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Normal and Abnormal Red Cell Membranes 1–23

# Topology of Amino-Phospholipids in the Red Cell Membrane

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The red cell membrane has an asymmetric arrangement of phospholipids. The amino-phospholipids are localized primarily on the inner surface of the membrane and the choline phospholipids are localized to a large extent on the outer surface of the membrane. Evidence is presented based on the use of covalent chemical probes in sequence that the red cell membrane contains heterogeneous domains of PE and PS and that the transport systems for Pi and $K^+$ are asymmetrically arranged. Certain amino groups of PE, PS, and/or protein localized on the outer membrane surface are involved in Pi transport and certain amino groups of PE, PS, and/or protein localized on the inner surface of the membrane are involved in $K^+$ transport.

Cross-linking studies with DFDNB show that the cross-linked PE-PE molecules are rich in plasmalogens. This suggests that clusters of plasmalogen forms of PE occur in the membrane. Both PE and PS are cross-linked to membrane protein. These PE and PS molecules contain 24–28% 16:0 and 18:0 fatty acids and 12% fatty aldehydes. PE and PS molecules are cross-linked to a spectrin-rich fraction. It is proposed that the binding of spectrin to membrane PE and PS may help anchor spectrin to the inner surface of the membrane and regulate shape changes in the cell.

$K^+$-valinomycin forms a complex with TNBS and converts it from a nonpenetrating probe to a penetrating probe. Valinomycin enhances $K^+$ leak and Pi leak in the red cells. SITS inhibits completely the valinomycin-induced Pi leak and inhibits partially the valinomycin induced $K^+$ leak. Valinomycin and IAA have additive effects on Pi leak. Ouabin has no effect on basal or valinomycin-induced Pi leak. These data suggest that Pi leak and $K^+$ leak occur by separate transport systems.

In summary, the amino-phospholipids in the red cell membrane are asymmetrically arranged; some occur in clusters and some are closely associated with membrane proteins. Amino-phospholipids also are believed to bind spectrin to the inner surface of the membrane and also may play a role in cation and anion leak.

**Key words: amino-phospholipids, chemical probes, red cell membrane, valinomycin, ion transport, membrane topology**

Abbreviations: TNBS, 2,4,6-trinitrobenzenesulfonic acid; FDNB, 1-fluoro-2.4-dinitrobenzene; MA, methylacetimidate; PE, phosphatidylethanolamine; PS, phosphatidylserine; DFDNB, 1,5-difluoro-2.4-dinitrobenzene; Pi, inorganic phosphate; TLC, thin layer chromatography; IAA, iodoacetate; BHT, butylated hydroxytoluene; DMA, dimethylacetal; DEGS, diethyleneglycolsuccinate; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid.

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Phospholipids are integral components of cell membranes, where it is generally agreed they are arranged in large part as a bilayer [1, 2]. The more detailed arrangement of phospholipids in cell membranes, such as asymmetry, clusters, and association with specific membrane proteins are relatively new ideas that are being explored. In previous studies evidence was provided for the asymmetric arrangement of amino-phospholipids in the red cell membrane using nonpenetrating chemical probes [3–7]. This work was supported by others using phospholipases [8] or phospholipid exchange proteins [9, 10]. We also provided data, using cross-linking probes, which indicated that PE and PS occurred as nonrandom clusters in the red cell membrane [11, 12]. Occurrence of lipid clusters in artificial membranes recently has been reported [13, 14].

In this paper we consider in more detail the topology of PE and PS in the red cell membrane. In particular it was our aim to examine the molecular species of PE which are localized on the outer surface of the membrane and compare it with the PE molecules localized on the inner surface. We also examined the molecular species of those PE molecules which are cross-linked to themselves by DFDNB and those PE and/or PS molecules which are cross-linked to membrane proteins.

We have found that valinomycin forms a complex with TNBS in the presence of $K^+$, $Rb^+$, or $Cs^+$ and that the complex permeates the red cell membrane, thereby converting TNBS from a nonpenetrating probe to a penetrating probe [15]. This allows one to use the same probe to examine the amino-phospholipids on both the outer and inner membrane surface and avoids comparison of data of whole cells to ghosts.

The effect of chemical probes and valinomycin on the labeling of PE and PS is compared to the effect of these probes on anion and cation leak in the red cell. Evidence is presented for an asymmetric arrangement of these transport systems in the red cell. This was established by the sequential use of penetrating and nonpenetrating probes. Evidence is also presented for different domains of PE and PS in the red cell membrane.

## MATERIALS AND METHODS

Human red cells were obtained from the Red Cross Blood Bank and stored at 4°C until used. Cells were used within 1–2 weeks. The extraction of lipids and the TLC analysis of the dinitrophenyl, trinitrophenyl derivatives, and amidine derivatives of PE and PS were carried out as described previously [5, 6, 7, 11, 16]. Inorganic phosphate was measured as previously reported [6]. $K^+$ and $Na^+$ were measured by flame photometry using an Instruments Lab, Inc. Model 143 flame photometer. Cell counting was performed by means of a haemocytometer. Hematocrits were done on a MSE Microhaemocrit reader. Protein was assayed by the Lowry procedure [17].

### Cross-Linking of Red Cell Lipids and Proteins at 0°C and 37°C With DFDNB

Human red cells were centrifuged for 10 min at 2,000 rpm and the plasma and buffy coat removed. The packed cells were washed twice with isotonic saline. Into each of nine tubes was pipetted 1 ml packed cells. Ghosts were prepared by a modification of the method of Dodge, Mitchell, and Hanahan [18] by lysis of cells at 21°C in 20 volumes of 10 mM Tris buffer (pH 7.4) containing 1 mM EDTA. The ghosts were spun down at 40,000g for 15 min and washed twice with lysing buffer. The ghosts were divided into three sets of three tubes. The first set was incubated at 37°C for 4 h in 20 ml 120 mM NaCl–40 mM $NaHCO_3$ buffer (pH 8.5) containing 100 $\mu$M DFDNB. The second set was

incubated at 0°C for 16 h with the same buffer containing 100 μM DFDNB. The third set was incubated 16 h at 0°C in buffer not containing DFDNB. The reaction was stopped by centrifugation for 15 min at 40,000g. The ghosts were washed with isotonic saline. The lipids were extracted with chloroform-methanol containing 0.01% BHT by the method of Folch et al [19] and dried under nitrogen. The lipids were analyzed by TLC. The plates (Merck-Darmstadt silica gel 60, 0.25 mm) were developed in chloroform:methanol: acetic acid:water (65:25:2:4). FDNP-PE and PE-DNP-PE were scraped from the plates from DFDNB-treated red cells. PE was scraped from the plates from control cells. The PE bands were treated for 1 h at 80°C in 3 ml anhydrous methanol containing 1% conc. HCl. The samples were cooled and 3 ml of water were added, followed by 6 ml hexane containing 0.01% in BHT. The hexane layers were removed and washed with 0.1% $Na_2CO_3$. The washed hexane layers were dried with anhydrous $Na_4SO_4$. The resulting extracts containing fatty acid methyl esters and dimethylacetals of fatty aldehydes were stored under nitrogen until read for analysis.

The analysis of the fatty acid methyl esters and dimethylacetals of fatty aldehydes was carried out on a Hewlett-Packard 5830A gas chromatograph equipped with a 18850 computer terminal. A 10% DEGS on an acid-washed, dimethylchlorosilane-treated chromosorb W column (6 ft, glass, 2 mm ID) was used at 190°C. The flow rate of helium was 30 ml/min.

### Reaction of Red Cells With TNBS

Human red blood cells were washed three times with isotonic NaCl. In each of three tubes were added 1.5 ml packed cells. The cells were reacted with 2 mM TNBS in 60 ml 40 mM $NaHCO_3$–120 mM NaCl buffer (pH 8.5) for 1 h. The cells were centrifuged and washed once with saline; ghosts were prepared and lipids extracted as described previously. The lipid extracts were dried under nitrogen, applied to TLC plates, and developed in chloroform:methanol:acetic acid:water (65:25:2:4). Unreacted PE (visualized by spraying with 5% rhodamine 6G in methanol) and TNP-PE were scraped and reacted with methanol-HCl as described above.

