CROSS-LINKING OF PHOSPHOLIPIDS IN HUMAN ERYTHROCYTE MEMBRANE

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SUMMARY

Treatment of human erythrocyte membranes with a short (5 Å) bifunctional reagent produced only monosubstituted products of amino-phospholipids. Treatment with longer reagents (9 Å and 18 Å) produced 24% and 33% of cross-linked PE molecules, respectively. No cross-linked products containing PS molecules could be isolated. These data are consistent with a view that PE and PS molecules are largely located in lipoprotein complexes at the inner surface of the membrane rather than in the lipid bilayer.

It has recently been reported (1) that 1,5-difluoro-2,4-dinitrobenzene, a bifunctional arylating reagent, was able to cross-link only 5% of the total amino-phospholipids in human erythrocyte membrane. The remaining amino-phospholipids were also modified by the reagent but the products were monosubstituted. The low yield of the cross-linked products was attributed to a possibility that amino-phospholipids were largely found as components of lipoprotein subunits at the inner surface of the membrane and only a small fraction was present in the bilayer.

We have conducted similar experiments with human erythrocyte membrane using three bifunctional reagents, FFDNB*, FFDS and FDPC, having different distances between the reactive centers, and have obtained with the latter two reagents a higher yield (24-33%) and a different distribution of the cross-linked products (2).

*Abbreviations: FFDNB, 1,5-difluoro-2,4-dinitrobenzene; DNP-, 2,4-dinitrophenyl group; DPE, 2,4-dinitrophenylene group; FFDS, 4,4'-difluoro-3,3'-dinitrodiphenylsulfone; FDPC, N-(3-fluoro-4,6-dinitrophenyl)-cystamine; PE, phosphatidyl ethanolamine; PS, phosphatidyl serine; PC, phosphatidyl choline; Sph, sphingomyelin; isotonic buffer, 5 mM sodium phosphate-0.15M sodium chloride, pH 8.0.

MATERIALS AND METHODS

Whole human blood or separated red blood cells were obtained from Northeastern New York Red Cross Blood Center, Albany, N. Y. Erythrocyte membranes were prepared according to the method of Fairbanks et al. (3). Samples of PE and PS were purchased from Sigma Chem. Co. Samples of FFDNB and FFDS were obtained from Pierce Chem. Co. and FDPC was synthesized in this laboratory. All other reagents used in this study were of analytical grade quality.

Treatment of lyophilized red blood cell membranes with bifunctional reagents. Samples (100 mg) of lyophilized membranes were suspended in 5 mM sodium phosphate-0.15 M sodium chloride, pH 8.0, buffer (100 ml) by magnetic stirring and were treated with an 0.1% methanolic solution of the bifunctional reagent. The solution of the reagent was added dropwise to a stirred reaction mixture during a period of five minutes. Final concentration of FFDNB (mol. wt. 204) was 382 μM and that of FFDS (mol. wt. 344) was 386 μM. These quantities of the two reagents correspond to a 2.7:1 molar ratio of the reagent to amino groups of PE and PS in the membrane. After addition of the reagent the reaction mixture was stirred at room temperature for 1 to 3 hours, then the contents were dialyzed first against the isotonic buffer at room temperature and then against water at 4°C and lyophilized.

The third reagent, FDPC (mol. wt. 336.2), as hydrochloride salt, was dissolved in 5 ml water and the resulting solution was added dropwise to a stirred membrane suspension. The final concentration of the reagent was 131 μM (1:1 molar ratio of the reagent to amino groups of PE plus PS). After 1 to 3 hours of stirring 1 ml of β-mercaptoethanol was added, the contents were kept at room temperature overnight, then dialyzed against water and against the isotonic buffer. The membrane suspension in the buffer was stirred in air in the presence of 50 μM o-phenanthroline and 10 μM $CuSO_4$ (4) during several hours and then dialyzed and lyophilized.

Treatment of PE and PS with bifunctional reagents. Samples (10 mg) of PE and PS were suspended separately in 20 ml of the isotonic phosphate buffer, pH 8.0, or in 20 ml of 0.2% sodium bicarbonate prepared in ethanol-water mixture (1:1, by volume) and treated with one of the bifunctional reagents using the same conditions as those described for the treatment of the erythrocyte membrane. After several hours of stirring at room temperature, the contents were concentrated by rotary evaporation. The residues were dissolved in ethanol and subjected to thin-layer chromatography (see below). The yellow bands corresponding to the reaction products were isolated and quantitated by spectrophotometry and by phosphorus analysis (5).

