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Chemical Synthesis of Dinitrodiphenylsulfone Derivatives of Ethanolamine and Serine and its Application to the Study of Neighbor Analysis of Amino-Phospholipids in the Erythrocyte Membrane

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Summary. The dinitrodiphenylsulfone derivatives of serine and ethanolamine have been prepared and their chromatographic and spectral properties are described. This cross-linking agent was used to determine the neighbor analysis of amino-phospholipids in the erythrocyte membrane. The results with erythrocyte ghosts show that at 50 μM probe 31–50% of the total phosphatidylethanolamine is cross-linked to itself and 10–12% of the phosphatidylethanolamine is cross-linked to phosphatidylserine. Approximately 10–12% of the phosphatidylserine is cross-linked to itself and 16–20% of phosphatidylserine is cross-linked to phosphatidylethanolamine. The cross-linking of amino-phospholipids of ghosts with difluorodinitrodiphenylsulfone (9 Å span) is compared with cross-linking of these phospholipids by difluorodinitrobenzene (5 Å span). It is important to use the same sample of ghosts for this type of study since biological variability was seen in ghosts prepared from different batches of stored blood.

In a previous paper we reported the chemical synthesis of dinitrophenyl derivatives of ethanolamine and serine (Baumgarten, Marinetti & Love, 1974). These derivatives were used as standard reference compounds for application to the study of the neighbor analysis of amino-phospholipids in the erythrocyte membrane (Marinetti, Baumgarten, Sheeley & Gordesky, 1973; Marinetti, Sheeley, Baumgarten & Love, 1974, Marinetti & Love, 1974). The use of difluorodinitrobenzene in the study of the erythrocyte membrane showed that a large fraction of the amino-phospholipids are cross-linked to themselves and to proteins. To continue the study of the mean distances separating these phospholipids in cell membranes we selected 4,4′-difluoro-3,3′-dinitrodiphenylsulfone (DDDS)[1] since this cross-linking agent spans a greater

1 *Abbreviations:* PE = phosphatidylethanolamine; PS = phosphatidylserine; DDDS = difluorodinitrodiphenylsulfone; DFDNB = difluorodinitrobenzene; BSA = bovine serum albumin; FDDS = fluorodinitrodiphenylsulfone; FDNP = fluorodinitrophenyl; FDNB = fluorodinitrobenzene; Et = ethanolamine; DNP = dinitrophenyl; DDS = dinitrodiphenylsulfone.

distance (9 Å) than does 1,5-difluoro 2,4-dinitrobenzene (DFDNB) (5 Å).

The synthesis of difluorodinitrodiphenylsulfone has been reported by Zahn and Zuber (1953). These workers used this probe for cross-linking silk fibroin (Zahn & Zuber, 1956). Zahn and Wegerle (1960) used this probe for cross-linking collagen. They also reported the synthesis of dinitrodiphenylsulfone derivatives of tyrosine, lysine and hydroxylysine.

Difluorodinitrodiphenylsulfone has been used subsequently for the intramolecular cross-linking of amino-tyrosyl residues at the active site of staphylococcal nuclease (Cuatrecasas, Fuchs & Anfinsen, 1969) and for intramolecular cross-linking of albumin (Wold, 1961). Recently the use of this probe for cross-linking phospholipids of the red cell has been published by Marfey and Tsai (1975).

Materials and Methods

I. 4-Ethanolamine-4'-fluorodinitrodiphenylsulfone (I) mol wt 385

344 mg (1 mmole) of DDDS (Pierce Chemical Co.), in 20 ml of acetone and 61 mg (1 mmole) of ethanolamine (Eastman Kodak) in 6 ml of water containing 200 mg of $NaHCO_3$ were slowly mixed. The ethanolamine solution was added slowly in increments to the solution of DDDS. After reacting for 2 hr at 23 °C, the mixture was treated with excess 6 N HCl and extracted two times with 40 ml aliquots of ethyl acetate. TLC analysis of the ethyl acetate extracts on silica gel plates (Merck & Co. Darmstadt SG 5763) using chloroform/methanol/water 65:25:4 v/v showed one major spot for the monoethanolamine product I [relative mobility (R_f) 0.84], a very minor spot for the bisethanolamine product (R_f 0.71), and a trace of reagent (R_f 0.56). The yield of I was 370 mg (96% yield). Product I purified by TLC had a major peak in methanol at 291 nm and a minor peak at 405 nm. The molar extinction coefficient in methanol at 291 nm was 1.59×10^4.

