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The Interaction of 1-Fluoro-2,4-Dinitrobenzene with Amino-Phospholipids in Membranes of Intact Erythrocytes, Modified Erythrocytes, and Erythrocytes Ghosts

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Summary. 1-Fluoro-2,4-dinitrobenzene (FDNB) has been used to study the availability of amino-containing phospholipids in erythrocyte membranes and ghosts in an aqueous, isotonic medium. It was found that the addition of bovine serum albumin (BSA) to the medium protects the cells from cation leak and protects some of the amino-phospholipids from reacting with the probe. In isotonic medium without BSA, 46% of the phosphatidylethanolamine and 12% of the phosphatidylserine of erythrocytes and 73% and 21% of these respective lipids of ghosts react with the probe. In the presence of 70 μM BSA, 31% of phosphatidylethanolamine and 6.5% of phosphatidylserine of erythrocytes and 59% and 16% of these respective lipids of ghosts react with the probe. The labeling of these lipids does not change under conditions of varying tonicity, or after treatment of erythrocytes with pronase or lysolecithin. The data suggest that 46% of phosphatidylethanolamine and 12% of phosphatidylserine of the erythrocyte membrane are free in a lipid bilayer; 27% and 9% of these respective lipids are loosely bound to proteins which are lost during the preparation of ghosts and 27% of the phosphatidylethanolamine and 79% of the phosphatidylserine are tightly bound to core proteins which remain part of the erythrocyte membrane even after hemolysis.

During the past few years a considerable amount of research has been directed toward determining the structure of cell membranes. Some proteins of the erythrocyte membrane have been isolated and characterized (Maddy, 1970; Marchesi, Steers, Marchesi & Tillack, 1970; Fairbanks, Steck & Wallach, 1971) and attempts have been made to determine their arrangement in this membrane (Rosenberg & Guidotti, 1969; Bender, Garan & Berg, 1971; Bretscher, 1971; Carraway, Kobylka & Triplett, 1971; Phillips & Morrison, 1971*a*, *b*). The lipid composition of this membrane has also been extensively studied (Sweely & Dawson, 1969; Cooper, 1970). Although

the gross lipid composition is well established, the arrangement of the lipids in the membrane is almost unknown. Phospholipases A and C have been used to determine the accessibility of the phospholipid molecules in the membrane. The results have shown that the phospholipids of the intact erythrocyte are refractory to these enzymes but that a major portion of the phospholipids in erythrocyte ghosts are hydrolyzed (Roelofsen, Zwaal, Comfurius, Woodward & VanDeenen, 1971; Laster, Sabban & Loyter, 1972).

Nonspecific chemical probes, such as 1-anilino-8-naphthalenesulfonate (ANS), whose fluorescence is altered under conditions of varying hydrophobicity, have been used to determine changes in the conformation of lipids in erythrocyte ghosts perturbed with enzymes (Weidekam, Wallach & Fischer, 1971) or organic solvents (Fortes & Hoffman, 1971; Metcalfe, Metcalfe & Engelman, 1971).

Physical methods have also been used to elucidate the structure of the erythrocyte membrane and it is generally agreed that the phospholipids exist mainly in a bilayer (Chapman, 1972).

Recently, Carraway, Kobylka, Summers and Carraway (1972) using radioactive acetic anhydride, and Gordesky, Marinetti and Segel (1972) using FDNB have begun to probe certain phospholipids of intact erythrocyte ghosts. These chemical probes react with the amino groups of proteins and the amino-phospholipids such as phosphatidylethanolamine (PE) and phosphatidylserine (PS). Both laboratories have concluded that the structure of the ghost membrane is different from the membrane of the intact erythrocyte.

The present report deals with the reactivity of PE and PS of the erythrocyte membrane with FDNB, under conditions in which the cell is osmotically swollen or crenated, treated with pronase, or altered by incubation with lysolecithin. Evidence that BSA protects erythrocytes as proposed by Furchgott and Ponder (1940) is also presented.

Materials and Methods

Reaction of Cells or Ghosts with FDNB

The reaction of FDNB with fresh human erythrocytes (0.4 ml packed cells) and erythrocyte ghosts in an isotonic medium, and the isolation and identification of the lipids were carried out as previously described by Gordesky *et al.* (1972).

The blood, obtained by venipuncture and collected in heparinized vacutainers, was centrifuged at 2,000 rpm for 10 min. The plasma and buffy coat were removed and the cells were washed twice with isotonic saline. A sample of 0.4 ml of packed cells, or ghosts made by a modification of the procedure of Dodge, Mitchell and Hanahan (1963) from 0.4 ml of packed cells, were added to 29.6 ml of a reaction medium containing

120 mM $NaHCO_3$, 40 mM NaCl, 4.4 mM glucose, 1.5 mM FDNB, and BSA (Sigma-Fraction V) of varying concentrations. The pH of the suspension remained at 8.5 to 8.6 throughout the incubation. The cells (or ghosts) were incubated for 2 hr at 23 °C and then centrifuged. The supernates were discarded and the pellets washed twice with the isotonic bicarbonate buffer.

