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ARRANGEMENT OF PHOSPHATIDYLSERINE AND PHOSPHATIDYLETHANOLAMINE IN THE ERYTHROCYTE MEMBRANE

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Summary

Cross-linking of phosphatidylethanolamine and phosphatidylserine in the erythrocyte membrane with the reagent difluorodinitrobenzene was studied as a function of temperature, time and concentration of difluorodinitrobenzene. The optimal extent of cross-linking of phosphatidylethanolamine to phosphatidylethanolamine, phosphatidylethanolamine to phosphatidylserine and phosphatidylserine to phosphatidylserine was expressed as molar ratios of these three different cross-linked species. The experimental results were compared to different models of a phospholipid monolayer containing phosphatidylethanolamine and phosphatidylserine in which phosphatidylserine was arranged primarily as singles (having 6 phosphatidylethanolamine neighbors) as clusters of dimers, trimer and tetramers or as large clusters. In the various model monolayers each lipid component has 6 neighbors. The models which are consistent with the experimental results are those in which phosphatidylserine and phosphatidylethanolamine occur as small clusters in a non-random array.

Introduction

The asymmetric arrangement of phosphatidylserine and phosphatidylethanolamine in the erythrocyte membrane has been indicated by use of chemical probes [1—5] and phospholipases [6]. The amino-phospholipids phosphatidylserine and phosphatidylethanolamine are localized primarily on the inner surface of the membrane. The degree of cross-linking of phosphatidylethanolamine to phosphatidylethanolamine, phoaphatidylserine to phosphatidylserine and phosphatisylserine to phosphatidylethanolamine by difluorodinitrobenzene was shown by Marinetti and Love [7] to be dependent on the concentration of diflurorodinitrobenzene. This work demonstrated that optimal cross-linking of

Abbreviations: FDnp, fluorodinitrophyl; FDnb, fluorodinitrobenzene.

these phospholipids in red cell ghosts occurred in the concentration range of 50—150 μM difluorodinitrobenzene. This type of study made it possible to test several models as to the arrangement of these aminophospholipids in the membrane. Our data are consistent with the occurrence of clusters of dimers, trimers and tetramers of phosphatidylserine and phosphatidylethanolamine in a monolayer on the inner surface of the membrane. These results concur with data from physical chemical measurements indicating that clusters or lateral phase separation of phospholipids occur in membranes [8—15].

Methods and Reagents

The isolation of ghosts from human red cells and their reaction with difluorodinitrobenzene were carried out as described previously [7]. The analysis of the various cross-linked species was obtained by HCl hydrolysis of the extracted lipids, their extraction from acid or basic medium with ethyl acetate and their separation by thin-layer chromatography [16].

Results

A typical profile of the amount of cross-linked species of phosphatidylethanolamine and phosphatidylserine as a function of the concentration of difluorodinitrobenzene is shown in Fig. 1. Three such experiments were performed and the amount of each derivative was analyzed. The mean ± S.D. of Dnp-bis ethanolamine, Dnp-bis-serine and serine-Dnp-ethanolamine representing respectively the optimal cross-linking of phosphatidylethanolamine to phosphatidylethanolamine, phosphatidylserine to phosphatidylserine and phosphatidylethanolamine to phosphatidylserine are given in Table I. Also shown in

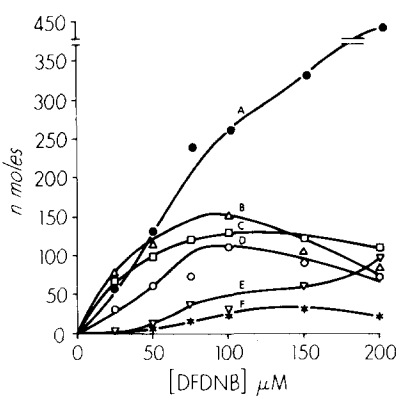

Fig. 1. Profile showing the extent of cross-linking of phosphatidylethanolamine and phosphatidylserine in the erythrocyte membrane as a function of the concentration of difluorodinitrobenzene. Ghosts from 1 ml of packed human erythrocytes were reacted with different concentrations of difluorodinitrobenzene in 20 ml of 120 mM $NaHCO_3$/40 mM NaCl pH 8.5 for 2 h. The analysis of the cross-linked species was carried out as described previously (7.16). A = FDnp-ethanolamine, B = Dnp-bisethanolamine representing phosphatidylethanolamine linked to phosphatidylethanolamine, C = phosphatidylserine cross-linked to protein, D = seryl-Dnp-ethanolamine, representing phosphatidylethanolamine cross-linked to phosphatidylserine. E = FDnp-serine, F = serine-Dnp-serine representing phosphatidylserine cross-linked to phosphatidylserine.