II. 4,4'-diethanolamine-3,3'-dinitrodiphenylsulfone (II) mol wt 426

A solution of 344 mg (1 mmole) of DDDS in 20 ml of acetone was added slowly to a solution of 244 mg (4 mmoles) of ethanolamine in water containing 200 mg of $NaHCO_3$. After reacting for 2 hr at 23 °C the mixture was treated with excess 6 N HCl and extracted three times with 40 ml aliquots of ethylacetate to obtain 410 mg of product (96% yield). TLC analysis as described in *I*, showed one major spot R_f 0.71, a minor spot for the monoderivative (R_f 0.84) and a trace of reagent (R_f 0.56). Product II purified by TLC had a major peak at 298 nm (molar extinction coefficient 3.81×10^4) and a minor peak at 406 nm.

III. 4-seryl-4'-fluoro-3,3'-dinitrodiphenylsulfone (III) mol wt 429

A solution of 105 mg (1 mmole) of serine (Sigma Chemical Co.) in 6 ml of water containing 400 mg of $NaHCO_3$ was added slowly to a solution of 344 mg (1 mmole) of

DDDS in 20 ml of acetone. After reacting for 2 hr at 23 °C the mixture was acidified with excess 6 N HCl and extracted twice with 40 ml aliquots of ethyl acetate. Yield was 400 mg (93%). TLC analysis as described in *I* showed on major spot for III (R_f 0.30), a minor spot for the bis-serine derivative (R_f 0.02) and a trace of reagent (R_f 0.56). The product III purified by TLC had a major peak at 294 nm (molar extinction coefficient 1.48×10^4) and a minor peak at 408 nm (in methanol).

IV. 4,4'-diseryl-3,3'-dinitrodiphenylsulfone (IV) mol wt 514

344 mg (1 mmole) of DDDS in 20 ml of acetone were added to 20 ml of water containing 420 mg (4 mmoles) of serine and 400 mg of $NaHCO_3$. After reacting for 2 hr at 23 °C the mixture was acidified with excess 6 N HCl and extracted three times with 40 ml aliquots of ethyl acetate. Yield was 485 mg (94%). TLC analysis as described in *I* showed one major spot for IV (R_f 0.02), a trace of III (R_f 30) and trace of reagent (R_f 0.56). The product IV purified by TLC had a major peak in methanol at 302 nm (molar extinction coefficient 2.73×10^4 in 90% methanol-10% water) and a minor peak at 409 nm.

V. 4-ethanolamine-4'-seryl-3,3'-dinitrodiphenylsulfone (V) mol wt 470

344 mg (1 mmole) of DDDS in 10 ml of acetone were reacted with 61 mg (1 mmole) of ethanolamine in 6 ml of water containing 200 mg of $NaHCO_3$ (*see I* above). After reacting for 2 hr at 23 °C, 210 mg (2 mmoles) of serine in 10 ml of water containing 200 mg of $NaHCO_3$ were added. The mixture was reacted for 2 hr more, acidified with excess 6 N HCl and extracted three times with 40 ml aliquots of ethylacetate. TLC analysis showed a micture of compounds, the major one being product V (R_f 0.20), plus some bis-serine derivative IV (R_f 0.02), some bis-ethanolamine derivative II (R_f 0.71), some mono-ethanolamine derivative I (R_f 0.84) and a trace of mono-serine derivative III (R_f 0.30). Product V purified by TLC showed a major peak at 298 nm (molar extinction coefficient 3.27×10^4) and a minor peak at 406 nm in methanol.