### Spectrin Preparation From Control and FDNB- and DFDNB-Treated Red Cells

Packed red cells (10 ml) were reacted with 0.5 mM FDNB or DFDNB in 190 ml of 120 mM NaCl–40 mM $NaHCO_3$ (pH 8.5) for 0.5 h at 23°C. Control cells were incubated in buffer alone. The cells were washed with the same buffer and lysed in 20 volumes of first 30mM $NaHCO_3$, then 15 mM $NaHCO_3$ pH 7.4. The spectrin plus soluble proteins were obtained by dialysis over night at 4°C against 0.2 mM EDTA (pH 7.4) [20]. The contents of the dialysis bags were centrifuged at 30,000 rpm for 30 min at 5°C. The supernatants contain the total soluble ghost proteins. Spectrin was isolated by precipitation at pH 5.3. Aliquots of the protein fraction were assayed for total protein and analyzed by SDS gel electrophoresis as described previously [21]. Aliquots also were reacted with antispectrin antibody. Antiserum to spectrin was supplied by Dr F. H. Kirkpatrick, University of Rochester (New York). Rabbit antiserum was prepared by subcutaneous injection of electrophoretically pure spectrin in Freund's adjuvant. The spectrin (bands 1 and 2) was purified by preparative SDS gel electrophoresis in an apparatus described by Ryan et al [22]. The antiserum had a single precipitin line by Orchterlony double diffusion. Cross-immunoelectrophoresis indicated that the antiserum was directed mainly against band 1 and had a lesser reaction against band 2.

## RESULTS

The asymmetric arrangement of PE and PS in a variety of cell membranes as determined in different laboratories by chemical probes, phospholipases, or phospholipid exchange proteins is summarized in Table I. Several membranes have an asymmetric arrangement of PE and PS. In most cases these amino-phospholipids face the interior of the cell. There are some discrepancies in the findings from different laboratories indicating that the type of probe or experimental conditions used may lead to different results.

In the present work it was our aim to explore the topology of amino-phospholipids in the erythrocyte membrane in greater detail. In order to see whether the small number of PE molecules (5–10% of the total PE) on the outer surface of the red cell were different from those on the inner surface, the fatty acids and aldehydes of the TNP-PE and of the unreacted PE were analyzed. Since TNBS is not permeable under our conditions, the TNP-PE molecules are localized on the outer surface, whereas the unreacted PE molecules (these PE molecules in part are buried within the membrane) are localized on the inner surface of the membrane. The results in Table II show that the fatty acid and fatty aldehyde patterns are very similar. Therefore there appears to be no significant difference in the molecular species of the small number of PE molecules which are localized on the outer membrane surface compared to the bulk of the PE molecules localized on the inner membrane surface or buried within the membrane.

We next wished to see if the PE molecules which are cross-linked to themselves by DFDNB differed from the PE molecules which were not cross-linked. We also compared

**TABLE I. Asymmetry of Amino-Phospholipids in Cell Membranes**

| | % on outer surface | |
|---|---|---|
| Membrane | PE | PS |
| Red cell[a] | 2–20 | 0–2 |
| Inner mitochondria[a] | 30–70 | – |
| Retinal rod disk[f] | 60 | 25 |
| Yeast[a] | 20 | 0 |
| E coli[a] | 20 | – |
| Platelet[b] | 10–20 | 0–2 |
| Endoplasmic ret. | 90[c](50)[e] | 85[c](50)[e] |
| Golgi | 90[c](50)[e] | 85[c](50)[e] |
| Lysosomal[c] | 80 | – |
| Nuclear membrane[c] | 90 | 83 |
| Inner mitochondria[c] | 90 | – |
| Influenza virus[d] | 50 | 3 |
| PM2 bacteriophage[d] | ∿20 | – |
| B megaterium[d] | 31–35 | – |

[a] Data from References 5, 7, 25, 28 using chemical probes and References 8, 9, 24 using protein probes.

[b] Data from References 26, 27.

[c] Data from Reference 29.

[d] Data from References 37, 41.

[e] Data from Reference 40.

[f] Crain and Marinetti, unpublished data.

**TABLE II. Fatty Acid and Fatty Aldehyde Composition of PE on the External and Internal Surfaces of the Red Cell Membrane***

| | External surface | Internal surface |
|---|---|---|
| | TNP-PE | Unreacted PE |
| 16:0 DMA | 15 ± 4 | 10 ± 3 |
| 16:0 | 11 ± 3 | 13 ± 3 |
| 18:0 DMA | 7 ± 1 | 12 ± 2 |
| 18:0 | 6 ± 1 | 8 ± 1 |
| 18:1 DMA | 2 ± 0.3 | 4 ± 1 |
| 18:1 | 13 ± 1 | 12 ± 2 |
| 18:2 | 4 ± 1 | 3 ± 0.4 |
| 20:4 | 23 ± 4 | 18 ± 4 |
| 22:4 | 8 ± 1 | 7 ± 1 |
| 22:6 | 6 ± 1 | 4 ± 1 |
| % Saturated fatty acids (16:0 + 18:0) | 17 ± 3 | 21 ± 3 |
| % Fatty aldehydes (16:0, 18:0, 18:1) | 24 ± 4 | 26 ± 4 |
| % Total saturation[a] | 39 ± 5 | 43 ± 5 |

* Values (mean ± SD) represent the area percentage of each fatty acid or fatty aldehyde relative to the total area of all the peaks obtained by gas chromatography. Details are given in the text.

[a] Percentage total saturation is the percentage of 16:0 + 18:0 fatty acids plus 16:0 + 18:0 fatty aldehydes. DMA indicates the dimethylacetal derivatives of the respective fatty aldehydes.

the fatty acid and fatty aldehydes of the PE molecules cross-linked to themselves with the PE and PS molecules which are cross-linked to membrane protein. The results in Table III show that the 30% of total PE molecules which become cross-linked to themselves have a much higher content of fatty aldehydes, a higher total saturation, and a lower content of 16:0 and 18:0 fatty acids than the non-cross-linked FDNP-PE or the total PE. This indicates that the plasmalogen PE molecules exist as clusters, and in particular, the clusters are favored at 37° compared to 0°C.

It is also noteworthy that with increasing temperature, the amino-phospholipids (PE + PS) which become cross-linked to protein contain a somewhat higher content of saturated fatty acids. Since PE contains a very high content of plasmalogen (about 50%) and PS has very little plasmalogen, we can estimate the percent of PE and PS cross-linked to protein. These calculations show that 30–50% of the cross-linked phospholipids is PE. This varies with different batches of red cells.

### Use of Chemical Probes in Sequence – Evidence for Heterogeneous Domains of PE and PS in the Membrane

Red cells were reacted with TNBS and MA singly and in sequence. The results in Table IV show that under these conditions of excess probe used singly and where available sites are saturated, only 10.7% of the total PE reacts with TNBS compared to the 84% which reacts with MA. More significant, however, is the observation that about 1% of the PE molecules are accessible to TNBS but not to MA. Therefore about 10% of the

**TABLE III. Nature of Fatty Acids and Aldehydes of Cross-Linked PE to PE and of Amino-Phospholipids Cross-Linked to Proteins in the Red Cell Membrane***

| | PE Cross-linked to PE[b] | | FDNP-PE | | Phospholipid Cross-linked to Protein[a] | | Control | Control |
|---|---|---|---|---|---|---|---|---|
| | 0°C | 37°C | 0°C | 37°C | 0°C | 37°C | PE | PS |
| % Saturated fatty acids (16:0 + 18:0) | 6 | 8 | 18 | 19 | 24 | 28 | 21–24 | 48 |
| % Fatty aldehydes (16:0, 18:0, 18:1) | 52 | 64 | 28 | 32 | 12 | 12 | 22–26 | 0.4 |
| % Total saturation[b] | 49 | 61 | 41 | 46 | 34 | 38 | 39–43 | 48 |

* Values represent the area percentage of each fatty acid or fatty aldehyde relative to the total area of all the peaks obtained by gas chromatography. Details are given in the text.

