidylcholine

Phospholipid-exchange proteins also provide a non-disruptive method for the measurement of the rate of transbilayer movement of lipid in membranes (flip-flop). Since only 50% of the phosphatidylcholine is accessible for exchange with exogenous lipid over a 3 h period, it is clear that flip-flop

Fig. 2. *Time course of exchange between right-side-out sealed sarcoplasmic-reticulum vesicles and unilamellar [^3H]dioleoyl phosphatidylcholine vesicles*

See the Experimental section for details. Exchange protein was present as follows: ○, 100 units; □, 300 units; ■, 800 units; ●, 1000 units.

must be occurring relatively slowly. By measuring changes in the amount of exchange over a period of several days it should be possible to estimate the rate of flip-flop. If subsequent to the equilibration of the sarcoplasmic reticulum and the exogenous phosphatidylcholine pools exchange of phosphatidylcholine takes place between the two sarcoplasmic-reticulum monolayers then re-equilibration will occur between the phosphatidylcholine in the sarcoplasmic-reticulum outer monolayer and the exogenous phosphatidylcholine pool. This will be detected as an increase in the [^3H]dioleoyl phosphatidylcholine entering the sarcoplasmic-reticulum membrane.

As shown in Fig. 3, no detectable increase in the amount of exchange occurred when samples were incubated for up to 96 h. From the experimental errors involved we can place a lower limit of $t_{1/2} > 15$ days on the rate of phosphatidylcholine flip-flop in the sarcoplasmic-reticulum membrane.

To exclude the possibility that the failure to see any increase in the exchangeable pool was due not to the lack of flip-flop of phosphatidylcholine but to a loss of the phospholipid-exchange activity during these extended incubations, the different components (right-side-out sealed sarcoplasmic-reticulum vesicles, dioleoyl phosphatidylcholine vesicles and phospholipid-exchange protein) were separately incubated at 37°C for the same periods. When they were subsequently combined, a constant equilibrium value of phosphatidylcholine exchange (during a 4 h incubation) was observed (see the legend to

Fig. 3. Measurement of the rate of flip-flop of phosphatidylcholine by the exchange of [^3H]dioleoyl phosphatidylcholine with right-side-out sealed sarcoplasmic-reticulum vesicles over 96 h

See the Experimental section for details. In control experiments the different components of the lipid exchange system were incubated separately for 0, 24, 48, 72 and 96 h before mixing. The proportions of total phosphatidylcholine found to be available for exchange during a subsequent 4 h incubation were 53%, 49%, 51%, 51% and 51% respectively.

Fig. 3). This indicates that during these experiments both populations of vesicles remained intact and the exchange protein retained its catalytic activity.

Transbilayer distribution of phosphatidylethanolamine measured by using trinitrobenzenesulphonate labelling

Since no exchange protein specific for phosphatidylethanolamine or phosphatidylserine has yet been isolated we investigated phosphatidylethanolamine and phosphatidylserine distribution in the sarcoplasmic-reticulum membrane with trinitrobenzenesulphonate, a reagent that reacts specifically with primary amino groups (Okuyama & Satake, 1960). Rothman & Kennedy (1977) demonstrated that at 3°C trinitrobenzenesulphonate reacted with lipids in the outer-membrane monolayer only, in

Fig. 4. Time course of the reaction of the phosphatidylethanolamine in right-side-out sealed sarcoplasmic-reticulum vesicles with trinitrobenzenesulphonate. See the Experimental section for details. Reaction temperature was as follows: ○, 3°C; ●, 37°C. These results are means for duplicate experiments.

their experiments with *Bacillus megaterium*. At higher temperatures it slowly permeated the membrane to react with all of the phosphatidylethanolamine present.

