EXTENT OF CROSS-LINKING OF AMINO-PHOSPHOLIPID NEIGHBORS IN THE ERYTHROCYTE MEMBRANE AS INFLUENCED BY THE CONCENTRATION OF DIFLUORODINITROBENZENE.

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SUMMARY: The degree of cross-linking of phospholipids to phospholipids and phospholipids to proteins in the erythrocyte membrane is dependent on the concentration of diflurodinitrobenzene. With ghosts isolated from human erythrocytes, the optimal extent of cross-linking of neighboring phosphatidylethanolamine molecules occurs at 50 μM difluorodinitrobenzene, the optimal extent of cross-linking of neighboring phosphatidylserine molecules occurs at 125 μM difluorodinitrobenzene and the optimal cross-linking of phosphatidylethanolamine to phosphatidylserine occurs at 75 μM difluorodinitrobenzene. Up to 37% of the total amino-phospholipids are cross-linked to membrane protein, the major part occurring with phosphatidylserine. Under optimal conditions of difluorodinitrobenzene concentration, 60% of the total phosphatidylethanolamine is cross-linked to phosphatidylethanolamine, 27% of the total phosphatidylethanolamine is cross-linked to phosphatidylserine, 24% of the total phosphatidylserine is cross-linked to phosphatidylserine and 44% of the total phosphatidylserine is cross-linked to phosphatidylethanolamine.

INTRODUCTION: We recently reported the cross-linking of amino-phospholipids in the human erythrocyte membrane with DFDNB[1] (1). Under the conditions employed in which the cross-linking agent was used in excess, we observed a small amount of cross-linking of the amino-phospholipids. However, when DFDNB is not used in excess we now report extensive cross-linking of amino-phospholipids in the erythrocyte membrane. These results lead to a different interpretation from our previous one and are consistent with an appreciable amount of lipid bilayer in the erythrocyte membrane.

Recently Marfey and Tsai (2) reported that DFDNB gave no cross-linking of PE[1] or PS[1] in the human erythrocyte membrane but difluorodinitrodiphenylsulfone cross-linked 24% of the PE of this membrane.

METHODS AND REAGENTS: The isolation of erythrocyte ghosts and the experimental

1 - DFDNB = 1,5-difluoro-2,4-dinitrobenzene; PE = phosphatidylethanolamine; PS = phosphatidylserine.

conditions of cross-linking and analysis of the dinitrophenyl products are given previously (1,3,4). The major change in this study is that DFDNB was used over the concentration range of 50-200 μM rather than 2.0 mM, and the reaction was allowed to go 16 hours at 23°C. Similar results are obtained at a 2 hour reaction.

Ghosts from 1.0 ml of packed human erythrocytes (Red Cross stored blood) were reacted with varying concentrations of DFDNB (50-200 μM) in 20 ml of 120 mM $NaHCO_3$-40mM NaCl buffer pH 8.5 for 16 hours at 23°C. The ghosts were washed twice with 15 ml of 0.5% bovine serum albumin (Sigma, Fraction V) in the same buffer. The ghosts lipids were extracted once with 8 ml and then with 2 ml of chloroform-methanol 1:1. The protein residues were digested with perchloric acid and analyzed for total phosphate.

The lipid extracts were dried under nitrogen and dissolved in 5 ml of chloroform-methanol 1:1. Duplicate aliquots of 0.25 ml were used for analysis of total lipid P, and 0.5 ml was used for thin layer chromatography (1,3). The remaining lipid extract was evaporated to dryness under nitrogen, suspended in

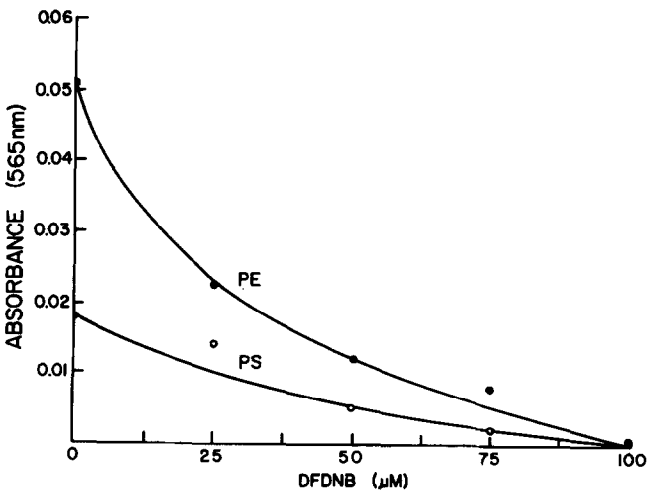

Figure 1. The extent of Reaction of PE and PS of the Erythrocyte Ghost With DFDNB.