Ghosts were made from the FDNB-treated cells and the lipids extracted with 20 volumes of $CHCl_3$/MeOH 1:1 (v/v). The lipid extracts were centrifuged and the supernates concentrated under nitrogen for spotting on thin-layer chromatography (TLC) plates. The plates, SG 5763, Merck & Co., Darmstadt, were developed in $CHCl_3$/MeOH/H_2O 130:50:7 (v/v). The reacted lipids were identified by their yellow color and by comparison to standards. Unreacted PE and PS were located by staining with ninhydrin. The areas of silica gel containing the specific lipids were scraped into tubes and extracted three times with 2 ml of methanol. The combined extracts were concentrated to 5 ml and the absorbance at 345 nm was determined. Total lipid P was determined by the method of Harris and Popat (1954).[1]

To perform the experiments in a hypotonic medium, 6 ml of a stock FDNB solution (7.5 mM FDNB in 5% $NaHCO_3$) was added to 39 ml of a solution containing glucose and BSA so that the final concentrations were: $NaHCO_3$ 80 mM, glucose 3.7 mM, FDNB 1.0 mM, and BSA 46 μM. For the isotonic and hypertonic controls in this experiment, the concentration of Na^+ was adjusted to 150 mM and 230 mM, respectively. The final osmolarities of the different media were as follows: hypotonic 145 mosm, isotonic 290 mosm, and hypertonic 445 mosm, as determined by a Precision Systems Osmometer Model 2007. The pH of these solutions remained at 8.5 to 8.6 during the incubation with cells.

Lysolecithin Treatment

Chromatographically pure egg lysolecithin (Koch-Light Laboratories, Ltd.) was added to the FDNB stock solution, or to the $NaHCO_3$ solution without FDNB, so that the final concentration of lysolecithin in the incubation medium containing the erythrocytes was 40 or 80 μM. The reactions were performed in a medium containing 120 mM $NaHCO_3$, 40 mM NaCl, 4.4 mM glucose, 1.5 mM FDNB, and 70 μM BSA.

Pronase Digestion

Washed, packed erythrocytes (0.5 ml) were suspended in 106 mM phosphate buffer, pH 7.4, at a hematocrit of 11% and then reacted with 25 and 100 μg of pronase (Calbiochem, B grade) for varying periods of time as described by Phillips and Morrison (1971*b*). The cells were centrifuged, washed with buffer and reacted in a medium containing 120 mM $NaHCO_3$, 40 mM NaCl, 4.4 mM glucose, 1.5 mM FCNB and 70 μM BSA.

Measurement of Na^+ and K^+ Concentration of Erythrocytes

Erythrocytes were labeled with 1.5 mM FDNB as described above except that BSA was added in varying amounts and the sodium and potassium concentrations of the medium were equimolar to the erythrocyte intracellular electrolytes (sodium = 8 mEquiv/liter, potassium = 90 mEquiv/liter). The cells were washed free of FDNB and BSA with

1 PS was not always cleanly separated from other phospholipids as was PE. Since the PE/PS molar ratio in normal erythrocytes is reasonably constant (Redd, Swisher, Marinetti & Eden, 1960; Harris & Kellermeyer, 1970), the calculation of total PS in these experiments was based on this ratio and the measured total PE value.

the same buffer free of BSA and FDNB, and then suspended in a Krebs-Henseleit bicarbonate buffer (sodium = 140 mEquiv/liter, potassium = 5 mEquiv/liter) for incubation as previously described by Feig, Segel, Shohet and Nathan (1972). Samples were taken at various times for determination of intracellular sodium and potassium by flame photometry (Feig *et al.*, 1972).

Results

In this study considerable attention will be devoted to the influence of BSA on the labeling of the erythrocyte membrane aminophospholipids by FDNB. Albumin is the major protein in plasma. Consequently, the erythrocytes are constantly surrounded by and interact with albumin. Moreover, albumin is generally known to protect erythrocytes from shape changes and lysis produced by surface forces on glass, and by lytic detergents such as lysolecithin. FDNB was selected as a probe because it is a neutral penetrating probe which rapidly equilibrates between the inside and outside of the cell (Krupka, 1972). FDNB also produces easily measurable yellow derivatives with amino-containing compounds.