TABLE I

CALCULATED MOLAR RATIOS OF CROSS-LINKED DERIVATIVES OF PHOSPHATIDYLETHANOLAMINE AND PHOSPHATIDYLSERINE DERIVED FROM DIFFERENT MODELS

The models consist of a planar grid containing 136 total components of phosphatidylethanolamine + phosphatidylserine or phosphatidylethanolamine + phosphatidylserine + *Z* arranged as a monolayer with each component having six neighbors (except at the edge). *Z* = sphingomyelin + phosphatidylcholine.

Model I Array where the molar ratio phosphatidylethanolamine/ phosphatidylserine = 12 : 5	(PE—PS)/(PS—PS) *	(PE—PE)/(PS—PS) *
(a) Phosphatidylserine as 28 singles, 6 dimers	33.2	27
(b) Phosphatidylserine as 19 dimers, 2 singles	9.1	9.1
(c) Phosphatidylserine as 8 dimers, 6 tetramers	3.9	4.5
(d) Phosphatidylserine as 6 dimers, 7 tetramers	3.2	4.5
(e) Phosphatidylserine as 10 tetramers	2.5	3.8
(f) Phosphatidylserine as 5 heptamers, 1 pentamer	1.46	3.0
(g) Phosphatidylserine as 2 clusters of 20	0.62	2.6
Model II Array where the molar ratios of phosphatidylethanolamine/ phosphatidylserine/*Z* = 12 : 5 : 2.4		
(a) Phosphatidylserine as 25 singles, 5 dimers	29	25
(b) Phosphatidylserine as 12 dimers, 2 tetramers, 3 singles	5.9	5.9
(c) Phosphatidylserine as 11 trimers, 1 dimer	3.2	3.8
(d) Phosphatidylserine as 6 tetramers, 5 dimers, 1 single	2.9	3.8
(e) Phosphatidylserine as 8 tetramers, 1 dimer, 1 single	2.3	3.3
(f) Phosphatidylserine as 5 hexamers, 1 pentamer	2.8	3.6
(g) Phosphatidylserine as 7 pentamers	1.8	2.0
Experimental results **	3.24 ± 0.36	4.2 ± 0.4

* Calculated in the models from the frequency of nearest neighbors.

** These values represent the mean ± S.D. of three experiments using the optimal values for each cross-linked species.

the table are the molar ratios of these derivatives. In order to determine what arrangement of phosphatidylethanolamine and phosphatidylserine in a monolayer was consistent with the experiment data, several model systems were constructed. In one model, (Model I) a grid was arranged consisting of circles representing phosphatidylethanolamine and phosphatidylserine. In this grid the diameter of the circles was fixed since studies on monomolecular films have shown that the packing area of phosphatidylethanolamine and phosphatidylserine having similar fatty acids is about the same (39—41 $Å^2$ per molecule) [17]. The arrangement of phosphatidylethanolamine and phosphatidylserine in the models is such that each molecule has 6 neighbors. The molar ratio of phosphatidylethanolamine and phosphatidylserine was set a 12/5. This ratio was chosen from the molar ratio of phosphatidylethanolamine/phosphatidylserine in the red cell membrane of 1.6 [18] and correcting for 33% of phosphatidylserine and 4% of phosphatidylethanolamine which is cross-linked to membrane protein [7]. This fraction of phosphatidylethanolamine and phosphatidylserine is considered to be fixed boundary phospholipid which is unavailable to the

lipid bilayer. Hence Model I is based on a binary monolayer of phosphatidylethanolamine and phosphatidylserine localized on the inner surface of the membrane. Phosphatidylserine was distributed in the monolayer system as singles (having 6 phosphatidylethanolamine neighbors), in clusters of dimers, trimers and tetramers, or in large clusters. The neighbor frequency of phosphatidylethanolamine and phosphatidylserine was then calculated and represented as molar ratios of (PE—PS)/(PS—PS) and (PE—PE)/(PS—PS). These ratios were calculated on the basis of the neighbor frequency of these lipids in the grids. These values were then compared to the observed experimental values.