In these experiments, right-side-out-sealed sarcoplasmic-reticulum-vesicle lipids were labelled with trinitrobenzenesulphonate (3.0 mM) at 3°C and 37°C. The trinitrobenzenesulphonate-labelled derivatives of phosphatidylethanolamine and phosphatidylserine, trinitrophenyl phosphatidylethanolamine and trinitrophenyl phosphatidylserine, were then isolated by t.l.c. The time course of the reaction of phosphatidylethanolamine is shown in Fig. 4. Although the initial rate of reaction was faster at 37°C than 3°C, in both cases a steady equilibrium level of labelling was observed. At 37°C this corresponded to the reaction of all of the membrane phosphatidylethanolamine; at 3°C, $50 \pm 4\%$ of the phosphatidylethanolamine in right-side-out sealed sarcoplasmic-reticulum vesicles reacted. The stable equilibrium attained at 3°C strongly suggests that trinitrobenzenesulphonate fails to penetrate the membrane at this temperature and the reacting pool of phosphatidylethanolamine is that in the outer-membrane monolayer.

EXPLANATION OF PLATE 1

Electron micrograph of right-side-out sealed sarcoplasmic reticulum before (a) and after (b) equilibration with trinitrobenzenesulphonate (30 mM) for 2 h at 3°C

Samples were negatively stained using phosphotungstic acid, as described in the Experimental section. The bars represent 1.0 μm .

Comparison of the morphology of right-side-out sealed sarcoplasmic-reticulum vesicles before and after (Plate 1) 2h incubation at 3°C with trinitrobenzenesulphonate (30mM) suggests that there is no disruption of the vesicles under these conditions.

These results strongly suggest that phosphatidylethanolamine is symmetrically distributed across the sarcoplasmic-reticulum bilayer. In addition very rapid exchange of phosphatidylethanolamine between the two membrane monolayers was shown not to take place. Since steady equilibrium values of labelling were observed over periods of 90min at 3°C no measurable flip-flop of phosphatidylethanolamine took place during this period.

The phosphatidylserine in the sarcoplasmic-reticulum membrane was labelled in the same way that phosphatidylethanolamine was labelled, but since phosphatidylserine comprises a smaller proportion of sarcoplasmic-reticulum phospholipid larger amounts of right-side-out sealed sarcoplasmic-reticulum vesicles were used. The labelling observed after equilibration at 37°C represented the reaction of only 5% of the total phosphatidylserine. When commercially obtained phosphatidylserine was reacted with trinitrobenzenesulphonate in chloroform/methanol/water containing 50mM-K₂HPO₄/KH₂PO₄, pH8.0, only 30% of it reacted; under these conditions phosphatidylethanolamine reacted to completion. This incomplete labelling means that we are unable to assign a transbilayer distribution for phosphatidylserine. Bishop *et al.* (1979) have also reported incomplete labelling of membrane lipids with trinitrobenzenesulphonate.

Discussion

The principal evidence for lipid asymmetry derives from the erythrocyte membrane. The erythrocyte has proved to be extremely convenient for measurements of transbilayer distribution of lipids. It only possesses a single membrane and inevitably that membrane is in intact cells sealed and correctly oriented. The conclusions from a great many studies are that the inner (cytoplasmic) leaflet of the bilayer contains most of the amino phospholipids (phosphatidylethanolamine and phosphatidylserine) and the outer leaflet contains most of the choline-containing phospholipids (phosphatidylcholine and sphingomyelin) (Bretscher, 1972; Rothman *et al.*, 1976; Chap *et al.*, 1977; Rothman & Kennedy, 1977; Sandra & Pagano, 1978). Other eukaryotic plasma membranes seem to have a broadly similar transbilayer distribution of lipids.

To identify lipid asymmetry in an intracellular membrane there are a number of problems. The methods of preparing intracellular membranes in-

volve homogenization of the intact cells followed by separation of fractions by centrifugation techniques. During these processes there is the possibility that a membrane that has been disrupted does not completely reseal, or it reseals in part with the opposite orientation. It is also probable that the membrane fraction chosen for study is contaminated by membrane deriving from other organelles.

In an attempt to fulfil the criteria (outlined by Rothman & Lenard, 1977) for a meaningful study of lipid asymmetry in sarcoplasmic reticulum, we have prepared a fraction that has the functional property of accumulating and retaining calcium oxalate. This fraction (right-side-out sealed sarcoplasmic-reticulum vesicles) is by definition sealed to Ca²⁺ ions, and it is probable that it is similarly sealed to the probes of phospholipid asymmetry, phospholipid-exchange protein and trinitrobenzenesulphonate. It must also have a single orientation, such that the ATP hydrolysis site of the Ca²⁺ pump is at the outer surface.