Preparation of the methyl ester derivatives of III, IV, and V

Treatment of III, IV and V with methanolic-H_2SO_4 at 50 °C for 1 hr or with thionyl chloride in methanol for 2 hr at 23 °C yielded the methyl ester derivatives which were easily extracted into ethyl acetate. The methyl ester of III had an R_f of 0.81, a major peak at 290 nm and a minor peak at 396 nm. The dimethyl ester of IV had an R_f of 0.74, a major peak at 296 nm and a minor peak at 393 nm. The methyl ester of V had an R_f of 0.65, a major peak at 296 nm and a minor peak at 398 nm.

Solubility Properties of I–V

All derivative I–V are readily extracted from acidified aqueous solution by ethyl acetate. However only the ethanolamine derivatives I and II are extracted from an alkaline aqueous bicarbonate solution (pH 8.5) by ethyl acetate. This was observed for the dinitrophenyl derivatives of serine and ethanolamine described previously (Baumgarten *et al.*, 1974). Compounds I and III are very soluble in acetone and methanol. Compounds II and IV are more soluble in acetone than in methanol. The ethanolamine derivatives I and II are less soluble in aqueous bicarbonate solution in contrast to the serine derivatives which are quite soluble in aqueous bicarbonate solution.

Reaction of Erythrocytes and Ghosts with DDDS

One ml aliquots of packed human erythrocytes were suspended in 20 ml of 120 mM $NaHCO_3$ – 40 mM NaCl buffer pH 8.5. DDDS was added in 50 µl of acetone to give a final concentration of 50 µM. The reaction was carried out at room temperature for 15 hr. Less than 5% of the cells undergo lysis under these conditions. The cells were spun down at 2000 rpm and washed with 15 ml of buffer containing 0.5% bovine serum albumin (BSA). The cells were lysed in 15 ml of 10 mM Tris buffer pH 7.4 containing 1 mM EDTA. The ghosts were obtained by centrifugation at 20,000 rpm for 20 min. The lipids were extracted 3 times with chloroform/methanol 1:1. The extracts were evaporated to dryness, the lipid residue dissolved in 5 ml of chloroform/methanol 9:1 and passed through a cellulose column. The column was eluted with 40 ml of chloroform/methanol 9:1 and with 20 ml of methanol. The solvent was evaporated and the lipid residue suspended in 3 ml of 3 N HCl and hydrolysed for 2 hr at 100 °C. The hydrolysate was made alkaline with $NaHCO_3$ and extracted with ethyl acetate to obtain the ethanolamine derivatives. The extract was then made acid with HCl and extracted with ethyl acetate to obtain the serine derivatives (Baumgarten *et al.*, 1974). The ethyl acetate extracts were evaporated to dryness, the residues dissolved in chloroform/methanol 1:1 and run on silica gel plates using chloroform/methanol/water 65:25:4 v/v. The authentic standard compounds were run on the same plate. The various bands were eluted with methanol and their absorbances determined at the wavelengths of the major peak of each derivative.

Ghosts from 1 ml of packed cells were treated for 2 or 15 hr at 23 °C with 50 µM DFDNB, with 50 µM DDDS or with a saturating level (about 100 µM) of DDDS. Lipid extraction and analysis of the various dinitrodiphenylsulfone or dinitrophenyl derivatives of serine and ethanolamine were carried out as explained above.

Results

The properties of the ethanolamine and serine derivatives of DDDS are given in Table 1. The various derivatives have characteristic absorp-

Table 1. Properties of the dinitrodiphenylsulfone derivatives of serine and ethanolamine[a]

Dervative	Major peak (nm)	Minor peak (nm)	Molar ext. coeff.	R_f[b]
I monoethanolamine	291	405	15,940	0.84
II bis-ethanolamine	298	406	38,100	0.71
III monoserine	294	408	14,800	0.30
IV bis-serine	302	409	27,300	0.02
V mixed (ethanolamine-serine)	298	406	32,700	0.20
Methylester of III	290	396	–	0.81
Dimethyl ester of IV	296	398	–	0.74
Methyl ester of V	296	398	–	0.65

[a] The spectra and extinction coefficients were determined in methanol for all compounds except IV which was analyzed in 90% methanol-10% water. The molar extinction coefficients were measured at the maximum absorption of the major peak.