[a] Cross-linked with 100 μM DFDNB for 16 h at 0°C or 4 h at 37°C. Experimental details are given in the text.

[b] Percentage total saturation is the percentage of 16:0 + 18:0 fatty acids plus 16:0 + 18:0 fatty aldehydes.

**TABLE IV. Sequential Reaction of Red Cell Phosphatidylethanolamine With MA and TNBS***

| Probe | TNBS | MA | MA then TNBS |
|---|---|---|---|
| Derivative | TNP-PE | MA-PE | TNP-PE |
| nmoles | 45 ± 4.6 | 353 ± 24 | 3.8 ± 1.4 |
| % reaction | 10.7 | 84 | 0.9 |

* Packed cells (0.5 ml) were reacted once with 2 mM TNBS for 1 h, or twice with 20 mM MA for 0.5 h each time, or twice with 20 mM MA for 0.5 h followed by reaction with 2 mM TNBS for 1 h. The cells were reacted in 20 ml of 40 mM $NaHCO_3$ – 120 mM NaCl buffer (pH 8.5) at 22°C. The cells were washed and lysed, ghosts were prepared, and the lipids were extracted with chloroform-methanol and analyzed by TLC as described in the text. Values represent the mean ± SD of six analyses. The total PE in 0.5 ml of packed cells is 419 ± 8 nmoles.

PE molecules available to react with TNBS are not available to MA. These are believed to represent PE molecules in a more hydrophobic domain which are reached by the hydrophobic TNBS but not by the hydrophilic probe MA. If this domain is positively charged it also would be more available to TNBS, which is anionic, rather than to MA, which is cationic.

Similar studies were done with MA and FDNB. As seen in Tables V and VI, a significant number of PE and PS molecules are available to react with FDNB but not with MA. These findings also suggest heterogeneous domains of PE and PS in the membrane.

The significance of the above results using probes in sequence is apparent when one examines the effect of these probes on anion and cation leak in the red cell. These results are given in Table VI and Figures 1–3. MA has no effect on Pi leak in intact red cells under these experimental conditions. However, FDNB and TNBS markedly inhibit Pi leak. Moreover, pretreatment of cells with MA only very slightly prevents the FDNB effect on Pi leak and only moderately prevents the TNBS effect on Pi leak. Therefore, a certain population of PE or PS molecules may be involved in anion leak and these PE or PS molecules are accessible to FDNB and TNBS but not accessible to MA. Since TNBS

**TABLE V. Sequential Reaction of Amino-Phospholipids of the Red Cell Membrane With FDNB and MA**

| Probe | FDNB[a] | MA[b] | MA then FDNB[c] |
|---|---|---|---|
| Derivative | DNP-PE | MA-PE | DNP-PE |
| nmoles | 166 ± 3 | 435 ± 9 | 10 ± 1 |
| % reaction | 37 | 98 | 2 |
| Derivative | DNP-PS | MA-PS | DNP-PS |
| nmoles | 37 ± 1 | 85 ± 8 | 30 ± 1 |
| % reaction | 14 | 31 | 11 |

[a] Packed red cells (0.5 ml) were suspended in a total volume of 20 ml of buffer containing 120 mM NaCl–40 mM $NaHCO_3$ (pH 8.5) at 22°C and reacted with 2 mM FDNB for 1 h. Ghosts were prepared and the lipids extracted and analyzed by TLC as given in the text. Values are the mean ± SD of four analyses.

[b] The cells were reacted twice for 0.5 h with 20 mM MA under conditions described above.

[c] The cells were first reacted twice with 20 mM MA, washed, then reacted with 2 mM FDNB as described above.

**TABLE VI. Sequential Reaction of Amino-Phospholipids of the Red Cell Membrane With FDNB and MA – Correlation With Pi and K+ Leak***

| | Control | FDNB | MA then FDNB | TNBS | MA then TNBS | MA |
|---|---|---|---|---|---|---|
| | | | Labeling of PE and PS (nmoles) | | | |
| XNP-PE | – | 307 | 120 | 28 | 6 | – |
| XNP-PS | – | 56 | 32 | 0 | 0 | – |
| | | | Effect on Pi and K+ leak (nmoles/liter/$10^{10}$ cells/h) | | | |
| Pi Leak | 360 | 1 | 48 | 6 | 125 | 341 |
| K+ Leak | 1,170 | 7,000 | 7,660 | 1,350 | 890 | 1,120 |

* Packed cells (25 ml) were suspended in 170 ml of 120 mM NaCl–40 mM $NaHCO_3$ buffer (pH 8.5) and reacted twice with 20 mM MA at 23°C for 0.5 h. Control cells were incubated in buffer alone. Aliquots (7 ml) of cells were taken from each and these were incubated with 2 mM TNBS, 2 mM FDNB, or buffer alone at 23°C for 1 h. From each of these samples, 1-ml aliquots were removed, ghosts were prepared, and the lipids were analyzed by TLC. The remaining cells were centrifuged, suspended in the same buffer at pH 7.4, and incubated for 15, 30, and 60 min at 37°C. The cells were centrifuged and the supernatants analyzed for Pi and K+ as given in the text. XNP-PE or XNP-PS designates the DNP or TNP derivative of PE or PS.

does not penetrate the cell membrane and it inhibits Pi leak nearly as much as does FDNB, it is apparent that these amino groups which are involved in Pi leak are localized on the outer membrane surface.

When the effect of these probes on $K^+$ leak is examined, the observations are analogous but in the opposite direction to those seen with Pi leak. As seen in Table VI and in Figure 2, MA has no effect on $K^+$ leak but FDNB markedly enhances $K^+$ leak. However, pretreatment of cells with MA has no effect on the FDNB-enhanced $K^+$ leak. Therefore the PE molecules which react with FDNB after cells are pretreated with MA may be involved with $K^+$ leak. Amino groups on certain proteins may also be involved.

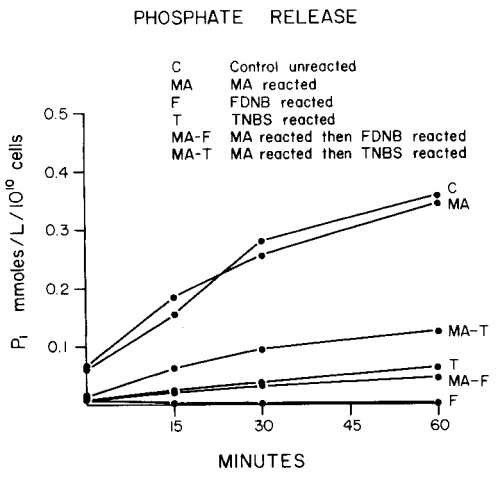



Fig 1. Effect of MA, FDNB, and TNBS alone and in combination on Pi leak in red cells. MA reaction: Packed cells (25 ml) were suspended in 170 ml of 120 mM NaCl–40 mM $NaHCO_3$ buffer (pH 8.5) containing 20 mM MA. The cells were reacted for 0.5 h at 23°C, spun down, and resuspended in fresh buffer containing 20 mM MA and reacted for another 0.5 h at 23°C. TNBS or FDNB reaction: packed cells (25 ml) were suspended in 170 ml of buffer containing either 2 mM TNBS or 2 mM FDNB and reacted for 1 h at 23°C. Sequential reaction of cells was done by first reacting cells with MA and then with either TNBS or FDNB as described above. Control cells were also used. These cells were incubated in buffer without probes for 2 h at 23°C. All cells were washed with buffer then 1-ml aliquots of packed cells were suspended in 120 mM NaCl–40 mM $NaHCO_3$ buffer (pH 7.4) and incubated for various times. The cells were spun down and aliquots were used for analysis of Pi or $K^+$ as given in the text. C) control cells; MA) cells reacted with MA; T) cells reacted with TNBS; F) cells reacted with FDNB; MA-T) cells reacted first with MA then with TNBS; MA-F) cells reacted first with MA then with FDNB.

