THE ASYMMETRIC ARRANGEMENT OF PHOSPHOLIPIDS IN THE HUMAN ERYTHROCYTE MEMBRANE

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SUMMARY. In erythrocytes treated with 2,4,6-trinitrobenzenesulfonate (a non-penetrating probe) for 24 hours, a maximum of 33% of the phosphatidylethanolamine and none of the phosphatidylserine reacts with this reagent. In erythrocyte ghosts, however, 95% of the phosphatidylethanolamine and over 50% of the phosphatidylserine reacts in 90 minutes under the same conditions. When extracted erythrocyte lipids are treated with 2,4,6-trinitrobenzenesulfonate in either a chloroform-methanol-bicarbonate or a sonicated aqueous bicarbonate system, both phosphatidylethanolamine and phosphatidylserine react essentially to completion within minutes. We interpret these results to indicate the localization of nearly all of the phosphatidylserine on the interior surface of the membrane thus demonstrating an asymmetric distribution of phospholipids in the erythrocyte membrane.

It is important to be able to selectively label the components on the inner and outer surfaces of cell membranes. Berg (1) used the non-penetrating probe [^{35}S]-labelled diazosulfanilic acid to distinguish components on the outside of the erythrocyte membrane. Although the potential of this type of approach was demonstrated, the conditions of the reaction were such that hemolysis ensued. Bonsall and Hunt (2), using 2,3,5-trinitrobenzenesulfonate, found that both the lipids and proteins of the human erythrocytes reacted with this probe and there was almost no penetration of the reagent into intact erythrocytes even after a twenty hour incubation. Godin and Wan Ng (3) also have studied the interaction of TNBS with erythrocyte membranes.

We have previously studied (4) the availability of the amino groups of PE and PS in the erythrocyte membrane using the penetrating probe FDNB. In this report using TNBS we provide evidence for the asymmetric arrangement of amino-phospholipids in the erythrocyte membrane.

Abbreviations: TNBS - 2,4,6-trinitrobenzenesulfonate; PE - phosphatidyl-ethanolamine; PS - phosphatidylserine; FDNB-1-fluoro-2,4-dinitrobenzene.

EXPERIMENTAL PROCEDURE

The reaction was carried out as previously described (4) using 1.5 mM TNBS instead of FDNB. However the incubation time was extended to 24 hrs. Phospholipids and the TNBS-derivatives of control and reacted cells were extracted and separated by TLC (4).

The ninhydrin reaction to estimate the extent of reaction of PS was carried out by spraying the TLC plates with a solution containing 200 mg of ninhydrin dissolved in 90 ml of acetone and 10 ml of 2,4-lutidine. After a development time of one hour the purple colored bands were scraped into 15 ml tubes and extracted for 10 min with 5 ml of methanol. The tubes were centrifuged for 5 min at 2000 RPM to sediment the silicic acid and the absorbance of the supernatants was determined in a Gilford spectrophotometer at 565 nm.

RESULTS AND DISCUSSION

The data in Figure 1 demonstrate that the TNBS-PE color is linearly proportional to the phospholipid concentration. The absorption maximum of TNBS-PE is 337 nm. A least squares analysis of the data demonstrated linearity with a zero order correlation coefficient of 0.997.

The reaction of TNBS with PE and PS in intact erythrocytes over a 24 hour period is shown in Figure 2. The data indicate that a maximum value of available PE on the outside of the erythrocyte membrane is about 33% of the total PE. On the other hand, PS of intact erythrocytes does not appear to react with TNBS during the 24 hour incubation. Assuming that TNBS does not penetrate the membrane, these observations suggest that either the exterior PS is masked and not able to react or PS is present only on the inner surface of the membrane. The ability of PS in erythrocyte membranes to react with TNBS is amply demonstrated in Figure 3. It is seen that in erythrocyte ghosts, PE is able to react maximally with TNBS within 1 hour at which time 95% of the total PE has reacted. Over half of the PS has also reacted but it requires more time to reach a plateau. It is interesting to note however that the PS does not begin to react until the reaction of TNBS with PE has nearly reached a plateau. Studies with isolated PE and PS show that at

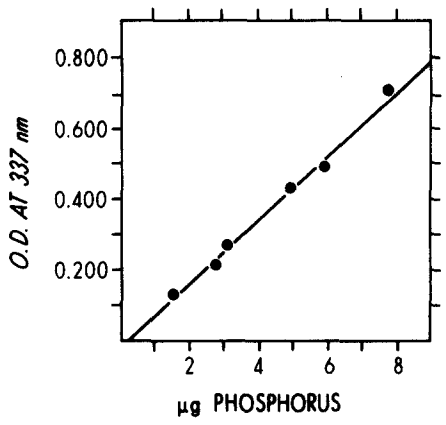

Fig. 1.