[b] The R_f values were determined by thin layer chromatography as explained in the text.

tion bands and extinction coefficients and all can be separated by thin layer chromatography. Furthermore, by ethyl acetate extraction from basic or acidic solutions, the serine derivatives can be separated from the ethanolamine derivatives, thereby facilitating their characterization and analysis.

The extent of cross-linking of phosphatidylethanolamine (PE) and phosphatidylserine (PS) in the membrane of the intact erythrocyte or in the isolated ghost is shown in Tables 2–5. DDDS at a final concentration of 50 μM is near optimal for cross-linking of PE to PE or PS to PS in ghosts but the reaction has not reached completion. However, with intact cells, the concentration of DDDS required for effective cross-linking is much higher since the hemoglobin acts as a large sink for the probe. Hence the absolute values in Table 2 for intact cells and ghosts are low since not all of the PE and PS has reacted. Indeed only 25% of the total aminophospholipids have reacted in the cells as compared to 67% in ghosts when the DDDS concentration is 50 μM. At saturating concentrations of DDDS, 95% of the PE and PS of ghosts reacts but the extent of cross-linking is decreased.

Table 2. Cross-linking of PE and PS in the membrane of intact erythrocytes and erythrocyte ghosts[a]

Derivative	nmoles[b]
Intact erythrocytes	
monoethanolamine I	17
bis-ethanolamine II	57
monoserine III	0
bis-serine IV	5
ethanolamine-serine V	30
protein bound PE+PS[c]	10
Ghosts	
monoethanolamine I	49± 3
bis-ethanolamine II	184±12
monoserine III	10± 1
bis-serine IV	27± 6
ethanolamine-serine V	88±37
protein bound PE +PS[c]	140

[a] The reaction conditions are explained in the text using 50 μM DDS. The reaction time was 15 hr at 23 °C.

[b] nmoles of each derivative I–V determined from their molar extinction coefficients. The values for intact erythrocytes (1 ml packed cells) are the average of duplicate analyses. The values for the ghosts (from 1 ml packed cells) are the mean ±SD of five analyses.

[c] The major phospholipid cross-linked to protein is PS.

Table 3. Distribution of dinitrodiphenylsulfone derivatives of PE and PS in Erythrocyte Ghosts

	Saturated[a] DDDS		50 μM DDDS	
	nmoles	percent[b]	nmoles	percent[b]
PE cross-linked to PE	292±20	40	368±24	50
PE cross-linked to PS	77± 8	10	88±37	12
PE as the mono-derivative	345±39	46	49± 3	7
PE cross-linked to protein[c]	15	2	15	2
PS cross-linked to PS	32± 4	7	54±12	12
PS cross-linked to PE	77± 8	17	88±37	20
PS as the mono-derivative	158±23	35	10± 1	2
PS cross-linked to protein[c]	155	34	140	31
PE not reacted[c]	14	2	23	30
PS not reacted[c]	29	6	159	15

[a] Saturated DDDS means excess undissolved probe was present in the reaction mixture. We estimate that saturation occurs at about 100 μM DDDS. The reaction time was 15 hr at 23 °C. The reaction conditions are given in the text.

[b] Percent of total nmoles of PE (743 nmoles) or PS (451 nmoles) in the ghosts from 1 ml of packed cells sample. Values represent the mean ±SD of five analyses.

[c] Estimated by the difference from the total of 1194 nmoles of PE plus PS present in the ghost sample (ghost from 1 ml of packed cells) and using a PE/PS molar ratio of 1.65. The amount of lipid P cross-linked to protein was determined by analysis for total P of ghosts extracted with chloroform/methanol 1:1 and subtracting the value of control ghosts from ghosts treated with cross-linking agent.