The same analysis was done using a different model (Model II) in which a ternary lipid system was employed. In this model, the phosphatidylethanolamine to phosphatidylserine molar ratio was 12/5 but the model also included a third lipid component *Z* representing lecithin and sphingomyelin so that the molar ratios of phosphatidylethanolamine/phosphatidylserine/*Z* was 12 : 5 : 2.4. This takes into account the fact that the inner layer of the red cell membrane contains about 30% of the total membrane phosphatidylcholine and 15% of the total membrane sphingomyelin [6]. Cholesterol is omitted in this model but is included in the third model. The third component was arbitrarily randomly arranged. In the third model, the ratio of phosphatidylethanolamine/phosphatidylserine/*Z* was changed to 12 : 5 : 17 to take into account the cholesterol content of the membrane [18]. In this model we assume that 1/3 of the cholesterol is on the inner half of the bilayer and 2/3 is on the outer half of the bilayer. This is based on the observation of Murphy [19] that cholesterol in the red cell membrane appears to be peripherally arranged on the cell membrane in a non-random fashion. In the fourth model the ratio of phosphatidylethanolamine/phosphotidylserine/*Z* is changed to 12 : 5 : 28 assuming that the cholesterol is divided evenly between the two halves of the lipid bilayer. In all models, the phosphatidylethanolamine and phosphatidylserine were placed into a variety of arrays and the neighbor frequency measured and the ratios shown in Tables I and II were calculated and compared to the experimental data. Two arrays are shown in Figs. 2 and 3. Fig. 2 shows an array of dimers and tetramers of phosphatidylserine whereas Fig. 3 shows an array of mainly single phosphatidylserines with a few dimers. Phosphatidylethanolamine also occurs as clusters.

The data shown in Tables I, II and III attempt to answer the question whether the experimental results on the cross-linking of the amino-phospholipids are consistent with a random or non-random array of these phospholipids on the inner half of the lipid bilayer. The experimental results were compared to several models in which the molar ratios of phosphatidylethanolamine/phosphatidylserine/*Z* were varied in a manner which considered only phosphatidylethanolamine and phosphatidylserine (Table I, model I) and which considered phosphatidylethanolamine, phosphatidylserine and a third component *Z* representing sphingomyelin, phosphatidylcholine and cholesterol. The ratios of phosphatidylethanolamine/phosphatidylserine varied from 12 : 5 to 12 : 8. The phosphatidylethanolamine/phosphatidylserine ratio of 12 : 5 considers only 2/3 of the total membrane phosphatidylserine to be in the monolayer, the other 1/3 being protein bound [7]. The phosphatidylethanolamine/phosphatidylserine ratio of 12 : 8 considers all the phosphatidylserine to be in the

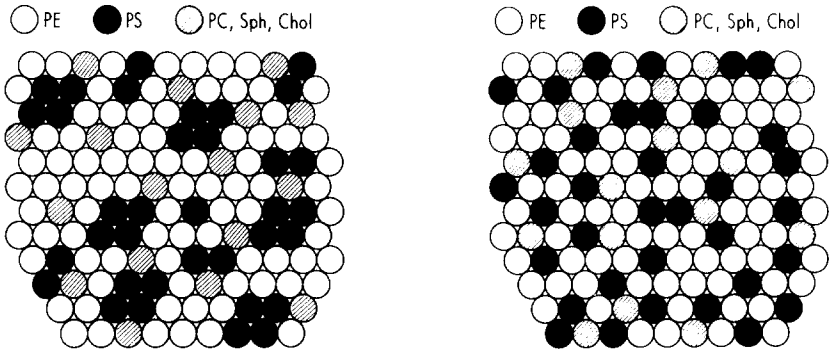

Fig. 2. A model of lipid monolayer containing phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (Sph), phosphatidylcholine (PC) and cholesterol (Chol) in which phosphatidylserine occurs as small clusters. This model shows an array of phosphatidylserine existing as 5 tetramers, 5 dimers and 1 single in a monolayer consisting or primarily phosphatidylethanolamine with some phosphatidylcholine, sphingomyelin and cholesterol. This is one example of models given in Tables I and II. Each lipid component has six neigbors (except at the edge).