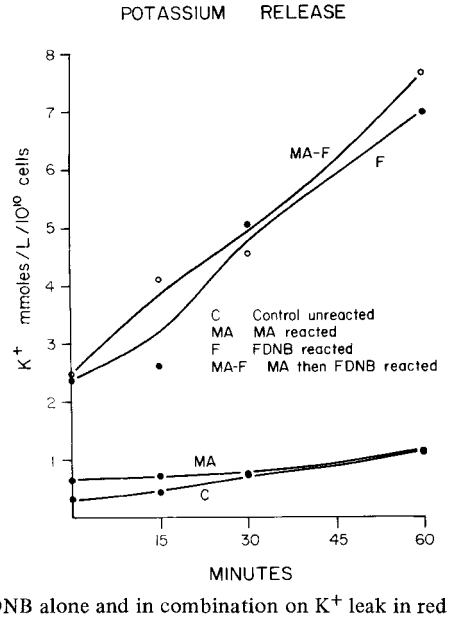



Fig 2. Effect of MA and FDNB alone and in combination on $K^+$ leak in red cells. Experimental conditions are given in Figure 1. Aliquots of the supernatants of control and treated cells were analyzed for $K^+$ as given in text. The symbols MA, F, C, and MA-F are explained in Figure 1.

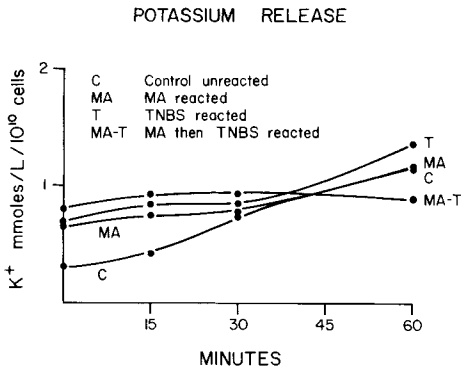



Fig 3. Effect of MA and TNBS alone and in combination on $K^+$ leak in red cells. Experimental conditions are given in Figure 1. Aliquots of the supernatants from control and treated cells were analyzed for $K^+$ as given in text. The symbols C, T, MA, and MA-T are explained in Figure 1.

As seen in Figure 3, TNBS has no significant effect on $K^+$ leak. Since TNBS is a nonpenetrating probe and FDNB is a penetrating probe, we conclude that a certain small population of amino groups* on either phospholipids or proteins located on the inner surface of the cell membrane play an important role in $K^+$ leak.

### Effect of Valinomycin on the Labeling of PE and PS of the Red Cell Membrane by TNBS and FDNB

We have found recently that $K^+$-valinomycin forms a complex with trinitrophenol (picric acid) and that $K^+$-valinomycin converts TNBS from a nonpenetrating to a penetrating probe [15]. Valinomycin has no effect without $K^+$. We have carried out these studies in greater detail. As seen in Table VII, valinomycin in the presence of $K^+$ increases the extent of labeling of PE in intact red cells from 9% to 52% but has no effect on the labeling of PE by FDNB. $K^+$ valinomycin also enhances the labeling of PS from 0% to

**TABLE VII. Effect of Valinomycin on the Labeling of PE and PS in Intact Red Cells by TNBS and FDNB***

| | TNBS | TNBS + valinomycin | FDNB | FDNB + valinomycin |
|---|---|---|---|---|
| XNP-PE (nmoles) | 34 ± 1 | 192 ± 35 | 184 ± 53 | 172 ± 37 |
| % reaction | 9.1 | 52 | 51 | 47 |
| XNP-PS (nmoles) | 0 | 20 ± 1 | 38 ± 20 | 43 ± 17 |
| % reaction | 0 | 11 | 18 | 19 |

* Packed red cells (0.5 ml) were reacted for 2 h in 20 ml of 120 mM NaCl–40 mM $NaHCO_3$ buffer (pH 8.5) at 23°C with 2 mM TNBS or 2 mM FDNB with and without 2 $\mu$M valinomycin. The cells were washed, ghosts prepared, and the lipids extracted and analyzed by TLC as explained in the text. XNP-PE and XNP-PS designate the DNP or TNP derivatives of PE and PS. The total PE in 0.5 ml of packed cells was 385 ± 75 nmoles and the total PS was 241 ± 47 nmoles. The percentage reaction is the percentage of total PE or PS which reacts with each probe. Values are the mean ± SD of these experiments from three different batches of red cells analyzed in duplicate.

*Since FDNB is not specific for amino groups, these may in part represent SH, imidazole, and tyrosine OH groups.

11% in intact cells. However, although valinomycin has no influence on the labeling of PS by FDNB, it is apparent that FDNB labels more PS than does TNBS in the presence of valinomycin (18% versus 11%). In this experiment three different batches of cells were used. These cells varied in storage time, and the data show large standard deviations due to this factor. As the cells age they become increasingly leaky to TNBS and react to a greater extent with TNBS (Fig 4). The increase in labeling of hemoglobin indicates penetration into the cell. However, the 40 nmoles of hemoglobin labeled represents a very small fraction of the total available amino groups (6,700 nmoles) in hemoglobin which react with FDNB [6]. (The labeling of hemoglobin by TNBS may represent a very small fraction of old or damaged cells which are leaky to TNBS.)

The significance of the valinomycin effect on TNBS is that it allows one to examine the localization of PE and PS on the inner and outer surfaces of the intact red cell membrane using the same probe. This avoids uncertainties or differences in chemical reactivities and in other chemical or physical properties of each probe when two different probes are used and it also avoids comparing intact cells to ghosts as is required when only a nonpenetrating probe is used.

### The Effect of Valinomycin and SITS on the $K^+$ and Pi Leak in Red Cells

Valinomycin enhances both the leak (net efflux) of Pi and $K^+$ in intact cells (Table VIII). This is accompanied by a net uptake of $Na^+$ from the medium. The net $Na^+$ uptake nearly equals the net $K^+$ leak. SITS alone markedly inhibits Pi leak and has little or no effect on $K^+$ leak and $Na^+$ uptake. SITS completely blocks the valinomycin-induced Pi leak, moderately inhibits the valinomycin-induced $K^+$ leak, and has no effect on the valinomycin-induced $Na^+$ uptake. These results indicate that the Pi and $K^+$ leak are uncoupled and occur by different channels. This is in agreement with our findings that Pi leak is mediated by components on the outer membrane surface, whereas $K^+$ leak is mediated by components on the inner membrane surface.

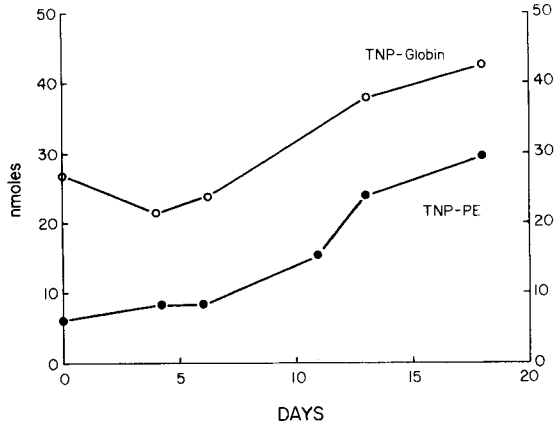



Fig 4. Effect of storage of red cells on the labeling of PS and hemoglobin by TNBS. Packed cells (0.5 ml) were reacted with 2 mM TNBS at 23°C in 20 ml of 120 mM NaCl–40 mM $NaHCO_3$ buffer (pH 8.5) for 1 h. Preparation of ghosts and lipid, and extraction and TLC of the lipids is given in text. Labeling of hemoglobin was carried out as described previously [6].