Marfey and Tsai (1975) reported 24% of crosslinking of PE to PE of red cell ghosts using DDDS. These workers found no reaction with PS. Under our experimental conditions using 50 μM DDDS we find that from 31–50% of the total PE molecules are cross-linked to themselves and 10–12% of the PE is cross-linked to PS. We also find cross-linking from 7–12% of PS to PS and some noncross-linked monoderivatives of PE and PS. The relative amount of cross-linked phospholipids *vs.* the noncross-linked phospholipids is dependent on the concentration of cross-linking agent as shown previously for DFDNB (Marinetti & Love, 1974). At saturating concentrations of DDDS, nearly all the PE and PS of ghosts react but the extent of crosslinking is decreased (Table 3). The differences seen in the extent of cross-linking of the aminophospholipids in Tables 2–5 is due primarly to biological variation in the age and condition of the cells since these cells were obtained from the Red Cross Blood Bank and they varied in storage time. Therefore, in comparing the cross-linking capability of DDDS with DFDNB under

Table 4. Distribution of dinitrodiphenylsulfone and dinitrophenyl derivatives of PE and PS in Erythrocyte Ghosts[a]

	DDDS		DFDNB	
	nmoles[b]	percent[c]	nmoles[b]	percent[c]
PE cross-linked to PE	298±10	31	416±22	44
PE as the mono-derivative	23± 1	2.4	60±13	6
PE cross-linked to PS	91± 5	10	153± 8	16
PE cross-linked to protein[d]	19	2	19	2
PE not reacted[d]	517	54	300	32
PS cross-linked to PS	38± 2	7	50± 4	9
PS cross-linked to PE	91± 5	16	153± 8	27
PS as the mono-derivative	8± 1.0	1.4	6± 1	1.1
PS cross-linked to protein[d]	197± 8	34	139±18	24
PS not reacted[d]	241	42	227	39

[a] Data from the reaction of ghosts from 1.0 ml packed cells with 50 μM DDDS or 50 μM DFDNB as explained in the text. Reaction time was 15 hr at 23 °C. The same batch of ghosts was used for both probes.

[b] These values (the mean ±SD of 4 analyses) represent the nmoles of PE or PS in the isolated derivatives from 1 ml of packed cells or ghosts from 1 ml of packed cells.

[c] These values represent the percent of total PE or PS present in the ghosts as obtained by P analysis and using a PE/PS molar ratio of 1.65. The total nmoles of PE and PS in the ghost sample are 948 and 575. At 50 μM probe there is insufficient probe for the reaction to go to completion. This concentration of probe was chosen to optimize cross-linking.

[d] Estimated by difference from total nmoles of PE plus PS present in the ghost sample and using a PE/PS molar ratio of 1.65. The amount of lipid P cross-linked to protein was determined by analysis of the total P of control ghosts and ghosts treated with each probe after the ghosts were exhaustively extracted with chloroform-methanol to remove noncovalently bound phospholipid.

similar conditions, we chose a concentration of 50 μM probe and did the experiments on ghosts from the same batch of red cells. It is seen in Table 4 that 31% of the total PE is crosslinked to itself with DDDS compared to 44% with DFDNB. Moreover, the extent of cross-linking of PE to PS is greater with DFDNB than with DDDS. The amount of cross-linking of PS to PS is about the same for both probes. With both probes the major phospholipid which is cross-linked to protein is PS, but with DDDS this accounts for 34% of the total PS as compared to 24% with DFDNB.

The reaction of ghosts with DDDS and DFDNB was carried out for 2 and 15 hr at two concentrations of each probe. The data in Table 5 shows that with both probes at either 50 or 100 μM concentration there is more PE crosslinked to PE at 15 hr as compared to the 2-hr time.