Fig. 3. A model of a lipid monolayer containing phosphatidylethanolamine, phosphatidylserine, spingomyelin, phosphatidylcholine and cholesterol in which phosphatidylserine occurs primarily as singles and a few dimers. This model is one example of Models given in Tables I and II. Abbreviations as in Fig. 2.

monolayer. The amount of Z was varied to include no cholesterol ($Z = 2.4$ representing only sphingomyelin and phosphatidylcholine) and $Z = 17$ representing sphingomyelin, phosphatidylcholine and 1/3 of the total membrane cholesterol and $Z = 28$ representing sphingomyelin, phosphatidylcholine and 1/2 of the total membrane cholesterol. The amount of sphingomyelin and phosphatidylcholine was chosen as 85 and 70%, respectively of the total content of each of these lipids based on the work of Verkleij et al. [6].

The data in Table I shows that Models Ic, d, e and Models IIc, d are in closer agreement withj the experimental results that the other models. The data in Table II show that Models IIId, e and Model IVd are in closer agreement with the experimental results that the other models. In order to determine whether these models represent random or non-random arrays, experiments were carried out in which spherical colored beads of equal diameter, representing phosphatidylethanolamine, phosphatidylserine and Z, were randomly mixed in various ratios representing phosphatidylethanolamine, phosphatidylserine and Z. The neighbor frequency and molar ratios were then calculated. The results are shown in Table III. It is apparent the Models Ic, d, e, Model IIc, d, Models IIId, e and Model IVd in Tables I and II do not fit random arrays shown in Table III, yet these specific models in Tables I and II are consistent with the experimental results. Random arrays were found to contain mainly singles, dimers, trimers, tetramers and very few higher clusters of the amino phospholipids but the frequency of dimers, trimers, tetramers and higher clusters was dependent on the molar composition of these amino-phospholipids.

The data in Table III show that random arrays do not fit the experimental results. The experimental results are consistent with a non-random array of phosphatidylserine and phosphatidylethanolamine.

The time course of reaction of phosphatidylethanolamine and phosphatidylserine in red cell ghosts with 100 μM difluorodinitrobenzene at 23 and 37° C is

TABLE II

CALCULATED MOLAR RATIOS OF CROSS-LINKED DERIVATIVES OF PHOSPHATIDYLETHANOLAMINE AND PHOSPHATIDYLSERINE DERIVED FROM DIFFERENT MODELS

The models consist of a planar grid containing 136 total components of phosphatidylethanolamine and phosphatidylserine + *Z* arranged as a monolayer with each component having six neighbors (except at the edge). *Z* = spingomyelin, phosphatidylcholine and cholesterol.

Model III Array where the molar ratios of phosphatidylethanolamine& phosphatidylserine/*Z* = 12 : 5 : 17	(PE—PS)/(PS—PS)*	(PE—PE)/(PS—PS)*
(a) Phosphatidylserine as 1 dimer, 18 singles; phosphatidylethanolamine as singles, dimers, and trimers	44	27
(b) Phosphatidylserine as 10 dimers, phosphatidylethanolamine as singles, dimers, trimers	4.4	1.1
(c) Phosphatidylserine as 5 tetramers, phosphatidylethanolamine as singles, dimers, trimers	0.96	1.25
(d) Phosphatidylserine as 9 dimers, 2 singles; phosphatidylethanolamine as trimers, tetramers	3.66	4.44
(e) Phosphatidylserine as 4 trimers, 3 singles, 1 tetramer phosphatidylethanolamine as pentamers, hexamers	3.1	4.18
Model IV **Array where the molar ratios of Phosphatidylethanolamine/ phosphatidylserine/*Z* = 12 : 5 : 28**		
(a) Phosphatidylserine as 14 singles, 1 dimer; phosphatidylethanolamine as 5 dimers, 31 singles	10	6
(b) Phosphatidylserine as 5 trimers; phosphatidylethanolamine mainly as singles	1.17	0.17
(c) Phosphatidylserine as 5 trimers; phosphatidylethanolamine as pentamers and hexamers	1.73	2.87
(d) Phosphatidylserine as 3 trimers, 2 dimers, 2 singles; phosphatidylethanolamine as pentamers, hexamers	3.1	4.2
Experimental results **	3.24 ± 0.36	4.2 ± 0.4

* Calculated in the models from the frequency of nearest neighbors.