The rates of reaction of FDNB with PE and PS, in cells and ghosts, in a medium containing 70 μM BSA, are shown in Fig. 1. All reactions

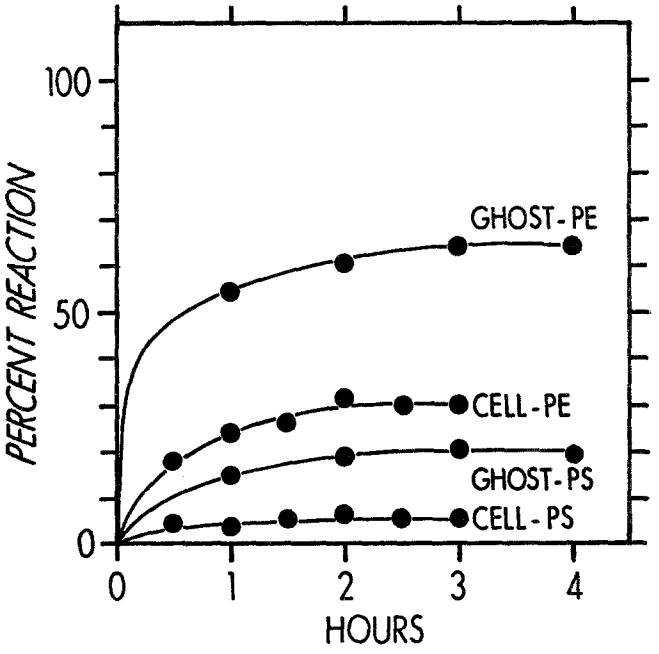

Fig. 1. Rate of reaction of PE and PS of erythrocytes and ghosts with FDNB. The reaction system of erythrocytes and ghosts with FDNB and the analysis of DNB-PE and DNB-PS are explained in the text. The system contained 70 μM BSA. The ordinate expresses the percent of the total PE and PS which reacted with FDNB

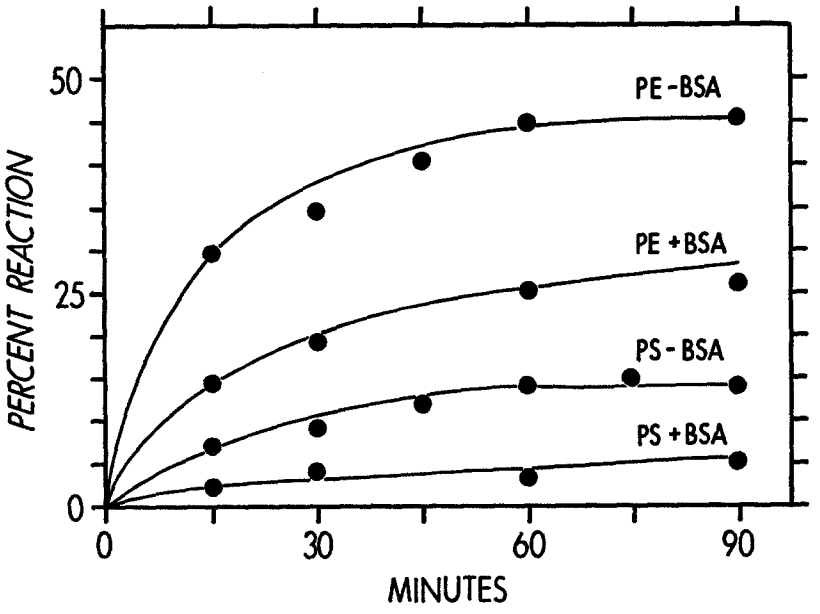

Fig. 2. The effect of BSA on the rate of reaction of PE and PS of erythrocytes with FDNB. The reaction system is explained in the text. When present, the BSA was 70 μM. The ordinate expresses the per cent of total PE and PS which reacted with FDNB

reach a plateau by 2 hr. PE and PS in ghosts react at a faster rate and to a greater extent than do PE and PS in cells. The effect of BSA on the reaction of the amino-phospholipids in cells is shown in Fig. 2. BSA allows less PE and PS to be available for reaction with FDNB. Increasing the concentration of BSA above 42 μM inhibits FDNB labeling of PE and PS in cells and ghosts as seen in Figs. 3 and 4, respectively. About 15% of the PE and 5% of the PS is protected by 70 μM BSA. Although the FDNB labeling of PE and PS in erythrocyte ghosts is greater than that seen with intact cells, 70 μM BSA causes a quantitatively similar decrease in relative FDNB labeling of each amino-phospholipid.

To be certain that the differential reactivity of PE and PS with FDNB in cells or ghosts was not due to a different chemical reactivity of these amino-phospholipids with the reagent, a sonicated dispersion of extracted red cell lipids (free from protein) was reacted with FDNB under the same conditions used for cells. It was found that PE and PS in these sonicated lipid dispersions react nearly to completion with FDNB within 60 min.