Table 5. Distribution of dinitrophenyl and dinitrophenylsulfone derivatives of PE and PS in erythrocyte ghosts[a]

	50 μM Probe				100 μM Probe			
	DFDNB		DDDS		DFDNB		DDDS	
	2 hr	15 hr	2 hr	15 hr	2 hr	15 hr	2 hr	15 hr
	(nmoles)		(nmoles)		(nmoles)		(nmoles)	
PE cross-linked to PE	252±12	416±22	142±5	298±10	286±44	320±20	194± 8	274±
PE as the mono-derivative	117±10	60±13	102±1	23± 1	268±25	213±12	216±32	103±2
PE cross-linked to PS	72± 8	153± 8	36±6	91± 2	131±25	135±11	53± 2	66±
PS cross-linked to PS	24± 4	50± 4	10±1	38± 2	78±13	64± 5	24± 1	25±
PS as the mono-derivative	16± 1	6± 1	10±2	8± 1	35± 7	30± 6	78± 5	27±

[a] The reaction conditions are explained in the text. The ghosts used for the 100 μM concentration of probe had a total of 1080 nmoles of PE and 650 nmoles of PS. The ghosts used for the 50 μM concentration of probe had a total of 948 nmoles of PE and 575 nmoles of PS. Appreciable cross-linking of PS to protein and a small amount of cross-linking of PE to protein also occurs. The 50 and 100 μM concentrations of probe are below the saturating level and thus not all the PE and PS react. These concentrations of probe were chosen to optimize cross-linking of the amino-phospholipids. Values represent the mean ±SD of four analyses. These values are the nmoles of PE or PS in each derivative obtained from ghosts from 1 ml of packed cells.

There is a concomitant drop in the mono-derivative of PE indicating that the reaction of the second fluorine with a neighboring PE molecule occurs at a slower rate than the reaction of the first fluorine. At the longer time there is also more PE cross-linked to PS and more PS cross-linked to PE but this is seen only at 50 μM probe. At 100 μM probe there is a greater total number of molecules of PE and PS which react as compared to 50 μM probe but the optimal amount of cross-linked products varies both with time and concentration and with the particular phospholipid. We have observed a similar finding for the cross-linking of PE and PS in the erythrocyte membrane by DFDNB (Marinetti & Love, 1974). It is obvious that a detailed study of the time of reaction at different concentrations of probe will be required to provide sufficient data which might elucidate the topology of these amino-phospholipids

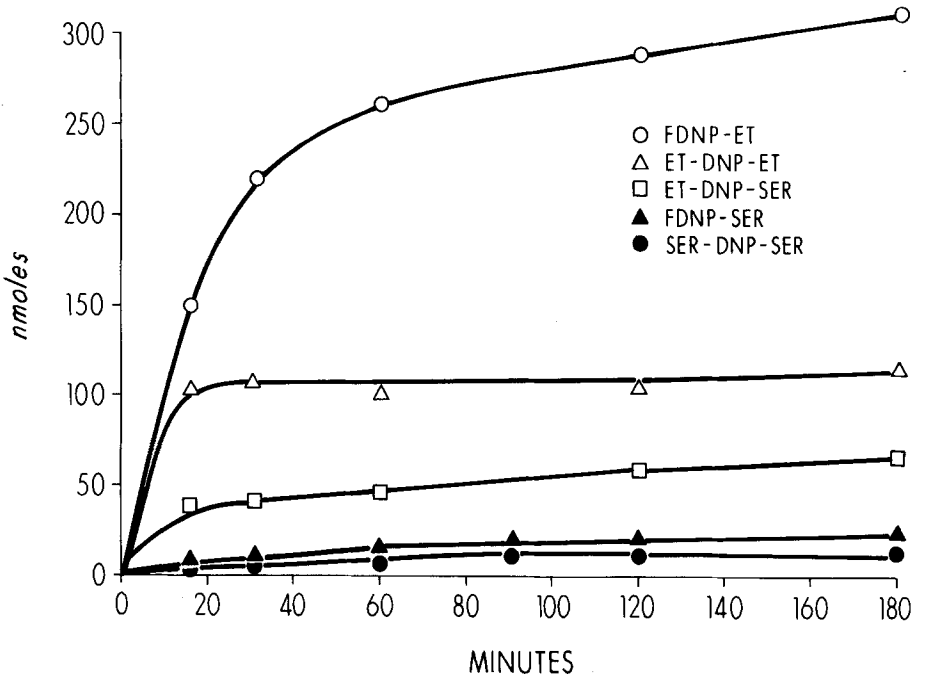

Fig. 1. Time-course reaction of PE and PS of erythrocyte ghosts with DFDNB. Ghosts from 1 ml of packed red cells were reacted with 100 μM DFDNB in 20 ml of 120 mM $NaHCO_3$–40 mM NaCl buffer pH 8.5 at 23 °C for different time periods. The analysis of the various derivatives of PE and PS with DFDNB was carried out as given in the text