** These values represent the mean ± S.D. of three experiments using optimal values for each cross-linked species.

shown in Figs. 4 and 5, respectively. At 23° C the reaction profiles show a rapid reaction in the first 30 min followed by a slower reaction up to 180 min. However at 37° C the profiles are different since the formation of FDnp-ethanolamine and FDnp-serine reach a plateau and then begin to decline as they are converted to cross-linked species. The higher temperature allows the reactions to proceed fast enough so that the influence of the reaction of the second fluorine is manifest.

In an attempt to compare the rate of reaction of the first fluorine with the second, ghosts were pre-labeled for 15 min at 23° C with 100 μM difluorodinitrobenzene, washed, and reincubated in fresh buffer. The reaction profiles are shown in Fig. 6. It can be seen that as FDnp-ethanolamine declines there is a concomitant increase in the ethanolamine-Dnp-ethanolamine and in the ethano-

TABLE III

COMPARISON OF CROSS-LINKING DATA ON ERYTHROCYTE MEMBRANE PHOSPHOLIPIDS TO MODELS WITH RANDOM ARRAYS

The random arrays were produced by random mixing of three different colored spherical beads of equal diameter representing phosphatidylethanolamine/phosphatidylserine/*Z* in the ratios shown, keeping the total number of beads equal or close of 136. After thorough mixing the beads were scattered as a group on to a grid and allowed to settle into a rectangular array as shown in Figs. 2 and 3. The neighbor frequency was calculated from which the ratios shown in the table were calculated. The values represent the mean ± S.D. of 12 independent throws. Model (a) assumes only phosphatidylethanolamine and phosphatidylserine in the monolayer; models b—f consider the addition of a third component *Z* representing sphingomyelin, phosphatidylcholine and cholesterol. In models a—d only 2/3 of the phosphatidylserine is included assuming that the remaining 1/3 is tightly bound to membrane protein. In models (e) and (f) all the phosphatidylserine is included in the monolayer. In model (b), the cholesterol is not included and assumed to be sequestered away from the phospholipids. In midels (c) and (e), 1/3 of the total membrane cholesterol is assumed to be localized on the inner half of the lipid bilayer whereas in models (d) and (f), 1/2 of the total membrane cholesterol is considered to be localized on the inner half of the lipid bilayer. The model assumes no special interactions between phosphatidylethanolamine, phosphatidylserine and *Z*.

Random arrays	(PE—PS)/(PS—PS)	(PE—PE)/(PS—PS)
I. (a) phosphatidylethanolamine/phosphatidylserine = 12 : 5	4.87 ± 0.62	5.97 ± 0.50
II. (b) phosphatidylethanolamine/phosphatidylserine/*Z* = 12 : 5 : 2 : 4	4.64 ± 0.77	5.4 ± 0.50
III. (c) phosphatidylethanolamine/phosphatidylserine/*Z* = 12 : 5 : 17	4.69 ± 1.26	5.32 ± 1.14
IV. (d) phosphatidylethanolamine/phosphatidylserine/*Z* = 12 : 5 : 28	4.2 ± 0.91	5.15 ± 1.33
V. (e) phosphatidylethanolamine/phosphatidylserine/*Z* = 12 : 8 : 17	3.55 ± 0.71	2.53 ± 0.52
VI. (f) phosphatidylethanolamine/phosphatidylserine/*Z* = 12 : 8 : 28	3.03 ± 0.75	2.82 ± 0.70
Experimental results	3.24 ± 0.36	4.2 ± 0.4