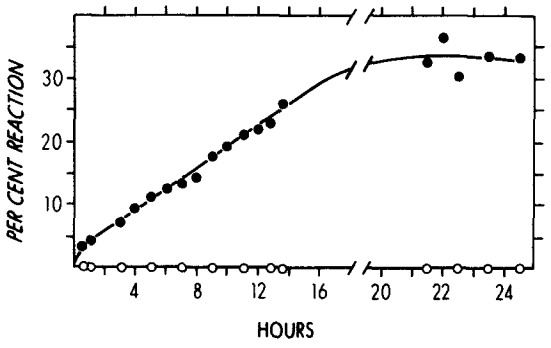

Fig. 2.

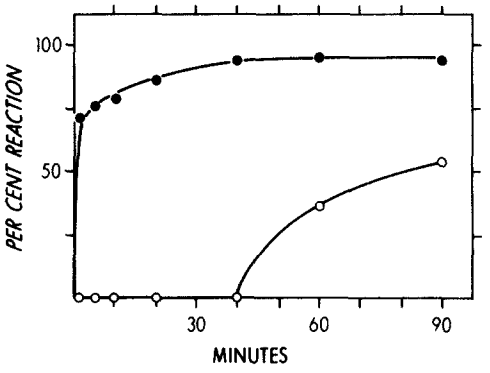

Fig. 3.

Figure 1 TNBS-PE Absorption as a Function of PE Phosphorus

Synthetic dipalmityl PE (Calbiochem.) was reacted with TNBS for two hours in 1 ml of $CHCl_3$:MeOH 1/1 containing 0.1 ml of 5% $NaHCO_3$. The TNBS-PE was isolated by TLC (4) and the absorbance was determined at 337 nm. Phosphate was measured by the method of Harris and Popat (7). Identical results were found with PE purified from erythrocytes.

Figure 2 TNBS Reaction with PE and PS of Intact Erythrocytes

Erythrocytes were reacted with TNBS and the phospholipids were isolated and quantitated as described previously (4). ●—● PE;
0—0 PS

Figure 3 TNBS Reaction with PE and PS of Erythrocyte Ghosts

Erythrocyte ghosts were reacted with TNBS and the phospholipids isolated and quantitated as described previously (4). ●—● PE;
0—0 PS

about 10 mM, PE reacts more rapidly with TNBS than does PS in a chloroform-methanol water medium containing bicarbonate but both reactions go to completion. PE reacts to completion within 20 minutes whereas PS requires up to 60 minutes for complete reaction. Treatment of a sonicated mixture of erythrocyte lipids, in the aqueous bicarbonate medium (4), with TNBS showed that PE and PS reacted to 95% and 90% respectively within one minute.

Arrotti and Garvin (8) have recently reported that erythrocytes incubated at 37° C in a phosphate buffer pH 8.0 are apparently permeable to TNBS. This finding differs from that of Bonsall and Hunt (2). The difference may be due to the different temperatures and experimental conditions used by these workers and may also be due to the methods used to measure penetration. Nevertheless, if one assumes that TNBS does penetrate the membrane to some extent, under our experimental conditions this penetration must be very slow in view of the slow reaction of PE and lack of reaction of PS in the intact cells, compared with the rapid reaction of PE and PS in ghosts. Our data support the finding of Bonsall and Hunt (2) that TNBS does not readily penetrate the erythrocyte membrane.

We conclude that with intact cells the incomplete reaction of PE and the lack of reaction of PS with TNBS indicates that all or nearly all of the PS and a minimum of 70% of the PE is on the inside surface of this membrane. These observations present strong evidence for an asymmetric arrangement of phospholipids in the erythrocyte membrane.

If the lipids of the erythrocyte are primarily in a bilayer then one can estimate that the outer layer contains primarily sphingomyelin, lecithin and cholesterol. This arrangement is supported by evidence from lipid exchange studies where the rate of exchange of lecithin, sphingomyelin and cholesterol between the membrane and plasma lipoproteins is very much greater than that of PS and PE, which is almost non-existent (5,6).

During the course of our investigation a paper appeared suggesting that PE is localized primarily on the inner surface of the erythrocyte membrane (9). Although we agree with this suggestion of Bretscher, data based only on initial rates of reaction are insufficient to warrant his conclusion.

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