in cell membranes. The rate of reaction of PE and PS with these probes must be rate limiting relative to the rate of lateral motion of these lipids in the bilayer. However, PE and PS may move as clusters in the bilayer as well as individual molecules. In any case, in order for these phospholipids to become cross-linked they must have been neighbors long enough for the probes to react.

The time course of reaction of DFDNB and DDDS with ghosts was studied. The reaction profiles are shown in Figs. 1 and 2. The initial 15 min net rate of formation of FDDS-Et is higher than that of FDNP-Et; however, after 120 min the yield of FDNB-Et is higher than the yield of FDDS-Et. The initial rates of formation of the other derivatives are approximately the same except for the bis-ethanolamine compound representing PE cross-linked to PE. The initial net rate of formation of Et-DNP-Et is higher than that of Et-DDS-Et although by 120 min the yields are about the same.

Although it is desirable to know the absolute rates of reaction of membrane-bound PE and PS with the first and second fluorine atom

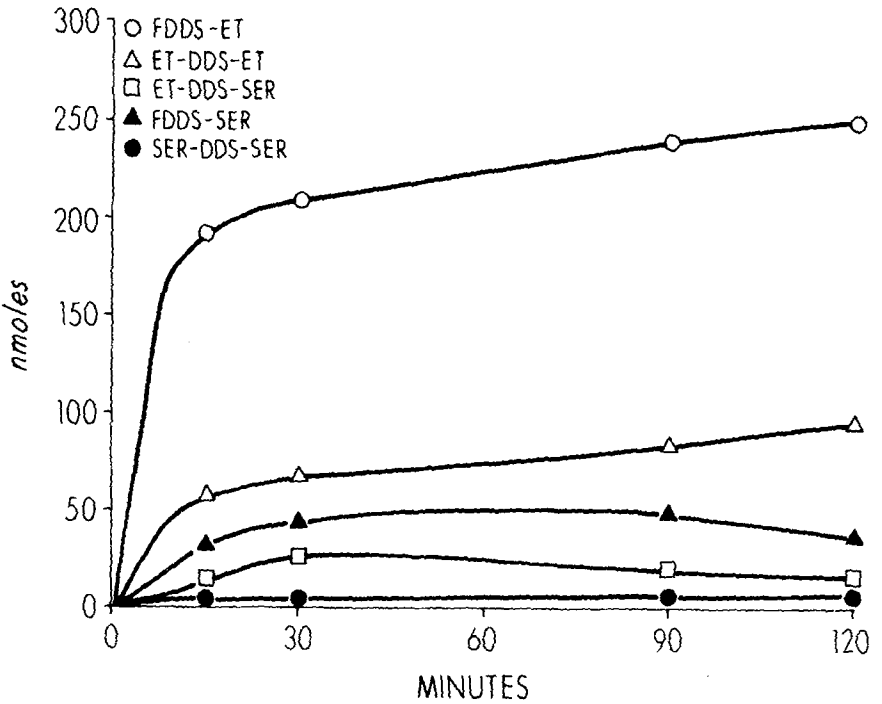

Fig. 2. Time-course reaction of PE and PS of erythrocyte ghosts with DDDS. Ghosts from 1 ml of packed cells were reacted with 100 μM DDDS as explained in Fig. 1

of DFDNB and DDDS, the complexity of the reactions shown in Fig. 3 make this very difficult to realize. Only time average net rates of formation of each compound can be obtained. Our previous work on the synthesis of the various derivatives of ethanolamine and serine with DFDNB and the present work on the synthesis of the derivatives of ethanolamine and serine with DDDS make it apparent that the amino group of these two compounds reacts very readily in bicarbonate buffer pH 8.5 with both the first and second fluorine atom of these chemical cross-linking agents. It was found that the first fluorine reacts faster than the second fluorine with both probes. Hence it appears safe to conclude that the differences seen in the net yield of the derivatives of PE and PS in the erythrocyte membrane is due to the unique arrangement of these lipids in the membrane. Studies on liposomes made only of pure PE and PS, with mixtures of PE and PS, and with mixtures of PE and PC and PS and PC may serve as good model systems to get more detailed information on rates of reaction of PE and PS with these cross-linking probes. It would be imperative to produce liposomes