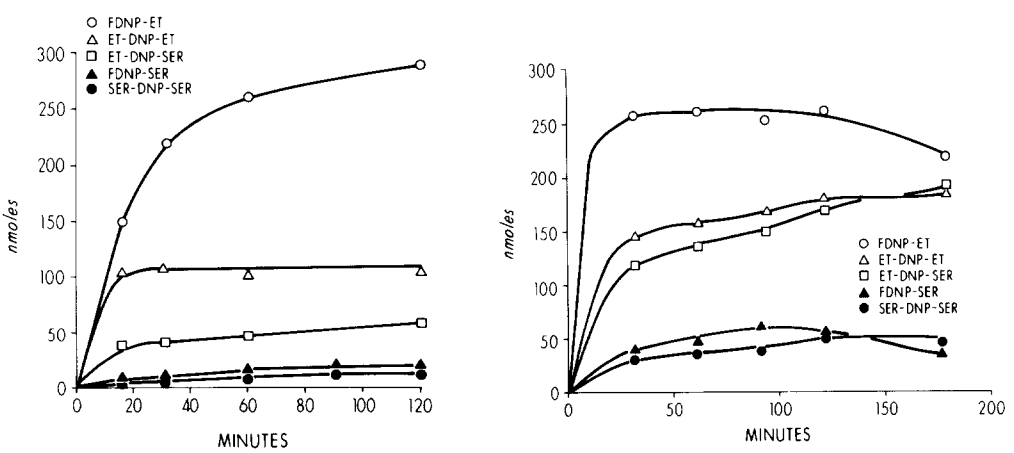

Fig. 4. Time course of reaction of phosphatidylethanolamine and phosphatidylserine of erythrocyte ghosts with difluorodinitrobenzene at 23° C. Aliquots of ghosts from 1 ml of packed cells were reacted with 100 μM difluorodinitrobenzene in 20 ml of $NaHCO_3$/NaCl buffer pH 8.5 at 23° C for periods up to 180 min. The ghosts were washed with buffer containing 0.5% bovine serum albumin and the lipids were extracted and analyzed as explained previously [7,16]. ET, ethanolamine; SER, serine.

Fig. 5. Time course of reaction of phosphatidylethanolamine and phpsphatidylserine of erythrocyte ghosts with difluorodinitrobenzene at 37° C. Aliquots of ghosts from 1 ml of packed cells were reacted as explained in Fig. 4 except the reaction temperature was 37° C, ET, ethanolamine; SER, serine.

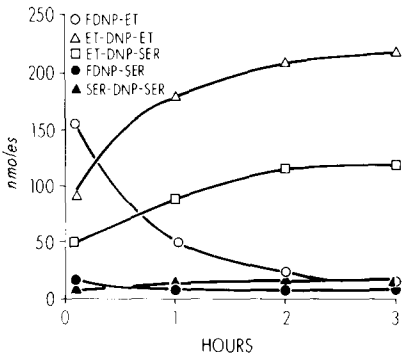

Fig. 6. Pulse-labeling of phosphatidylethanolamine and phosphatidylserine of erythrocyte ghosts with difluorodinitrobenzene. Aliquots of ghosts from 1 ml of cells were pulsed for 15 min at 23°C with 100 μm difluorodinitrobenzene in 20 ml of $NaHCO_3$/NaCl buffer pH 8.5. The ghosts were washed with buffer, resuspended in buffer and incubated at 37°C. Aliquots were analyzed at different times as explained previously [7,16].

lamine-Dnp-serine. Also FDnp-serine declines there is an increase in the serine-Dnp-serine.

It would be highly desirable to obtain the reaction rates of phosphatidylethanolamine and phosphatidylserine with the first and second fluorine of difluorodinitrobenzene. This is very difficult to do since multiple reactions are occurring simultaneously. Moreover, since the reactions of phosphatidylethanolamine and phosphatidylserine occur with membrane bound components in which there can occur variation in local domains and different degrees of masking, one can only obtain a net time average value of all of these processes. If one examines the ability of these phospholipids to react in solution then it is necessary to use a polar organic solvent such as methanol. This creates a problem since the change in dielectric constant of this solvent will influence the ionization of the polar groups of phosphatidylethanolamine and phosphatidylserine and thus influence their rate of reaction. If one sonicates the phosphatidylethanolamine and phosphatidylserine in buffer, the size and arrangement of the sonicated particles must be considered. Possibly the simplest system for comparison of the inherent reactivity of the amino group of phosphatidylethanolamine and phosphatidylserine would be the reaction of difluorodinitrobenzene with ethanolamine and serine or with phosphorylethanolamine or phosphorylserine. Ethanolamine and serine have been studied and both react readily and to completion with difluorodinitrobenzene to form the mono or bisubstituted products [16]. There is not a marked difference in reactivity of the amino group of these two compounds.