BSA decreases the cation permeability induced by FDNB. Increasing concentrations of BSA provide greater protection (Fig. 5). The cells initially contained 8 mEquiv/liter sodium and 90 mEquiv/liter potassium. Time zero

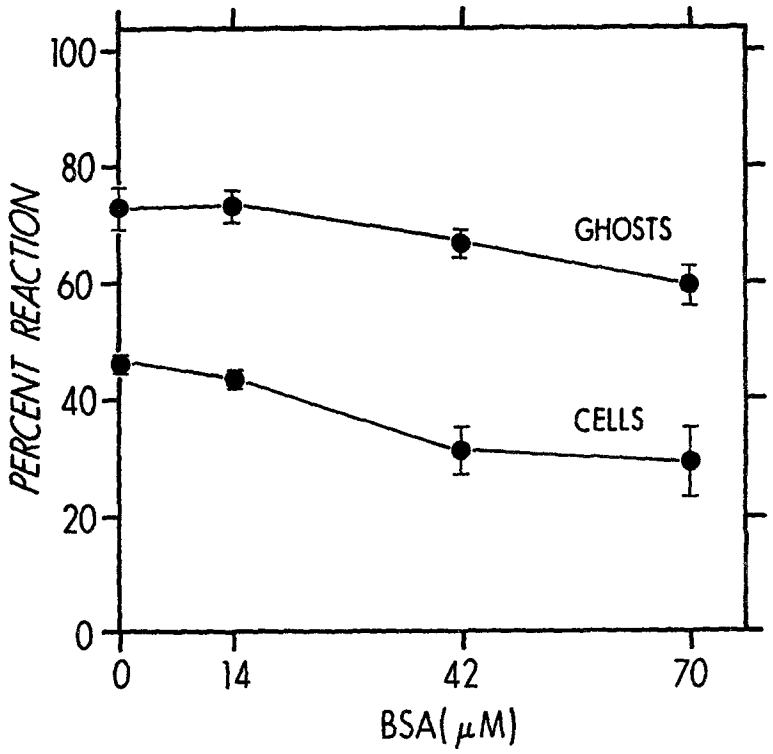

Fig. 3. The effect of BSA on the extent of reaction of PE of erythrocytes and ghosts with FDNB. The reaction of erythrocytes and ghosts with FDNB was carried out as explained in the text using different concentrations of BSA. The ordinate expresses the per cent of total PE which reacted with FDNB. The number of experiments done in duplicate at concentrations of BSA of 0, 14, 42 and 70 μM are, respectively, 4, 2, 2, 6 with ghosts and 2, 3, 7, 7 with cells. Values are mean ± SD

values, although taken immediately upon resuspension of treated cells, already show changes in internal sodium in all FDNB-treated cells, and in potassium when BSA was less than 70 μM during the prelabeling period.

In contrast to the protective effect of BSA, the presence of hemoglobin and intracellular proteins from lysed cells has no effect on the FDNB labeling of the membrane amino-phospholipids (Table 1). The relative per cent reaction of PE and PS of ghosts in the presence of their lysates was the same as that of ghosts washed free from these lysate proteins.

The erythrocyte membrane can be modified by incubating cells in hypotonic or hypertonic media so that the cells are swollen or crenated and the membrane is stretched or condensed. Neither of these treatments alters the amount of PE or PS which is available to react with FDNB (Table 2).

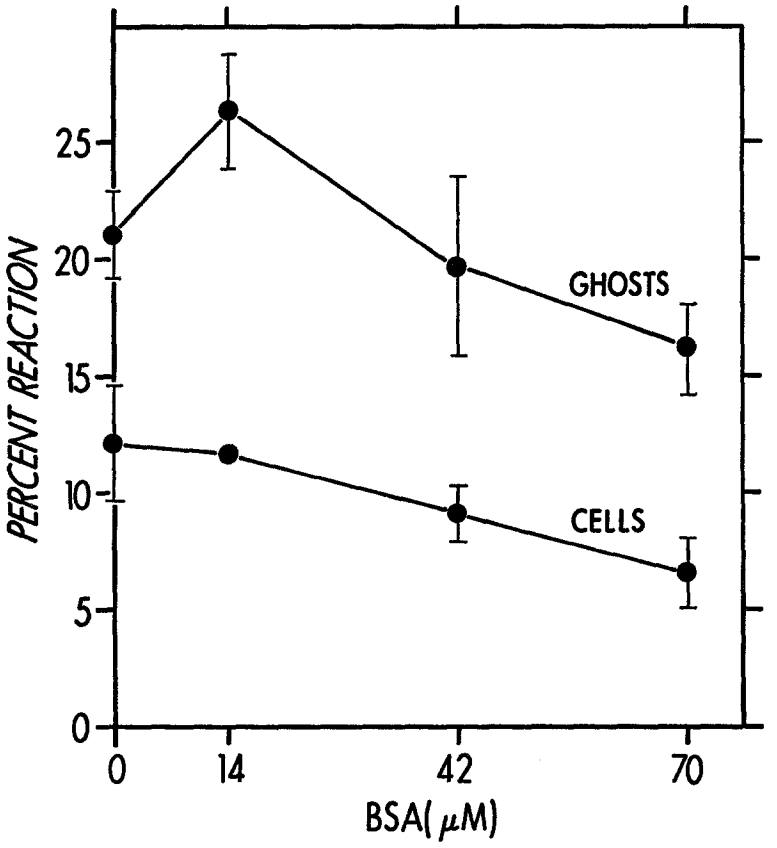

Fig. 4. The effect of BSA on the extent of reaction of PS of erythrocytes and ghosts with FDNB. The reaction of erythrocytes and ghosts with FDNB was carried out as explained in the text using different concentrations of BSA. The ordinate expresses the per cent of total PS which reacted with FDNB. The number of experiments done in duplicate at concentrations of BSA of 0, 14, 42 and 70 μM are, respectively, 2, 2, 2, 7 with ghosts and 2, 1, 5, 7 with cells. Values are mean ± SD