The extent of reaction of PE and PS was followed by reaction of these phospholipids with ninhydrin after separation by thin layer chromatography as explained in the text.

3 ml of 3N HCl and hydrolyzed for 2 hours at 100°C. The hydrolysate was made basic with excess $NaHCO_3$ and extracted with ethyl acetate to obtain the dinitrophenyl derivatives of ethanolamine. The hydrolysate was then acidified with HCl and extracted with ethyl acetate to obtain the dinitrophenyl derivatives of serine. The dinitrophenyl derivatives were separated by thin layer chromatography on silica gel using chloroform-methanol-water 65:25:4 v/v (3). The various dinitrophenyl derivatives were scraped off the plates and extracted into methanol. The absorbance of each derivative was measured at its λ_{max} and the amount of each was determined from its extinction coefficient (3).

The thin layer chromatogram of the total lipids run on silica gel in chloroform-methanol-water 65:24:4 v/v was dried and sprayed with a ninhydrin reagent. After 1 hour the purple spots of unreacted PE and PS were scraped off the plate and extracted into 5 ml of methanol. The absorbance of each extract was determined at 565 nm (5).

RESULTS: It became apparent that the differences in the rate of reaction of the first fluorine group on these probes as compared to the second fluorine group would make it important not to add the cross-linking agent in excess since it would react readily with all the available amine groups to yield primarily the mono-N-substituted derivatives and thereby prevent or minimize cross-linking of the nearest neighbors. In this report we show the effect of concentration of DFDNB on the yield of cross-linked phospholipids.

The data in Figure 1 show the extent of reaction of PE and PS with DFDNB as determined by ninhydrin reaction of these phospholipids separated by thin layer chromatography. It can be seen that at 100 μM DFDNB essentially all the PE and PS have reacted.

The results in Figure 2 show the amount of each dinitrophenyl derivative of ethanolamine and serine obtained at varying concentrations of DFDNB. The extent of cross-linking of PE and PS under optimal conditions of DFDNB concentration is shown in Table 1. The optimal cross-linking (60%) of PE to PE occurs at 50 μM DFDNB, whereas the optimal cross-linking (27%) of PE to PS oc-

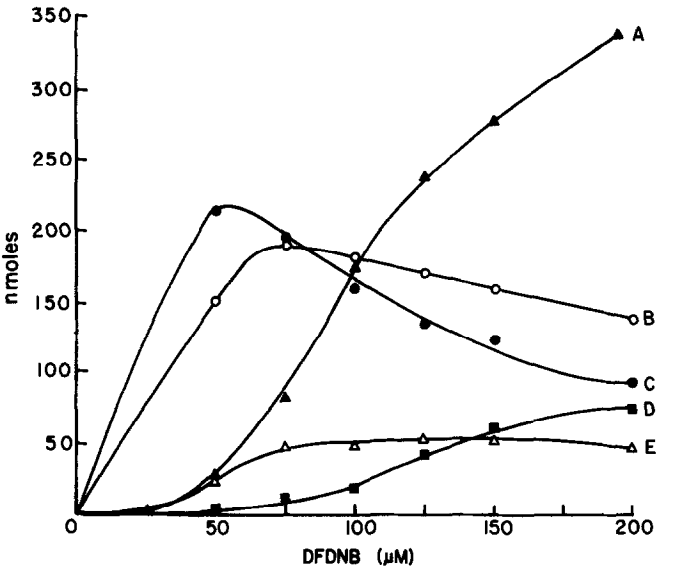

Figure 2. The Reaction of PE and PS of the Erythrocyte Ghost With Different Concentrations of DFDNB.

The reaction conditions are explained in the text. The curves represent the various dinitrophenyl derivatives of PE and PS after hydrolysis with 3N HCl for 2 hours. A = fluorodinitrophenylethanolamine; B = serinedinitrophenyl-ethanolamine; C = dinitrophenyl-bis-ethanolamine; D = fluorodinitrophenylserine; E = dinitrophenyl-bis-serine.

curs at 75 μM DFDNB. However, the optimal cross-linking (24%) of PS to PS occurs at 125 μM DFDNB. The optimal cross-linking (44%) of PS to PE occurs at 75 μM DFDNB.

The measurement of lipid P bound covalently to membrane protein was obtained by analysis of the protein residues after lipid extraction. These analyses are given in Table 2 and show that optimal cross-linking (37%) of phospholipids to proteins occurs at about 75 μM DFDNB although between 75 - 200 μM DFDNB the differences are small indicating a broad plateau region for optimal cross-linking of PE and PS to protein.