Thin-layer chromatography and quantitation of the modified amino-phospholipids. Thin-layer plates were prepared from a slurry of Silica G powder (18 g) and Florisil (2 g) in 65 ml of water. This quantity was sufficient for three 20 x 20 cm thin-layer plates. The plates were activated by heating at 120°C for 20 min, then cooled at room temperature for 30 min before use. 100 μg to 10 mg samples of the chloroform-methanol (2:1, by volume) lipid extract was applied per plate and the chromatography was performed with chloroform-methanol-water mixture (65:25:4, by volume) during 50 to 70 min. The yellow bands corresponding to the reaction products of PE or PS were scraped from the TLC plates, extracted with ethanol, the extract centrifuged, and the supernatant was used for spectrophotometric quantitation of the reaction products. Appropriate derivatives of the model compounds prepared from commercial PE and PS by treatment with the three bifunctional reagents were used as markers for TLC and as standards for spectrophotometry. Unreacted PE and PS were visualized on thin-layer plates by ninhydrin spraying followed by brief heating at 90-95°C.

RESULTS

The results obtained after treatment of the erythrocyte membrane with the three reagents are shown in Fig. 1 and in Table I. With FFDNB only monosubstituted products of PE and PS were isolated in 22% and 12% yield, respectively. The second fluorine atom in these products was hydrolyzed to phenolic hydroxyl groups whose ultraviolet absorption spectra were sensitive to pH (curves 1 and 2, Fig. 1). The yields of the reaction products were based on the total contents of PE and PS in the membrane (9.6% and 2.6% by weight of the dry membrane, respectively) (6).

With FFDS, a cross-linked product containing two PE molecules was isolated in 24% yield. Its R_F value on thin-layer chromatograms and the ultraviolet

TABLE I. Lipid derivatives isolated from treated human erythrocyte membrane

Compound	R_F	EtOH λ_{max}	$\varepsilon_{max} \times 10^{-4}$ ($M^{-1} \cdot cm^{-1}$)	Yield (%)
(OH)DNP-PE	0.98	332	1.5	22
(OH)DNP-PS	0.54	332	1.5	12
N,N-DS-bis-PE	0.96	290	3.4	24
N,N-DPC-bis-PE	0.30	320	3.2	33

Samples (100 mg) of intact or cholesterol-depleted erythrocyte ghosts were suspended in 100 ml of 5 mM sodium phosphate - 0.15 M sodium chloride, pH 8.0, buffer and treated with 0.1% methanolic solution of FFDNB or FFDS (molar ratio of the reagents to PE + PS was 2.7:1) or with an aqueous solution of FDPC hydrochloride (molar ratio approximately 1:1). After completion of the reaction (1-3 hours) the contents were dialyzed and lyophilized. Lipids were extracted with chloroform-methanol (2:1, by volume) and the extracts chromatographed on Silica G thin-layer plates with chloroform-methanol-water (65:25:4, by volume). The bands corresponding to the reaction products were isolated and quantitated spectrophotometrically. The reaction mixture containing FDPC reagent was treated overnight with 1 ml β-mercaptoethanol, dialyzed, and subsequently stirred in air in the presence of 50 μM o-phenanthroline and 10 μM $CuSO_4$. After dialysis and lyophilization, lipids were extracted and analyzed by TLC and by spectrophotometry. The yields of the products are based on the total contents of PE (9.6%) and PS (2.6%) of the dry membrane (6).

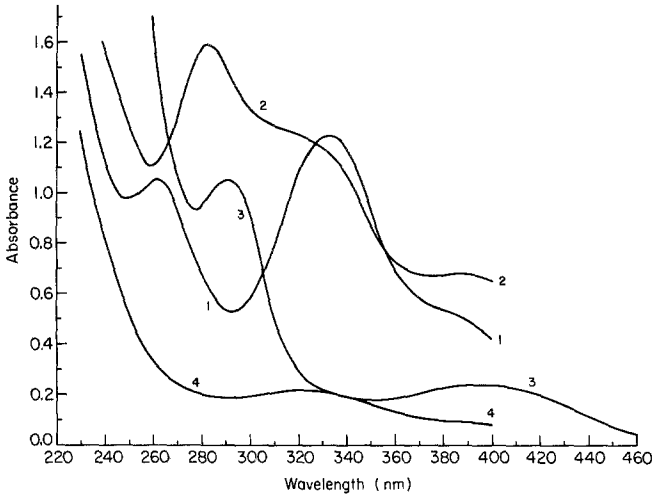


Figure 1 - Absorption spectra of the lipid derivatives isolated from treated erythrocyte membranes. Spectra were recorded in ethanol. Curve 1, 82 μM (OH)DNP-PE; curve 2, 82 μM (OH)DNP-PE in basic ethanol, pH 10; curve 3, 32 μM N,N-DS-bis-PE; curve 4, 7.5 μM N,N-DPC-bis-PE.

absorption spectrum which was insensitive to H^+ ion concentration in the 4-10 pH range (curve 3, Fig. 1), was identical with that of the model compound, N,N-DS-bis-PE (ε at 290 nm was 3.4×10^4 $M^{-1} \cdot cm^{-1}$ in ethanol). No cross-linked product containing two PS molecules or a product containing one PE and one PS molecule could be isolated.