Pronase, a nonspecific proteolytic enzyme, was used to digest a portion of the external proteins of the erythrocyte membrane (Phillips & Morrison, 1971*b*). At enzyme concentrations of 25 and 100 μg/ml there was no change in the amount of PE which reacts with FDNB (Table 3).

Erythrocyte membranes were also modified with lysolecithin, a surface active agent capable of lysing erythrocytes (Wilbur & Collier, 1943). As seen in Table 4, no appreciable difference in FDNB labeling of PE or PS was found between the control cells and cells reacted in the presence of 40 and 80 μM lysolecithin under conditions where the cells changed shape but no hemolysis occurred. The cells exposed to the lower concentration of

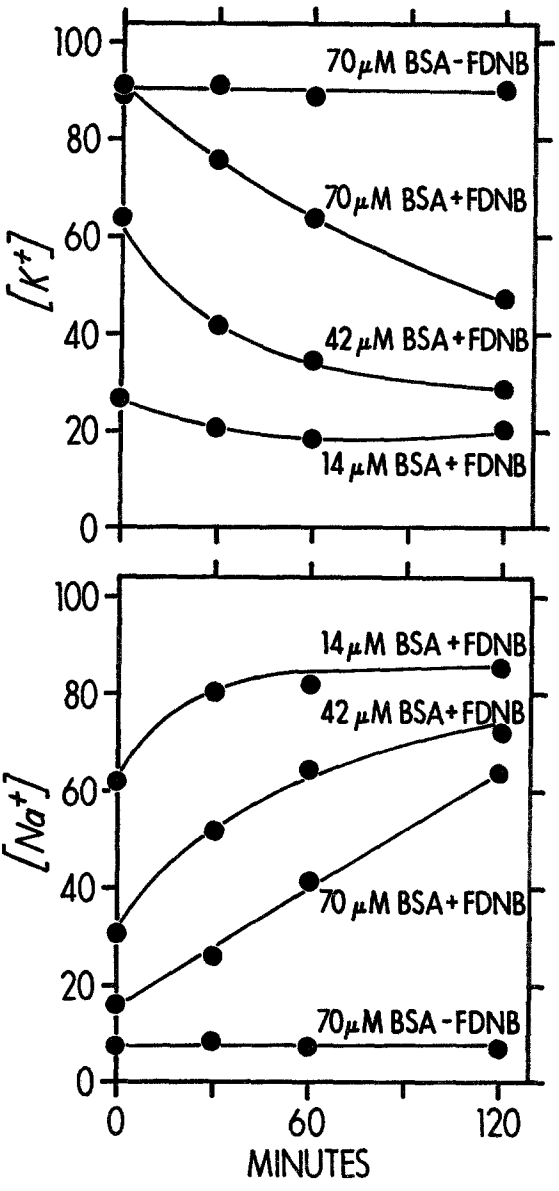

Fig. 5. The effect of BSA on Na^+ and K^+ leak in erythrocytes treated with FDNB. The Na^+ and K^+ leak measurement and the FDNB treatment were done as explained in the text. The values are mEquiv/liter cells

lysolecithin became crenated (echinocytes) whereas cells exposed to the higher concentration became spherocytes.

Erythrocyte hemolysis serves as an indicator of the membrane permeability to large molecules. Less than 2% hemolysis was observed in the hyper-

Table 1. The effect of the cell lysate on the per cent of total PE and PS of erythrocytes which reacts with FDNB[a]

System		PE	PS
I	Intact cells	30.5±4.1	8.4±2.2
II	Lysed cells with Hb present	52.8±4.8	14.0±2.0
III	Washed ghosts	52.8±4.5	12.2±3.2

[a] The reactions were carried out in an isotonic medium as given in the text. The FDNB concentration was 1.5 mM. The BSA concentration was 70 μM. The values represent the mean ± SD of duplicate analyses of four experiments. System I represents the normal reaction. In system II the cells were lysed and the reaction carried out with the ghosts and the lysate both present. In system III the cells were lysed and the ghosts washed once with the lysing medium (5 mM Tris, 1 mM EDTA, pH 7.4).