We have not directly measured how much of the amino phospholipid cross-linked to membrane protein is PE or PS. We can obtain this number by difference analysis after summation of PE and PS in the various dinitrophenyl derivatives and by using a PE/PS molar ratio of 1.65 determined previously (6). The

Table 1

Extent of Cross-Linking of Amino-Phospholipids Under Optimal Conditions of DFDNB Concentration [a]

Cross-linked species	Conc.DFDNB μM	Percent
PE to PE	50	60 [b]
PE to PS	75	27 [b]
PS to PS	125	24 [c]
PS to PE	75	44 [c]

a Taken from the data in Figure 2.
b Percent of the total amount of PE.
c Percent of total amount of PS.

Table 2

Cross-linking of Amino-Phospholipids to Proteins in the Erythrocyte Membrane*

Conc. of DFDNB μM	Cross-linked phospholipids nmoles
50	137
75	187
100	170
125	162
150	172
200	173

* The experimental methods are given in the text. The values represent the total nmoles of PE + PS which are cross-linked to proteins from ghosts prepared from 1.0 ml of packed cells.

distribution of the PE and PS in the various dinitrophenyl derivatives including cross-linking of PE to PE, PE to PS, PS to PS, PE to protein and PS to protein is shown in Table 3. This data was calculated for the reaction with

Table 3

Distribution of Dinitrophenyl Derivatives of PE and PS in Erythrocyte Ghosts Reacted with 100 μM DFDNB[a].

Cross-linked species	nmoles	Percent
PE as PE-DNP-PE	348	49[b]
PE as PE-DNP-PS	178	25[b]
PE as FDNP-PE	159	22[b]
PE as Protein-DNP-PE	27[d]	4[b]
PS as PS-DNP-PS	92	21[c]
PS as PS-DNP-PE	178	41[c]
PS as FDNP-PS	19	4[c]
PS as Protein-DNP-PS	143	33[c]

a These calculations are based on the data in Table 2 and Figure 2 at the DFDNB concentration of 100 μM. At this concentration all the amino groups of PE and PS have reacted with DFDNB.
b Percent of the total amount of PE.
c Percent of the total amount of PS.
d Obtained by difference from the total of 1144 nmoles of amino-phospholipids present in the ghost sample as determined by analysis for total lipid P and using a PE/PS molar ratio of 1.65 (6).

100 μM DFDNB since all the PE and PS react with the probe although this concentration does not represent the optimal one for the various types of cross-linking of PE and PS. The data show that under these conditions 49% of the total PE is cross-linked to itself in contrast to 21% of the total PS which becomes cross-linked to itself. Cross-linking of PE to PS accounts for 25% of the total PE and 41% of the total PS. At 100 μM DFDNB only 22% of PE and 4% of PS remain as the mono-N-substituted derivatives. It is noteworthy that much more PS becomes cross-linked to protein than does PE. Thus only 4% of PE is cross-linked to protein in comparison to 33% of PS. Therefore of the total amount of amino-phospholipids which becomes cross-linked to protein ap-

proximately 84% is PS. This shows that in the erythrocyte membrane PS is more closely associated with protein than is PE. Thus a non-random distribution of PE and PS occurs in the erythrocyte membrane.

Previous work has concluded that PE and PS are asymmetrically distributed in the erythrocyte membrane, these phospholipids being localized primarily on the inner surface of the membrane (7,8). The present studies now indicate that one-third of the PS is closely associated with protein. This type of nearest neighbor analysis is consistent with an appreciable amount of lipid bilayer in the erythrocyte membrane and that the bilayer contains more PE than PS. Our results demonstrate that PE and a certain fraction of PS are sufficiently close to each other in the membrane to become cross-linked but that an appreciable fraction of PS is closely associated with protein and does not mix freely with PE. These results are consistent with a non-random distribution of PE and PS in the membrane. The erythrocyte membrane appears to contain an asymmetric lipid bilayer rich in PE. The close association of PS with proteins indicates certain proteins which have a high affinity for PS and which may have distinct functional or structural properties.

Similar results on phospholipid cross-linking are obtained with difluorodinitrodiphenylsulfone at a concentration of 50 μM. This concentration represents the limit of solubility of this probe in the bicarbonate buffer system used in these studies. These studies and the synthesis of the dinitrodiphenylsulfone derivatives of serine and ethanolamine will be submitted for publication elsewhere.

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