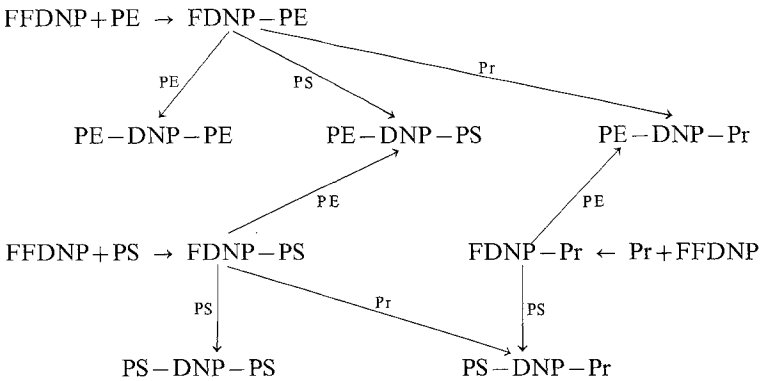

Fig. 3. Multiple Reactions of PE, PS and proteins of erythrocyte ghosts with DFDNB. This figure points out the complexity of reactions of DFDNB with phosphatidylethanolamine, phosphatidylserine and proteins of the erythrocyte membrane. FFDNP depicts difluorodinitrobenzene (DFDNB) to show the two reactive fluorine atoms. PE, PS, and Pr are symbols for phosphatidylethanolamine phosphatidylserine and membrane protein, respectively

of uniform size and of known composition. Even with these model systems the extrapolation of the results to cell membranes will be difficult.

The difference in distance spanned by the reactive groups on these cross-linking agents (5 Å *vs.* 9 Å) appears to give a significant difference in the neighbor analysis. This may be due in part to a difference in the rate of reaction of PE and PS with each probe and also to steric factors involving the access of the probe to the aminophospholipids. These parameters are under current investigation. We conclude that these probes may be useful for determining the topological arrangement of phospholipids in cell membranes and for studying the alteration of this arrangement in cells of different age, and in cells perturbed by chemical and physical agents. DFDNB has the major advantage over DDDS since it is more soluble and can be used over a much wider concentration range.

The biological variation seen with stored red cells is most likely due to an effect of age on the integrity of the cell membrane. During aging the cells lose an appreciable amount of phospholipid and cholesterol (Weed & LaCelle, 1969). This loss can be expected to alter the membrane structure. The red cell lipids also vary between young and old cells (Westerman, Pierce & Jensen, 1963) and are lost by incubation at 37 °C (Cooper & Jandl, 1969). Incubation at 37 °C leads to a much greater loss of cholesterol than phospholipid. Approximately 18% of the red cell cholesterol can be lost after 24 hr incubation at 37 °C. Thus, during our longer time of incubation of 15 hr, some unavoidable alter-

ation may have occurred in the red cell membrane. For this reason we also ran experiments at 2 hr. The time of incubation becomes more critical with whole cells since the reaction of PE and PS is slower in whole cells due to competition between hemoglobin and phospholipids for the probe. With ghosts the shorter time interval is desirable and feasible since the reaction of PE and PS can be made to go near completion under conditions where cross-linking is optimized. One must keep in mind, however, that during the preparation of ghosts the cell membrane may have been altered. These are inherent obstacles and limitations which researchers must deal with when studying biological membranes. Our work, therefore, represents a preliminary excursion with chemical probes to elucidate the finer details of phospholipid topology in the red cell membrane.

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