**TABLE VIII. Effect of SITS on Basal and Valinomycin-Induced Phosphate and K+ Efflux and Na+ Influx in Human Red Cells***

| | mmoles/liter/$10^{10}$ cells/0.5 h | | |
|---|---|---|---|
| | Pi | $K^+$ | $Na^+$ |
| Control (basal) | 0.75 ± 0.016 | 0.15 ± 0.15 | 163 ± 4.8 |
| + Valinomycin (1 μM) | 1.27 ± 0.024 | 8.72 ± 1.2 | 154 ± 0.6 |
| + SITS (0.1 mM) | 0.08 ± 0.014 | 0.31 ± 0.18 | 159 ± 1.2 |
| + SITS and valinomycin | 0.06 ± 0.003 | 5.49 ± 0.12 | 155 ± 0.01 |

* Packed cells (1 ml) were incubated in 4 ml of 140 mM NaCl–40 mM $NaHCO_3$ buffer (pH 7.4) with and without 1 μM valinomycin or 0.1 mM SITS. Incubation was carried out at 37°C for 30 min. The cells were centrifuged and the supernatant analyzed for Pi, K+, and Na+ as given in the text. Values are the mean ± SD of six (Pi) or three ($K^+$, $Na^+$) experiments analyzed in triplicate.

The temperature dependency of the valinomycin effect on Pi and $K^+$ leak is shown in Figures 5 and 6. The control Pi leak is greatly enhanced at 37°C compared to 23°C. Valinomycin enhances the Pi leak at both temperatures but the effect is greater at 37°C. The $K^+$ leak shows a different temperature dependence. The control leak is very small at both 23°C and 37°C. Valinomycin enhances the $K^+$ leak at both temperatures, but the effect is much greater at 37°C. The temperature dependence of the valinomycin effects on $K^+$ and Pi leak are consistent with a carrier-mediated transport system [23].

### The Effects of Ouabain and Iodacetic Acid (IAA) on Pi and $K^+$ Leak

In order to elucidate the nature of the Pi leak, cells were treated singly or in combination with valinomycin, IAA, and ouabain. The results in Table IX show that IAA enhances Pi leak as does valinomycin and that when used together the effects are additive. Ouabain has no effect on Pi leak whether added alone or in combination with either

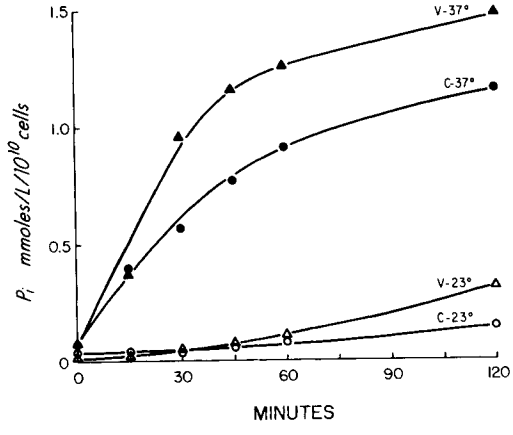



Fig 5. Effect of temperature on basal and valinomycin-enhanced leak of Pi in the red cell. Packed red cells (1 ml) were suspended in 4 ml of 120 mM NaCl–40 mM $NaHCO_3$ buffer (pH 7.4) with and without 1 μM valinomycin. The cells were incubated in a shaker bath at 37°C for different times up to 2 h. The cells were spun down and the supernatants analyzed for Pi. V) valinomycin; C) control.

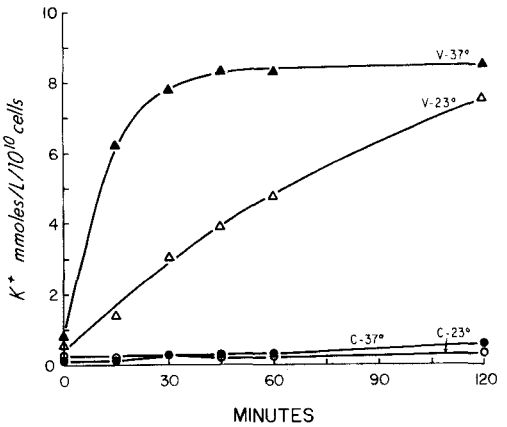



Fig 6. Effect of temperature on the basal and valinomycin-enhanced leak of $K^+$ on the red cell. The same cells given in Figure 4 were used for $K^+$ determinations of the supernatants after incubation. V) valinomycin; C) control.

**TABLE IX. Effect of Valinomycin, Ouabain, and Iodoacetate on Pi Leak in Human Erythrocytes**

| | mmoles Pi/liter/$10^{10}$ cells/h |
|---|---|
| Control | 0.99 ± 0.03 |
| + Valinomycin | 1.25 ± 0.03 |
| + IAA | 1.12 ± 0.02 |
| + Ouabain | 0.99 ± 0.07 |
| + Valinomycin and IAA | 1.33 ± 0.05 |
| + Valinomycin and ouabain | 1.28 ± 0.04 |
| + IAA and ouabain | 1.17 ± 0.05 |

Packed red cells (1 ml) were incubated in 4 ml of 140 mM NaCl–40 mM $NaHCO_3$ buffer (pH 7.4) with and without 1 μM valinomycin or 1 μM SITS, 1 μM ouabain or 1 mM iodoacetate (IAA) for 1 h at 37°C. The cells were centrifuged and the supernatants analyzed for Pi as given in the text. Values are the mean ± SD of six analyses from two experiments done in triplicate.

valinomycin or IAA. Therefore, valinomycin and IAA exert independent effects on Pi leak and the leak is not related to the ouabain sensitive ATPase. Studies on the effect of ouabain on $K^+$ leak showed no significant effect of this agent, indicating that the valinomycin-induced $K^+$ leak is not mediated by its action on the ouabain-sensitive ATPase.

### Effects of pH on the Labeling of PE and PS in Intact Cells by TNBS and FDNB

The effects of pH on the labeling of PE and PS by TNBS and FDNB are shown in Figure 7. The labeling of PE by FDNB reaches a plateau between pH 7.5 and 8.5 but above 8.5 there is a sharp increase in the labeling of PE. The labeling of PS by FDNB is nearly constant over the pH range 6–8.5 but undergoes a sharp rise at pH 9.0. Therefore some alteration has occurred at pH 9.0 which exposes PE and PS amino groups. This

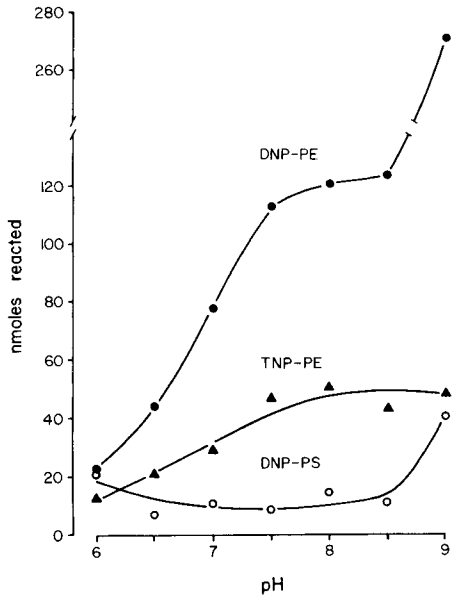



Fig 7. Effect of pH on the labeling of PE and PS in the red cell by TNBS and FDNB. Packed cells (1 ml) were suspended in 20 ml of Krebs-Ringer bicarbonate buffer adjusted to different pH values from 6 to 9. The cells were reacted for 0.5 h at 23°C with either 2 mM FDNB or 2 mM TNBS. The cells were washed, ghosts were prepared, and lipids were extracted and analyzed by TLC as given in text.

occurs under conditions where cell lysis is negligible. However, with TNBS the results are very different, since the labeling of PE reaches a plateau at pH 8.0 and does not increase at pH 9.0. Moreover, little or no PS is labeled with TNBS at all pH values. Therefore the pH effect in making more PE and PS molecules accessible to FDNB occurs on the inner surface of the cell membrane. We suggest that this is due to an unmasking of PE and PS molecules which are tightly bound to protein. A protein likely involved in masking these PE and PS molecules is believed to be spectrin. The next experiments suggest that spectrin may be tightly associated with amino-phospholipids on the inner surface of the membrane.