Table 2. Per cent of total PE and PS of intact erythrocytes which reacts with FDNB in media of different tonicity[a]

Medium	PE	PS
Hypotonic medium	25.1±2.7	5.4±1.2
Isotonic medium	27.5±1.6	8.0±1.6
Hypertonic medium	30.7±5.9	6.2±1.3

[a] The systems contained 0.4 ml packed erythrocytes, 1.0 mM FDNB, 46 μM BSA, 80 mM $NaHCO_3$, 3.7 mM glucose and varying amounts of NaCl as follows: hypotonic medium — none, isotonic medium — 70 mM NaCl, hypertonic medium — 150 mM NaCl. The values are the mean ± SD of duplicate analyses of two experiments.

Table 3. Effect of pronase on the per cent of total PE of erythrocytes which reacts with FDNB[a]

Time of Reaction (min)	Per cent reaction
0	29.9
15	28.2
30	28.9
60	28.5

[a] Erythrocytes were treated with pronase, washed and then reacted with FDNB as described in the text. Values are the average of two numbers which varied no more than 1.5% from the mean.

tonic, isotonic, or hypotonic media during the reaction of the erythrocytes with FDNB in the presence of 70 μM BSA for 4 hr at room temperature. After resuspension of the FDNB-treated cells for 6 more hours in hypertonic, isotonic or hypotonic media containing 3.7 mM glucose, there is less than

Table 4. Effect of lysolecithin on the per cent of total PE and PS of erythrocytes which reacts with FDNB[a]

Lysolecithin conc. (μM)	PE	PS
0	29.4±3.4	8.0±2.1
40	27.0±0.4	7.8±1.8
80	27.1±3.4	7.2±2.8

[a] Erythrocytes were reacted with FDNB in the presence of lysolecithin and BSA as described in the text. Values are the mean ±SD of four experiments.

2% hemolysis. Only after 24 hr does extensive (25 to 35%) hemolysis occur. Therefore, in these studies which utilize a completely aqueous medium, the erythrocytes are stable during the important 2-hr period of exposure to FDNB, so that this probe is interacting with an intact membrane.

Discussion

FDNB freely permeates the erythrocyte membrane and dinitrophenylates protein and lipid amino groups (Krupka, 1972). Krupka provided evidence that FDNB rapidly equilibrates between the inside and outside of the erythrocyte. In these studies we examined the reaction of amino-phospholipids of the erythrocyte membrane with FDNB. The reaction of FDNB with membrane PE and PS of intact cells was maximal in 2 hr (Gordesky *et al.*, 1972) and caused less than 2% hemolysis during this time. Using very different conditions of pH and hematocrit, Poensgen and Passow (1971) found, in three different experiments, 10 to 47% of PE and no PS of erythrocytes reacts with FDNB.

Erythrocyte membrane proteins, intracellular proteins and added BSA also react with FDNB .To insure that the plateau observed in Fig. 1 was not due to the exhaustion of FDNB, the yellow supernatant from an initial labeling experiment was reacted with fresh erythrocytes and the lipids were extracted in the usual way. Additional DNB-PE was demonstrated by TLC, thus showing that the plateau is not due to exhaustion of FDNB but rather is due to saturation of available PE and PS in the membrane. Intracellular proteins, primarily Hb, did not alter the FDNB labeling of PE and PS of ghosts (Table 1). Hemoglobin, therefore, does not act as a sink for FDNB in this system and FDNB remains in excess. Moreover, unlike albumin, the intracellular proteins do not protect PE and PS from reaction with FDNB.

A calculation can be made of total amino groups in the incubation mixture with intact erythrocytes to show that FDNB labels but a small number of these groups. The amount of protein and lipid in 0.4 ml of packed erythrocytes can be estimated from the values of Harris and Kellermeyer (1970) as follows: PE + PS – 0.5 μmoles, membrane protein[2]—0.002 μmoles, hemoglobin—2.2 μmoles. The maximum amount of BSA added was 2.1 μmoles. Therefore, a total of 4.3 μmoles of protein are available to react with FDNB. Each hemoglobin molecule has 80 free amino groups. The total hemoglobin in this reaction contains 175 μmoles of free amino groups. The 2.1 μmoles of BSA contribute approximately 103 μmoles of free amino groups. Membrane protein and nonhemoglobin intracellular protein[3] contain approximately 0.15 μmoles of free amino groups and PE and PS contribute 0.5 μmoles of free amino groups. Thus, a total of approximately 279 μmoles of free amino groups exist in the reaction medium. Since 45 μmoles of FDNB are added and excess FDNB is present when the reaction has reached a plateau, these results demonstrate that most of the amino groups (as well as SH, OH, tyrosyl, guanidinium and histidyl groups) which could potentially react with FDNB are masked and not available to FDNB.

When FDNB is used as a probe for the amino groups of PE and PS in the intact erythrocyte membrane, 46% of the PE and 12% of the PS amino groups are labeled in contrast to 73% and 21%, respectively in ghosts[4]. It is clear that in the preparation of ghosts an alteration has occurred in the membrane which leads to an increase in the availability of PE and PS for FDNB.