Red cell lipids were sonicated in buffer and reacted with varying concentrations of difluorodinitrobenzene at 23°C for 1 h. The results are shown in 7. The formation of ethanolamine-Dnp-ethanolamine reaches a plateau at about 60 μM difluorodinitrobenzene. The formation of the serine-Dnp-ethanolamine and serine-Dnp serine reach an apparent plateau at about 80—100 μM difluorodinitrobenzene.

In order to examine the effect of the time and temperature of the reaction and of the concentration of difluorodinitrobenzene on the yield of the three

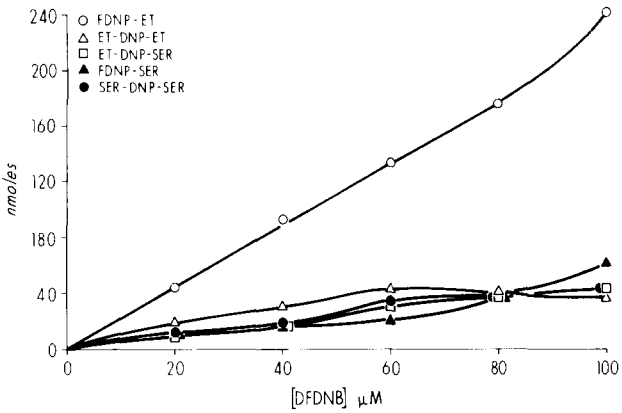

Fig. 7. Reaction of phosphatidylethanolamine and phosphatidylserine of sonicated erythrocyte lipids with different concentration of difluorodinitrobenzene. Aliquots of total erythrocyte lipids extracted from ghosts from 1 ml of packed cells were sonicated in 40 mM NaCl for 5 min at 0°C using a Bronson Sonifier set at 9 amps. The media of the sonicated lipids were adjusted to contain 120 mM $NaHCO_3$/40 mM NaCl pH 8.5 in a 10 ml volume. The sonicated lipids were reacted with different concentrations of difluorodinitrobenzene for 1 h at 23°C. The lipids were extracted by the method of Bligh and Dyer [21] and analyzed as explained previously [7,16]. ET, ethanolamine; SER, serine.

cross-linked species, the molar ratios of these species were calculated from the data given in Figs. 1, 4, 5 and 7. The results of these calculations are prsented in Tables IV and V. These calculations do not represent the values obtained under optimal cross-linking conditions as given in Fig. 1 and Tables I, II and III. It is apparent that the molar ratios are dependent on time, temperature and concentration as expected but the major finding seems clear, namely that the data are more consistent with the existence of clusters of phosphatidylserine and phosphatidylethanolamine. At early time intervals at 37°C or at 200 μM concentrations of difluorodinitrobenzene the results indicate a "random" array

TABLE IV

EFFECT OF DIFLUORODINITROBENZENE CONCENTRATION ON THE MOLAR RATIOS OF CROSS-LINKED SPECIES OF PHOSPHATIDYLETHANOLAMINE AND PHOSPHATIDYLSERINE

Ghosts from fresh cells reacted with difluorodinitrobenzene for 2 hours at 23°C (Fig. 1). Total lipids extracted from 1.0 ml of packed red cells were sonicated at 0°C for 5 min and reacted with difluorodinitrobenzene as explained in Fig. 7.

Ghosts	(PE—PS)/(PS—PS)	(PE—PE)/(PS—PS)
(Probe) μM		
50	6.2	12.5
100	3.4	4.8
150	2.8	3.7
200	2.8	3.1
Sonicated Lipids		
(Probe) μM		
25	4.4	9.1
50	3.0	5.3

TABLE V

EFFECT OF TIME AND TEMPERATURE OF THE DIFLUORODINITROBENZENE REACTION ON THE MOLAR RATIOS OF CROSS-LINKED SPECIES OF PHOSPHATIDYLETHANOLAMINE AND PHOSPHATIDYLSERINE

The ghosts were reacted with 100 μM difluorodinitrobenzene as described in Figs. 4 and 5.