### Cross-Linking of Amino-Phospholipids to Membrane Proteins

In order to determine whether membrane proteins, and in particular, spectrin, is closely associated with PE and PS molecules on the inner surface of the membrane, red cells were treated with DFDNB, and the spectrin fraction was isolated and analyzed for convalently bound phosphate and compared to the control spectrin fraction and the spectrin fraction isolated from cells treated with FDNB. First, as seen in Table X, DFDNB treatment led to a loss of total soluble protein obtained from ghosts. This was reflected by a loss in extractable spectrin, which decreased from 20–22% to 8% of the total ghost protein. As seen in Figure 8, the spectrin fraction obtained from control and FDNB-treated cells has less Pi than the spectrin fraction obtained from DFDNB treated cells. DFDNB treatment leads to a cross-linking of approximately a net of 75 nmoles of Pi per milligram protein. This represents about 38 molecules of Pi cross-linked to each molecule

**TABLE X. Effect of FDNB and DFDNB on the Extraction of Soluble Proteins and the Spectrin Fraction From the Red Cell Membrane***

| | Percentage of ghost protein | |
|---|---|---|
| | Total soluble proteins | Spectrin fraction |
| Control | 29 ± 3.6 | 20 ± 8.9 |
| FDNB treatment | 28.2 ± 3.9 | 22 ± 5.4 |
| DFDNB treatment | 10.3 ± 4.7 | 8 ± 5.5 |

* Human red cells (10 ml packed cells) were reacted with 0.5 mM FDNB or DFDNB for 30 min at 23°C in 190 ml of 40 mM $NaHCO_3$ – 120 mM NaCl buffer (pH 8.5). Control cells were incubated for 30 min in buffer alone. The preparation of total soluble ghost protein and spectrin fraction is given in the text. Values are the mean ± SD of four experiments.

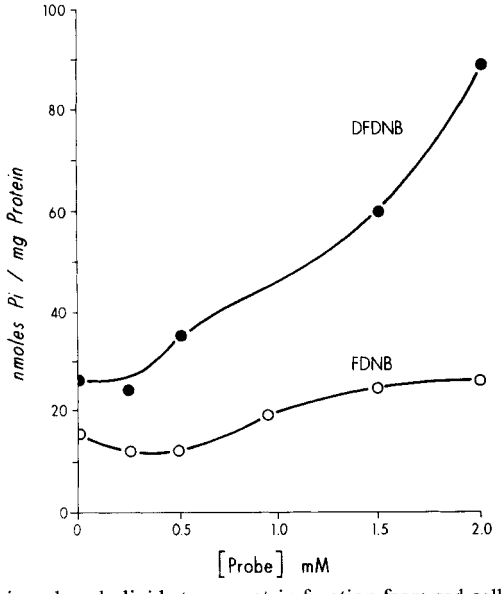



Fig 8. Cross-linking of amino-phospholipids to a spectrin fraction from red cell ghosts. Erythrocytes were reacted with 0.2–2.0 mM FDNB or DFDNB and the spectrin fraction was isolated as explained in Table X. Phosphate determinations were performed as described in text. Each point is the average of duplicate analyses and represents phosphate not extractable with chloroform-methanol.

of spectrin, since 1 mg of spectrin represents about 2 nmoles of spectrin. SDS gel electrophoresis showed that the spectrin from control cells and from FDNB-treated cells gave the identical typical doublet band (bands 1 and 2). The spectrin fraction from DFDNB-treated cells, however, showed most was cross-linked to form high-molecular-weight aggregates which did not enter the gel. It is noteworthy that this cross-linked spectrin was extracted from ghosts and was precipitated at pH 5.1 just like normal spectrin. The spectrin samples from normal and from FDNB- and DFDNB-treated cells did react with purified spectrin antibody. This test showed that the cross-linked proteins, which were obtained by DFDNB treatment and which did not enter the acrylamide gel on electropho-

resis, did contain spectrin. However, other proteins may also be present so that we cannot state unequivocally whether the cross-linked phospholipids are wholly or only partly cross-linked to spectrin.

## DISCUSSION

It is widely accepted that phosphilipids occur in large part as a bilayer in cell membranes [1, 2]. However, the more detailed structure of the bilayer has been investigated only recently. Bretscher [3, 4] and Gordesky and Marinetti [5] suggested that the aminophospholipids are asymmetrically arranged in the red cell membrane. Bretscher used a short-time pulse-labeling experiment with formylmethionine methylphosphate and compared the labeling of PE in cells to PE in ghosts. Gordesky and Marinetti labeled both cells and ghosts with excess TNBS and FDNB under conditions of saturation of available sites. These studies indicated that at least 70–90% of the PE and nearly 100% of the PS were localized on the inner surface of the membrane. Later, using phospholipases [8] or phospholipid exchange proteins [9, 10, 24] several laboratories confirmed the observations with chemical probes and extended them to include other phospholipids such as phosphatidylcholine and sphingomyelin. These choline phospholipids were presumed to be localized to the extent of 70–80% on the outer surface of the red cell membrane. Soon after these studies, other cell lines and intracellular membranes were studied. The picture which emerged indicated that the asymmetric arrangement of phospholipids was not confined to the red cell membrane but occurred in Golgi membranes [29] and membranes from yeast cells [7], E coli [25], platelets [26, 27], mitochondria [28], microsomes [29], viruses [37], and bacteria [37]. None of these studies gave an unequivocal conclusion, since some of the PE and PS could be localized on either membrane surface and be masked by protein. These findings also raised the question of how the asymmetry is generated in the cell and how it is maintained. Recently the asymmetric arrangement of PE in the microsomal and Golgi membranes has been challenged by Sundler et al [40].

In order to determine whether the phospholipids on each membrane surface occurred in a random array or occurred as clusters, Marinetti studied nearest neighbors of PE and PS in the red cell membrane by cross-linking with DFDNB [11, 12, 30]. These studies indicated that PE and PS occurred in part as nonrandom clusters. Inasmuch as phospholipids have a very rapid lateral mobility in lipid bilayers [31], one has to resolve the difficult question whether the cross-linking pattern observed with DFDNB represents natural partners having a relatively long lifetime or whether the pattern represents cross-linking due to rapid random collision. This is an important problem with cross-linking probes such as DFDNB, which require several minutes to achieve appreciable cross-linking. With artificial bilayers, this time is presumed to be very large relative to the time of lateral mobility of the phospholipids. However, there is uncertainty whether lateral mobility of all phospholipids is rapid, especially in natural membranes where proteins can influence this mobility. Another uncertainty in the problem of nonrandom arrays of lipids in natural membranes is the effect of calcium ions, and the effect of special interactions between the multiple types of phosopholipids. Do studies on phospholipid mobility in artificial bilayers having no proteins and having a simple lipid composition have direct relevance to natural membranes?

In our previous studies on lipid clusters [12] we examined the pattern of cross-linked species of PE-PE, PE-PS, and PS-PS to random and nonrandom arrays based on the

lipid composition of the membrane. This approach is a reasonable first approximation but is not definitive. We reasoned that more useful information could be obtained if we were to examine the nature of the molecular species of PE cross-linked to PE. In these studies we found that these cross-linked PE-PE molecules are rich in fatty aldehydes, especially at 37°C. Since the amino group of the diester form of PE should have the same chemical reactivity as the amino group of the plasmalogen form of PE and since these forms of PE occur in about equal amounts and should have the same intrinsic mobility in a bilayer, our observation provides clear evidence of the existence of clusters of plasmalogen PE in the red cell membrane.

The fatty acid and fatty aldehyde analysis of the amino phospholipids cross-linked to membrane protein shows a high concentration of 16:0 and 18:0 fatty acids, suggesting cross-linked PS. Appreciable amounts of fatty aldehydes were also found, indicating cross-linked PE. Therefore both PE and PS are cross-linked to membrane protein. We originally reported that about 33% of the total red cell PS and 3% of the total red cell PE were cross-linked to membrane protein. This was based on phosphate analysis. Our present studies indicate that 30–50% of the amino-phospholipids cross-linked to membrane protein is PE. The amount of PE and PS which becomes cross-linked to membrane protein varies with different samples of red cells, depending in large part on the age of the cell. Which specific proteins become cross-linked to PE and PS remains to be determined definitely. Our preliminary studies suggest that PE and PS become cross-linked to spectrin. This amounts to 38 molecules of amino-phospholipid cross-linked per molecule of spectrin. We suggest that this represents a tight association of spectrin with these PE and PS molecules and that this interaction helps to anchor spectrin to the inner surface of the membrane and may play a role in the spectrin-dependent shape changes of the cell. Our studies on the effect of pH on the labeling of PE and PS by FDNB is consistent with this concept. High pH is reported to release spectrin from the red cell membrane [32].