Although right-side-out sealed sarcoplasmic-reticulum vesicles have been shown to be functionally asymmetric it is theoretically possible that such vesicles might contain protein and lipid scrambled during the muscle homogenization procedure used in the initial isolation of sarcoplasmic reticulum. Due to the absence of ATP inside the vesicles the protein molecules pointing the wrong way would not function and would therefore not be detected. However, freeze-fracture replicas show that all intramembranous particles in isolated sarcoplasmic reticulum membranes are found on the cytoplasmic monolayer (Scales & Inesi, 1976). In addition, it is known that lipid asymmetry is retained when erythrocyte membranes are broken and re-sealed during 'ghost' formation (Bloj & Zilversmit, 1976).

Although right-side-out sealed sarcoplasmic-reticulum vesicles have a similar lipid composition to unfractionated sarcoplasmic reticulum the protein content indicates a purification with respect to the Ca²⁺-pump protein (Plate 1). The other main protein components of sarcoplasmic reticulum are Ca²⁺-binding proteins, and it has been shown that these are localized in the terminal triads (Meissner, 1975; Lau *et al.*, 1977). Thus our preparation derives largely from a single source, the longitudinal reticulum region of sarcoplasmic reticulum.

Several groups have investigated the transbilayer distribution of phosphatidylethanolamine in sarcoplasmic reticulum (Hasselbach & Migala, 1975; Hidalgo & Ikemoto, 1977; Vale, 1977), but in every case unfractionated sarcoplasmic reticulum was used. The finding that more than 50% of the phosphatidylethanolamine was apparently in the outer half of the bilayer may therefore be due to the presence in those preparations of unsealed vesicles where both halves of the bilayer were accessible to

the probe used. The data in Table 1 suggest that up to 35% of unfractionated sarcoplasmic reticulum may be unsealed. Sarzala & Michalak (1978) used sarcoplasmic reticulum that had been separated into two fractions, but since considerable doubt has now been put on the original identification of those fractions (Michalak *et al.*, 1980) it is impossible to interpret their results.

The data in the present paper suggest that there is no significant asymmetry in right-side-out sealed sarcoplasmic-reticulum vesicles in the distribution of either phosphatidylcholine or phosphatidylethanolamine. It therefore seems that the phospholipid asymmetry that has been generally observed for plasma membranes may not be a feature of intracellular membranes.

The lack of phospholipid asymmetry in sarcoplasmic reticulum may be associated with its specialized nature. Sarcoplasmic reticulum contains almost entirely a single protein (the Ca^{2+} pump), so that there are no differential enzymic functions associated with the two halves of the bilayer. Although the Ca^{2+} -pump protein is known to interact with the membrane phospholipid through its 'lipid annulus' (Warren *et al.*, 1975; Hesketh *et al.*, 1976), this annulus appears to be symmetrical across the bilayer (Bennett *et al.*, 1978).

The intracellular site of phospholipid synthesis *de novo* is at the cytoplasmic surface of the endoplasmic reticulum (Vance *et al.*, 1977), and the lipid content of other cellular membranes ultimately derives from the endoplasmic reticulum (Jelsema & Morré, 1978). Lipid newly inserted into the outer half of the endoplasmic-reticulum bilayer must be translocated in part to the inner half, otherwise distortion of the membrane would result. In agreement with this, a relatively fast rate of flip-flop has been measured in endoplasmic reticulum (Zilvermit & Hughes, 1977), much faster than the rate of flip-flop we have observed in right-side-out sealed sarcoplasmic-reticulum vesicles. The implication is that this flip-flop is an endoplasmic-reticulum function, presumably protein-mediated.

The newly formed bilayer at the endoplasmic reticulum can be utilized to form either (in muscle) sarcoplasmic reticulum, which shows no phospholipid asymmetry, or plasma membrane, which does show asymmetry. It therefore seems likely that phospholipid asymmetry is necessary for plasma-membrane function and is imposed on the membrane during its biogenesis, presumably by the action of an enzyme. A function for phospholipid asymmetry has never been demonstrated; however, Zwaal *et al.* (1977) have pointed out that the normal inner-leaflet location of phosphatidylserine means that its accessibility to blood cells might act as a physiological means of identifying damaged tissue.

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