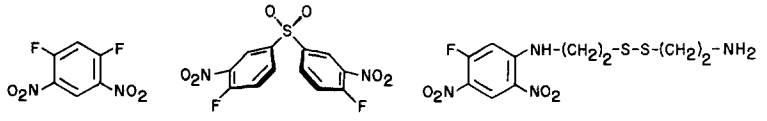
The highest yield (33%) of the cross-linked product containing two PE molecules was produced by the treatment of the membrane with FDDC reagent. Its R_F value on thin-layer plates and the ultraviolet absorption spectrum, which was also insensitive to H^+ ion concentration in the 4-10 pH range (curve 4, Fig. 1), was identical to that of the model compound, N,N-DPC-bis-PE (ε at 320 nm was 3.2×10^4 $M^{-1} \cdot cm^{-1}$). No cross-linked products containing PS molecules could be isolated.

When aqueous suspensions of either PE or PS were treated with one of the three reagents both types of products, monosubstituted and disubstituted, were isolated by thin-layer chromatography. The relative amounts of the two products

depended on the molar ratio of the reagent to PE or PS used during the reaction. For example, using a 0.4:1 molar ratio of FFDNB to PE or PS approximately 70% of N,N-DPE-bis-PE (λ_{max} in ethanol was 327 nm with an ε value of 3.0×10^4 $M^{-1} \cdot cm^{-1}$) and about 20% of N,N-DPE-bis-PS (λ_{max} in ethanol was 328 nm with an ε value of 3.0×10^4 $M^{-1} \cdot cm^{-1}$) was isolated. With a 1.7:1 molar ratio of the reactants approximately 30% of (OH)DNP-PE, 80% of (OH)DNP-PS and trace amounts of the corresponding disubstituted products were isolated.

DISCUSSION

The low yield (5%) of the cross-linked products obtained from the amino-phospholipids after treatment of the erythrocyte membrane with FFDNB (molar ratio of FFDNB to amino groups of PE plus PS, 19:1; final concentration of FFDNB in the reaction mixture, 2 mM) was attributed by Marinetti et al. (1) to a predominant location of PE and PS molecules in the lipoprotein complexes at the inner surface of the membrane rather than in the phospholipid bilayer. In our cross-linking experiments of the membrane with the same reagent but under different experimental conditions (molar ratio of FFDNB to amino groups of PE plus PS, 3:1; final concentration of the reagent in the reaction mixture, 380 μM) we were unable to isolate cross-linked products but only monosubstituted products (OH)DNP-PE and (OH)DNP-PS, in 22% and 12% yield, respectively. On the other hand, when FFDNB was used for cross-linking of PE or PS in aqueous suspensions, both monosubstituted and the cross-linked products were isolated.



FFDNB FFDS FDPC

Figure 2 - Amino group specific bifunctional reagents with different separations between the two reactive centers. FFDNB, 5 Å; FFDS, 9 Å; FDPC (after cleavage of β-mercaptoethylamine fragment and reoxidation, see Fig. 4), 18 Å.

With a 0.4:1 molar ratio of FFDNB to PE or PS predominantly cross-linked products (70% of PE to PE and 20% PS to PS) were obtained. With a 1.7:1 ratio predominantly monosubstituted products (30% from PE and 80% from PS) were produced. The absence of the cross-linked products after treatment of the membrane under conditions of low (3:1) molar ratio of the reagent to amino-phospholipids supports the conclusion reached by Marinetti *et al.* concerning the location of amino-phospholipids in the membrane (1).

However, it was possible to obtain cross-linked products of amino-phospholipids after treatment of the membrane with bifunctional reagents having greater distances between the reactive centers than FFDNB. The structures of the three reagents used are shown in Fig. 2. The distances between the two fluorine atoms in FFDNB, FFDS and FDPC are approximately 5 Å, 9 Å and 18 Å. FFDNB, the shortest bifunctional reagent, was unable to cross-link amino-phospholipids but FFDS and FDPC did cross-link PE molecules to the extent of 24% and 33%, respectively. No cross-linked products between two PS molecules or between PE and PS could be