The molecular species of PE molecules which are localized on the outer surface of the membrane are the same as those on the inner surface. The amount of PE which is localized on the outer surface as determined by labeling with TNBS is about 1.7% at pH 7.4 and 9% at pH 8.5. In both cases, this represents saturation of available PE molecules. Therefore at the higher pH more PE molecules are exposed. Whether this represents unmasking from bound protein or flip-flop of internal PE remains to be determined. At pH 9.0, an additional number of PE and PS molecules on the inner membrane surface become exposed and available to react with FDNB. This effect, we suggest, is due in large part to unmasking of PE by dissociation from bound spectrin. It is noteworthy that the major part of PS and about 15–50% of PE in intact cells are not available to react with certain permeable chemical probes such as MA and FDNB.

The use of probes in sequence has provided new insight into the topology of the phospholipids in the red cell membrane. Reaction of cells to saturation of available sites with MA still allows a certain population of PE molecules to be accessible to TNBS and a different population of PE and PS molecules to be available to FDNB. This indicates heterogeneous domains of PE and PS which differ in hydrophobicity since the hydrophobic neutral probe such as FDNB and to a lesser extent the hydrophobic anionic probe such as TNBS are able to react with these PE molecules whereas the hydrophilic cationic probe MA cannot. Recent work by Bevers et al [33] indicates different pools of phosphatidylglycerol in the plasma membrane of acholeplasma.

Of added interest is our finding that these very small number of PE and PS molecules and/or certain amino groups on specific proteins are involved in the transport of anions (Pi) and cations ($K^+$). The major evidence for this is that pretreatment of cells to saturation of available sites with MA, which has no effect on Pi and $K^+$ leak from red cells, does not prevent FDNB or TNBS from exerting their effects on inhibiting Pi leak and enhancing $K^+$ leak. These results also show that the amino groups regulating Pi leak are localized on the outer surface of the cell membrane, whereas the amino groups regulating $K^+$ leak are localized primarily on the inner surface of the membrane. Therefore an asymmetric arrangement of anion and cation transport systems occurs in the red cell membrane and it may be related to the asymmetric arrangement of amino phospholipids and proteins. This idea was suggested earlier by Knauf and Rothstein [34], who also provided evidence for SH groups in cation transport.

Valinomycin has been employed to convert the nonpenetrating probe TNBS into a penetrating probe in intact red cells [15]. $K^+$, $Rb^+$, or $Cs^+$ is required for this valinomycin effect. The penetration of TNBS was monitored by the enhanced labeling of PE, PS, and hemoglobin. It is noteworthy that even under these conditions, only 11% of the total membrane PS reacts with TNBS compared to 18% with FDNB. Moreover, the level of labeling of PE by TNBS and FDNB becomes the same (51–52%). These studies are done in intact cells which show very little or no hemolysis and thus the membrane is studied in the native state with minimal perturbation. This is to be contrasted with studies using phospholipases or phospholipid exchange proteins, which must compare cells to ghosts or which can only be done on ghosts.

### Alternative Models for Phospholipids in the Red Cell Membrane

The work in this paper suggests that the simple model of a lipid bilayer for cell membranes is not adequate to explain our observations. It is difficult to explain the effects of probes used in sequence on the labeling of PE and PS by the bilayer model. We therefore suggest alternative modifications of the membrane structure to account for our results. These are shown in Figures 9 and 10. In Figure 9, the phospholipids are arranged in a seagull model as suggested earlier by Deamer [42]. Only a relatively small part of the lipid membrane is postulated to have this arrangement, which may be in equilibrium with the major bilayer structure. In the seagull model, some polar head groups of the phospholipids are buried and may be accessible to certain probes and not to others, depending on their charge and hydrophobicity. In the other model, a certain fraction of the phospholipids are shown with polar head groups burried. These phospholipids are considered to be in transit during flip-flop. Again, this would represent a relatively small number of phospholipid molecules whose polar head groups would have access primarily to hydrophobic probes.

In Figure 10 are shown protein arrays in the bilayer which lead to masking of polar head groups of phospholipids or which may mask entire phospholipid molecules. These arrangements could explain why an appreciable fraction of PS molecules are refractory to several chemical probes.

Finally in Figure 11 is shown a model in which a cluster of PE and PS is tightly associated with spectrin. The PS molecules, because of their negative carboxyl groups, can be bound to spectrin via $Ca^{++}$ bridges. This may explain why EDTA is required to

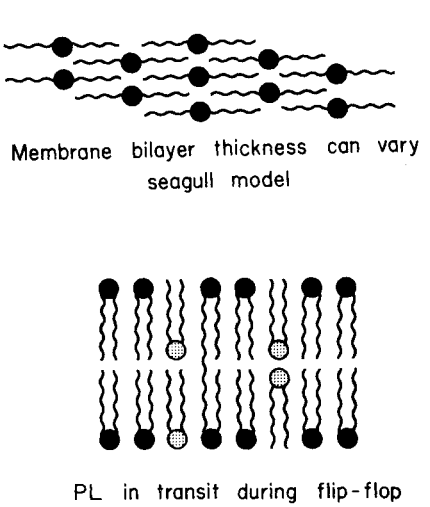



Fig 9. Provisional models of phospholipid arrangements in the red cell membrane. A) Model fashioned after Deamer [42]. We call this the seagull model. The fatty acid chains of the phospholipids run parallel to the plane of the membrane. This model is proposed to account for a small fraction of the phospholipid arrangement in the cell membrane. It may explain the variation in membrane thickness seen on electron micrographs of cell membranes and it shows that certain polar head groups of phospholipids can be buried. B) This model is the typical lipid bilayer and shows certain phospholipid molecules inverted. These represent phospholipid molecules in transit during flip-flop. The symbols —•— or ≈•≈ represent phospholipid molecules.

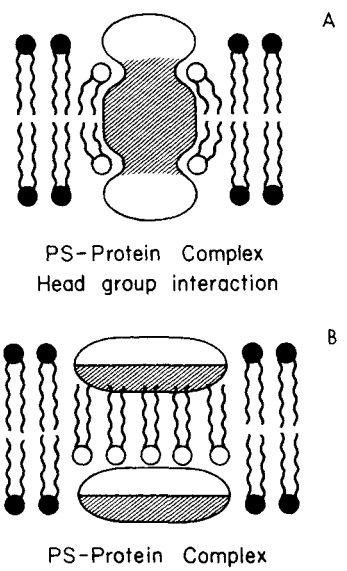
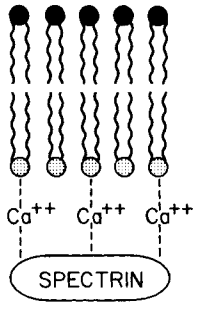



Fig 10. Provisional models of protein-phospholipid interactions in the red cell membrane. A) certain polar head groups of phospholipids (such as PS) interacting with membrane proteins, leading to a masking of these polar head groups. B) Array of amphipathic membrane proteins which are separated by a monolayer of phospholipid. The shaded area represents the hydrophobic part of the protein and the nonshaded area represents the polar portion of the protein which can interact with the polar head group of the phospholipids. Symbols as in Figure 9.




PS-$Ca^{++}$-Spectrin Complex

Fig 11. Provisional model of the interaction of spectrin with phospholipids of the red cell membrane. This model is suggested to depict how spectrin may interact with clusters of PS and/or PE that are located on the inner surface of the cell membrane. The $Ca^{++}$ bridges are presumed to occur between carboxyl groups of spectrin and carboxyl groups of PS. Symbols as in Figure 9.

release spectrin from the red cell membrane. This model and the one in Figure 10 offer possible roles for phospholipid clusters in cell membranes.