Evidence is available that when ghosts are prepared from intact red cells an alteration of the membrane occurs. This is supported by the observations of Roelofson *et al.* (1971) and Laster *et al.* (1972), that phospholipids in the membrane of intact erythrocytes are not attacked by phospholipase A or phospholipase C but in ghosts these phospholipids are readily attacked.

Moreover, proteins of ghosts are extensively hydrolyzed by trypsin whereas proteins of the intact erythrocyte are not extensively hydrolyzed (Triplett & Carraway, 1972).

In the presence of 70 μM BSA, intact erythrocytes and ghosts exhibit a similar relative decrease in PE and PS labeling. The 46% of reactive PE in intact cells is reduced to 31% and the 73% of reactive PE in ghosts is

2 This calculation assumes an average molecular weight of 100,000.

3 This calculation assumes 75 amino groups per 100,000 mol wt.

4 Values previously published in an abstract (Gordesky & Marinetti, 1972) were high due to an error in the slope of the standard curve for DNB-PE.

reduced to 59% by BSA. The reactive PS in erythrocytes is reduced from 12% to 6.5% and in ghosts it is reduced from 21% to 16%. Therefore, the decrease in reactivity of PE in both intact cells and ghosts caused by BSA is 14 to 15%. Furthermore, the decrease in reactivity of PS in both intact cells and ghosts caused by BSA is 5%. These data strongly suggest not only that BSA has a protective effect on PE and PS but that BSA protects the PE and PS at specific membrane sites present in both the intact cells and ghosts.

The protective action of BSA might be due to its binding to the membrane in such a way that some of the polar groups of PE and PS are masked. It is noteworthy that Ponder (1948) has demonstrated the binding of albumin to the erythrocyte. He postulated that albumin became embedded in the membrane and formed a specific part of the membrane structure.

The FDNB-induced change in cation permeability of erythrocytes has been described by Poensgen and Passow (1971). We have confirmed and extended their finding that cation leak is increased by FDNB. Added BSA protected the cells from the FDNB-induced cation leak, possibly due to the preservation of positively charged amino groups of PE and PS in the membrane.

When the cell membrane of intact cells was stretched in a hypotonic medium or condensed in a hypertonic medium, no significant change in the reactivity of PE and PS with FDNB was seen. When the membrane was altered by lysolecithin to cause sphering and crenation (Lichtman & Marinetti, 1972), or by pronase, which partially degrades but does not release proteins from the erythrocyte surface (Phillips & Morrison, 1971*b*), there was no effect on the FDNB labeling of PE. Albumin protected the cells from lysis by lysolecithin. This effect of albumin is twofold. Albumin binds lysolecithin thus making less lysolecithin free to interact with the red cell membrane. Albumin also binds to the cell membrane giving it partial protection from lysis by lysolecithin and protecting some amino-phospholipids from reacting with FDNB.

We suggest since 46% of the PE molecules and 12% of the PS molecules of the intact erythrocyte react freely with FDNB, they are not associated with protein and are in a lipid bilayer in the membrane. One-third of these PE molecules and one-half of these PS molecules may be protected by BSA. Moreover, in ghosts an additional 27% of the PE molecules and an additional 9% of the PS molecules are made available to FDNB. We postulate that these newly exposed amino-phospholipids are associated loosely with proteins which are lost during the preparation of these ghosts (Dodge *et al.*, 1963). The remaining 27% of the PE and 79% of the PS molecules which

are refractory to FDNB in ghosts are considered to be strongly bound to core proteins which remain part of the membrane structure even after hemolysis. These results and postulates are consistent with the Singer and Nicolson (1972) model of membrane structure.

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References

Bender, W. W., Garan, H., Berg, H. C. 1971. Proteins of the human erythrocyte membrane as modified by pronase. *J. Mol. Biol.* **58**:783

Bretscher, M. S. 1971. Human erythrocyte membranes: Specific labelling of surface proteins. *J. Mol. Biol.* **58**:775

Carraway, K. L., Kobylka, D., Summers, J., Carraway, C. A. 1972. Chemical modification of erythrocyte membranes: Double labeling with acetic anhydride. *Chem. Phys. Lipids* **8**:65

Carraway, K. L., Kobylka, D., Triplett, R. B. 1971. Surface proteins of erythrocyte membranes. *Biochim. Biophys. Acta* **241**:934

Chapman, D. 1972. Recent physical studies on the structure of bio-membranes. *Chem. Phys. Lipids* **8**:259

Cooper, R. A. 1970. Lipids of human red cell membrane: Normal composition and variability in disease. *Seminars in Hematology* **7**:296

Dodge, J. T., Mitchell, C., Hanahan, D. J. 1963. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **100**:119

Fairbanks, G., Steck, T. L., Wallach, D. F. H. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**:2606