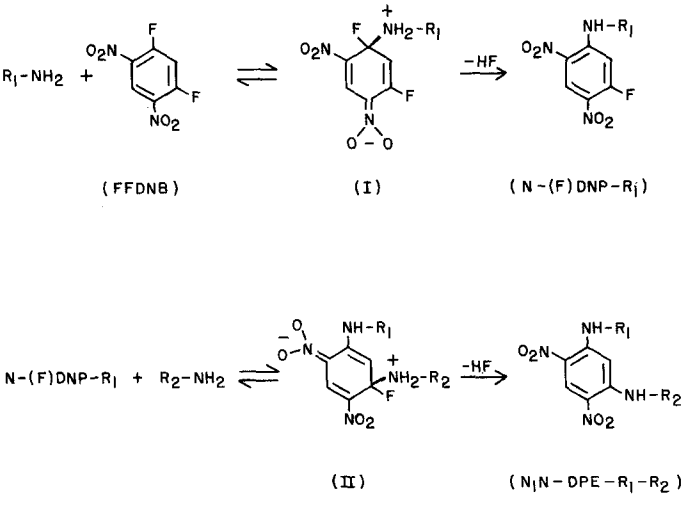


Figure 3 - Two consecutive SN_2-type reactions of FFDNB with amino groups of phospholipids (R_1 = R_2 = PE or PS) showing the nature of the transition states I and II.

isolated. This result suggests that the distribution of PE and PS molecules might be different in the membrane.

Cross-linking of the amino-phospholipids with the three reagents occurs by two distinct mechanisms. FFDNB and FFDS are neutral molecules and are able to penetrate the thickness of the membrane. Their reaction with amino groups of phospholipids occurs by the mechanism outlined in Fig. 3. It consists of two consecutive SN_2-type reactions (7). The FDPC reagent is monofunctional and it is positively charged. Therefore, it would not be expected to penetrate the membrane as easily as the other two reagents. Its reaction with amino-phospholipids is likely to be confined to the membrane surfaces. The reaction steps of the FDPC reagent leading ultimately to a cross-linked product are outlined in Fig. 4. The cross-linking step occurs at the level of the oxidation of a pair of neighboring sulfhydryl groups to a disulfide bond. This air-oxidation reaction

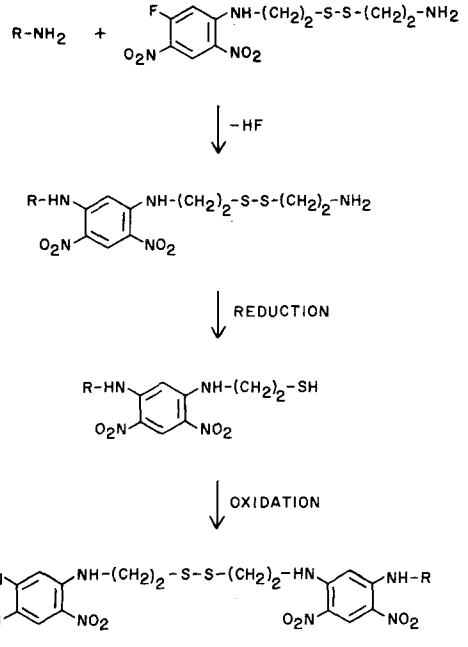


Figure 4 - Reaction scheme leading to cross-linking of amino groups of phospholipids (R = PE or PS) with FDPC reagent (see text for details).

can be accelerated by o-phenanthroline-Cu^{2+} chelate as was shown originally by Kobashi and Horecker (8) for oxidation of mercaptans to disulfides.

Several types of studies summarized by Bretscher (9) have indicated that phospholipids, like proteins, are distributed asymmetrically across the erythrocyte membrane. PC and Sph were predominantly found in the outer surface whereas PE and PS were found in the inner surface of the membrane. The latter phospholipids did not seem to be located in the lipid bilayer because very little cross-linked products could be isolated after treatment of the membrane with FFDNB (1). The results obtained with the longer bifunctional reagents (FFDS and FDPC) showed that PE molecules but not PS molecules could be cross-linked. In order to achieve such a cross-linking the lipoprotein complexes carrying PE molecules must be able to approach one another in the membrane to within approximately 9 Å since this was the distance between the reactive centers of the successful cross-linking reagent.

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REFERENCES

1. Marinetti, G. V., Sheeley, D. S., Baumgarten, R. and Love, R. (1974) Biochem. Biophys. Res. Commun. 59, 502-507.
2. Marfey, S. P. and Tsai, K. H. (1974) Federation Proc. 33, 1592.
3. Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606-2617.
4. Steck, T. L. (1972) J. Mol. Biol. 66, 295-305.
5. Bartlett, G. R. (1959) J. Biol. Chem. 234, 466-468.
6. Hanahan, D. J. (1969) in Red Cell Membrane, Structure and Function (Jamieson, G. A. and Greenwalt, T. J. eds.) p. 84. J. B. Lippincott Co., Philadelphia.
7. Berg, H. C., Diamond, J. M. and Marfey, P. S. (1965) Science 150, 64-67.
8. Kobashi, K. and Horecker, B. L. (1967) Arch. Biochem. Biophys. 121, 178-186.
9. Bretscher, M. S. (1973) Science 181, 622-629.