### Origin of Phospholipid Asymmetry in Cell Membranes

With very small liposomes made up of two or more phospholipid types, steric and charge factors can lead to asymmetric bilayers. This is not likely to be important with cell membranes except at certain loci where the membrane may have sharp bends and where the radius of curvature can influence packing of specific phospholipids. It is likely that binding to specific membrane proteins may play an important role in maintaining membrane asymmetry. In the case of the red cell we suggest that interaction of PE and PS with spectrin plays a major role in producing and maintaining the asymmetry of PE and PS in the membrane. Base exchange reactions or specific proteins which cause phospholipid translocation may play a role in modifying membrane asymmetry. For example, if the enzyme-catalyzed base exchange of serine and ethanolamine for choline is favored, this can lead to an asymmetry of PE and PS on the surface of cell membranes which face the cytoplasm where these enzymes are located. A balance between phospholipase activity and resynthesis might also lead to an asymmetric membrane. For example, if a phospholipase C specific for lecithin is situated in the cytoplasm and the enzyme which adds phosphorylcholine is located on the opposite side of the membrane, this may lead to an asymmetric arrangement for lecithin, since the diglyceride may readily rotate in the membrane and be converted back to lecithin only on one surface. Lysolecithin is reported to enhance phospholipid flip-flop [35]. Therefore phospholipases which act specifically on certain phospholipids to produce lyso-phospholipids may regulate membrane asymmetry.

### Biologic Signficance of Phospholipid Asymmetry and Clusters

The asymmetric arrangement of PE and PS in the red cell membrane and platelet membrane may be important for the control of intravascular clotting. This idea was suggested by several researchers and tested recently by Zwaal, Comfurius, and van Deenen [43]. Their data show that when made available to the exterior surface of inside-out

ghosts or disrupted platelets, PE and PS, phospholipids which are important in blood clotting, are able to activate the conversion of prothrombin to thrombin. This is the only case known to the authors where phospholipid asymmetry has a fairly clear biologic role.

Other functions of the phospholipid asymmetry may be related to the maintenance of the membrane potential. PS may play an important role in this regard because of its carboxyl group. This may in part tend to neutralize the carboxyl group of neuraminic acid residues of glycolipids and glycoproteins which are localized on the outer surface of the red cell membrane.

Phospholipid clusters can offer highly charged domains for interaction with specific proteins (eg, anchoring spectrin to the inner surface of the membrane). Phospholipid clusters may also offer channels for transport of ions and small molecules. How this can lead to specific transport is not readily apparent. Acidic clusters of phospholipids may also play a role in binding and sequestering of basic compounds such as histamine, serotonin, or catecholamines. This may be important in the platelet and mast cells which sequester these amines. Clusters of phospholipids may also be involved in the attachment of microfilaments and microtubules to the inner surface of the plasma membrane.

The above ideas are speculative. Similar ideas have been discussed by others [35, 37]. It is hoped that they will generate new concepts of membrane structure and function and lead to new and productive experiments.

### Limitations to the Use of Probes for Analysis of Membrane Topology

The following factors should be considered when using either small organic chemical probes or large protein probes.

1. The solubility of the probe in the medium – this is important with chemical probes when attempting to saturate available sites.

2. The stability of the probe in the medium – the probe can undergo hydrolysis or other chemical change which may not allow it to react fully with available sites; the probe may also be modified by enzymes in the membrane preparation.

3. The size and permeability of the probe – this presents different problems for small chemical probes and large protein probes. Small probes may readily penetrate the membrane. It may be difficult to find a chemical probe which does not penetrate the membrane. Large protein probes are likely not to penetrate the membrane; however, their large size may limit their accessibility to all sites on the outer membrane surface. Certain sites may be partially buried and be available to small chemical probes but not to large protein probes.

4. The requirement of other factors for activity of the probe – protein probes such as phospholipases require $Ca^{++}$ ions for activity and are influenced by pH and ionic strength. Chemical probes are less likely to require $Ca^{++}$ ions for activity but may also be influenced by pH and ionic strength. The pH and ionic composition near or on the membrane surface can change markedly from the pH and ionic composition of the bulk phase in the medium.

5. How much the probe perturbs the membrane – all probes will unavoidably perturb the membrane to varying degrees; chemical probes modify functional groups and usually attach other groups of varying size, charge, and hydrophobicity. Phospholipases may induce drastic changes in the phospholipid structure such as removing polar head groups or fatty acids. These changes can lead to rearrangement of membrane components to varying degrees. These changes may induce phospholipid flip-flop, especially when

polar head groups of the phospholipid are removed. Removal of phospholipid polar head groups also will alter the membrane potential and cause rearrangement of membrane components.

6. How stable the membrane components modified by the probes remain – we have found in E coli [25] and with mitochondria mitoplasts [28] that dinitrophenyl and trinitrophenyl derivatives of PE undergo degradation by enzymes and that the fragments such as dinitrophenyl-glycerophosphoryl-ethanolamine and dinitrophenyllysophosphatidylethanolamine can be utilized to resynthesize DNP-PE. Resynthesis may also occur with the degradation products of phospholipids (lysophosphatides, diglycerides, or phosphatidic acid) produced by the action of phospholipases.

7. Specificity of the probe – it would be desirable to have probes which act specifically on certain phospholipids in order to distinguish these from the bulk phospholipids.

8. Time of reaction of the probe – it is desirable to use probes in as short a time as possible to saturate available sites but to minimize membrane alteration; this time factor becomes much more critical in nearest-neighbor analysis in order to distinguish normal partners from random collision of rapidly moving components.

9. The charge and hydrophobicity of the probe – chemical probes can be neutral, cationic, anionic, hydrophobic, or hydrophilic. FDNB and DFDNB are neural and hydrophobic, TNBS is anionic and hydrophobic, MA is cationic and hydrophilic. Differences in charge and hydrophobicity of various regions of the membrane will influence the accessibility and reactivity of each probe to a different extent. These factors can be used to advantage to sense various domains in cell membranes. From the above discussion it is obvious that the judicious use of all the probes will be required to elucidate the fine details of membrane topology.

10. Isolation of cell membranes from disrupted cells – in this case the purity and physical state of the membrane must be determined. Are the membranes isolated entirely as closed vesicles with the same sidedness? Can lipid rearrangement occur independently of protein rearrangement? Are endogenous lipases and proteases present which are activated during cell disruption and these alter the membrane structure?

The advantages and disadvantages of protein and chemical probes has been discussed by Bergelson and Barsukov [35]. Some problems inherent in the use of phospholipases as probes for lipid asymmetry have been pointed out by Martin et al [36]. Carraway [38] and Peters and Richards [39] have discussed the use of chemical probes to study membranes. The combined use of these chemical and protein probes will in the long run provide new insights into membrane structure and function.

## ADDENDUM

Combined results of our studies on the human red cell using chemical probes leads us to postulate the following percentage distribution of PE and PS in the membrane:

| | Outside surface | Internal membrane matrix | Inside surface |
|---|---|---|---|
| PE | 5–10 | 45–50 | 40–50 |
| PS | 0–2 | 78–89 | 11–20 |

The above distribution of PE and PS is obtained from data on the differential labeling of these amino-phospholipids with FDNB and with TNBS in intact cells. The PE and

PS available on the outer surface are those molecules which react readily with TNBS. The PE and PS which are located on the inner surface are those additional molecules which react with TNBS plus valinomycin or those molecules which react with FDNB minus those molecules which react with TNBS. The molecules of PE and PS which do not react with FDNB or with TNBS + valinomycin are considered primarily to be buried in the membrane matrix and closely associated with protein or possibly to exist to some extent as inverted micelles. The readily reacting molecules of PE and PS are presumed to be in a mobile bilayer. The PE and PS molecules which become cross-linked to protein by DFDNB are considered to be boundary lipids closely associated with membrane protein. Particularly noteworthy is our new finding that clusters of plasmalogen PE occur on the inner surface of the membrane. The above model offers more details than the model proposed by Singer [2].

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