Feig, S. A., Segel, G. B., Shohet, S. B., Nathan, D. G. 1972. Energy metabolism in human erythrocytes. II. Effects of glucose depletion. *J. Clin. Invest.* **51**:1547

Fortes, P. A. G., Hoffman, J. F. 1971. Interaction of the fluorescent anion 1-anilino-8-naphthalene sulfonate with membrane charges in human red cell ghosts. *J. Membrane Biol.* **5**:154

Furchgott, R. F., Ponder, E. 1940. Disc-sphere transformation in mammalian red cells. II. The nature of the anti-sphering factor. *J. Exp. Biol.* **17**:117

Gordesky, S. E., Marinetti, G. V. 1972. 1-Fluoro-2,4-dinitrobenzene (FDNB) as a lipid probe of membrane structure. *Fed. Proc.* **31**:861

Gordesky, S. E., Marinetti, G. V., Segel, G. B. 1972. Differences in the reactivity of phospholipids with FDNB in normal RBC, sickle cells and RBC ghosts. *Biochem. Biophys. Res. Commun.* **47**:1004

Harris, J. W., Kellermeyer, R. W. 1970. The Red Cell. Harvard Press, Cambridge, Mass. p. 282.

Harris, W. D., Popat, P. 1954. Determination of the phosphorus content of lipids. *J. Amer. Chem. Soc.* **31**:124

Krupka, R. M. 1972. Combined effects of maltose and deoxyglucose on fluorodinitrobenzene inactivation of sugar transport in erythrocytes. *Biochim. Biophys. Acta* **282**:326

Laster, Y., Sabban, E., Loyter, A. 1972. Susceptibility of membrane phospholipids in erythrocyte ghosts to phospholipase C and their refractiveness in the intact cell. *F.E.B.S.* **20**:307

Lichtman, M. A., Marinetti, G. V. 1972. Erythrocyte shape alteration *in vitro*: Relationship to plasma and cellular factors. *Nov. Rev. Franc. d'Hematol.* **12**:755

Maddy, A. H. 1970. Erythrocyte membrane proteins. *Seminars in Hematology* **7**:275

Marchesi, S. L., Steers, E., Marchesi, V. T., Tillack, T. W. 1970. Physical and chemical properties of a protein isolated from red cell membranes. *Biochemistry* **9**:50

Metcalfe, S., Metcalfe, J. C., Engelman, D. M. 1971. Structural comparisons of native and reaggregated membranes from *Mycoplasma laidawii* and erythrocytes using a fluorescence probe. *Biochim. Biophys. Acta* **241**:422

Phillips, D. R., Morrison, M., 1971*a*. Exposed protein on the intact human erythrocyte. *Biochemistry* **10**:1766

Phillips, D. R., Morrison, M. 1971*b*. Exterior proteins on the human erythrocyte membrane. *Biochem. Biophys. Res. Commun.* **45**:1103

Poensgen, J., Passow, H. 1971. Action of 1-fluoro-2,4-dinitrobenzene on passive ion permeability of the human red blood cell. *J. Membrane Biol.* **6**:210

Ponder, E. 1948. Hemolysis and Related Phenomena. Grune and Stratton, N. Y. p. 42.

Reed, C. F., Swisher, S. N., Marinetti, G. V., Eden, E. G. 1960. Studies of the lipids of the erythrocyte. *J. Lab. Clin. Med.* **56**:281

Roelofsen, B., Zwaal, R. F. A., Comfurius, P., Woodward, C. B., Van Deenen, L. L. M. 1971. Action of pure phospholipase A_2 and phospholipase C on human erythrocytes and ghosts. *Biochim. Biophys Acta.* **241**:925

Rosenberg, S. A., Guidotti, G. 1969. The proteins of the erythrocyte membrane: Structure and arrangement in the membrane. *In*: The Red Cell Membrane. Structure and Function. G. A. Jamieson and T. J. Greenwalt, editors. p. 93. J. B. Lippincott Co., Philadelphia, Pa.

Singer, S. J., Nicolson, G. L. 1972. The fluid mosaic model of the structure of cell membranes. *Science* **175**:720

Sweely, C. C., Dawson, G. 1969. Lipids of the erythrocyte. *In:* The Red Cell Membrane. Structure and Function. G. A. Jamieson and T. J. Greenwalt, editors. p. 172. J. B. Lippincott Co., Philadelphia, Pa.

Triplett, R. B., Carraway, K. L. 1972. Proteolytic digestion of erythrocytes, resealed ghosts and isolated membranes. *Biochemistry* **11**:2897

Weidekamm, E., Wallach, D. F. H., Fischer, H. 1971. The effect of phospholipase A upon the interaction of 1-anilino-8 naphthalenesulfonate with erythrocyte membranes. *Biochim. Biophys. Acta* **241**:770

Wilbur, K. M., Collier, H. B. 1943. A comparison of the hemolytic actions of lysolecithin and saponin. *J. Cell Comp. Physiol.* **22**:233