Ghosts (23°C)	(PE—PS)/(PS—PS)	(PE—PE)/(PS—PS)
Reaction time (min)		
30	7.6	20
60	6.3	14.3
120	6.0	11.1
180	5.6	9.1
Ghosts (37°C)		
Reaction time (min)		
30	3.9	4.5
60	3.4	3.6
90	3.7	3.6

of phosphatidylserine and phosphatidylethanolamine. However, these conditions cannot be used to assess a fit to the models since only a small fraction of phosphatidylethanolamine and phosphatidylserine has reacted.

This cross-linking work provides new insight into the architecture of the red cell membrane and coupled with the previous results on the asymmetric arrangement of phosphatidylethanolamine and phosphatidylserine in the red cell membrane [1—6] indicates that the amino-phospholipids occur in a non-random array on the inner half of the lipid bilayer. Approximately one-third of the phosphadylserine molecules are tightly clustered about membrane proteins and the remaining thow-thirds appear to exist as small clusters on the inner half of the lipid bilayer. The data are also consistent with the occurrence of clusters of phosphatidylethanolamine.

Discussion

The major fatty acids of human red cell phosphatidylserine are 18 : 0 (about 42%), 18 : 1 (about 10%), 20 : 4 (about 21%) and 16 : 0 (about 7%) [20]. The major fatty acids of human red cell phosphatidylethanolamine are 16 : 0 (about 24%), 18 : 0 (about 10%), 18 : 1 (about 20%) and 20 : 4 (about 20%). Hence phosphatidylethanolamine and phosphatidylserine have a similar content of polyunsaturated acid (20 : 4), saturated acids (16 : 0 and 18 : 0), and mono-saturated acid (18 : 1). It is unlikely that the nature of the fatty acids in these phospholipids accounts for the cluster formation.

Phosphatidylserine and phosphatidylethanolamine are acidic phospholipids and can bind cations such as K^+, Na^+, Ca^{2+} and Mg^{2+}. Phosphatidylserine has a strong capacity to bind Ca^{2+} [17]. Ion pair interactions (salt bridges) between the amino groups and carboxyl groups or phosphate groups of phospholipid neighbors reinforced by Ca^{2+} bridges across the ionic phosphate groups may stabilize dimers, trimers or tetramers of thse phospholipids. These clusters are visualized as moving laterally in the plane of the monolayer as discrete entities

having a sufficiently long half life to be sensed by physical or chemical probes.

In order to use cross-linking probes as tools for assessing the arrangement of phosphatidylethanolamine and phosphatidylserine in cell membranes one has to consider the reaction time of the probe with phosphatidylethanolamine and phosphatidylserine and the rate at which phosphatidylethanolamine and phosphatidylserine move laterally in the membrane. The rate profiles in Fig. 4 indicate that phosphatidylethanolamine reacts faster than phosphatidylserine and that the reaction reaches equilibrium within 2 h for FDnp-phosphatidylethanolamine and FDnp-phosphatidylserine but that the cross-linking of phosphatidylethanolamine to phosphatidylethanolamine and phosphatidylethanolamine to phosphatidylserine reaches equilibrium within 20—30 min. The cross-linking of phosphatidylserine to phosphatidylserine requires about 2 h to reach equilibrium. The rate profiles are complex, as one would expect, since multiple reactions are occurring simultaneously. Previous work using spin labels [22] has indicated that certain phospholipid neigbors such phosphatidylcholine exchange rapidly, with times measured in μs. However, this work was done mainly on artificial liposomes in which proteins are absent. To the author's knowledge the rate of lateral mobility of intact phosphatidylethanolamine and phosphatidylserine (having naturally occurring fatty acids not perturbed by spin labels) in the red cell membrane has not been studied. Moreover, its is important to determine what fraction of the total phospholipid for each species undergoes this rapid lateral mobility. One can expect that phospholipid molecules closely associated with proteins will have different mobilities than the phospholipids in the lipid bilayer. Furthermore, phospholipid clusters can be stabilized by electrostatic forces and may have long life times relative to their lateral mobility in the membrane. Thus one can visualize the rapid movement of these clusters in the membrane. The evidence for the existence of clusters in lipid bilayers is discussed by Lee et al. [9] and is supported by the work of Pagano et al. [15] and Murphy [19].

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