DIFFERENCES IN THE REACTIVITY OF PHOSPHOLIPIDS WITH FDNB* IN NORMAL RBC, SICKLE CELLS AND RBC GHOSTS

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SUMMARY. The reaction of FDNB with phospholipid amino groups in RBC, sickle cells, and RBC ghosts was performed in a completely aqueous isotonic medium containing $NaHCO_3$, BSA, glucose and NaCl. During and after the reaction no hemolysis occurred. Within 120 min. the FDNB labeling of PE and PS reached a plateau. 32% of the PE in the normal RBC reacted with FDNB, compared to 21% in the sickle cell and 56% in the RBC ghost. 7% of the PS in the normal RBC reacted compared to 5% in the sickle cell and 15% in the RBC ghost. Thus, the availability of PE and PS amino groups may reflect structural differences in RBC membranes.

FDNB, a well-known reagent for protein end group analysis, was shown by Wheeldon and Collins (1) to react with the amino groups of PE and PS. This chemical probe is small enough to penetrate the membrane, and reacts under conditions where the membrane remains essentially in its native state.

This paper describes a technique in which FDNB reacts with PE and PS in membranes of intact RBC under conditions in which no hemolysis occurs during or after the incubation. Changes in the accessability of phospholipid polar groups after modification of the membrane are reported.

EXPERIMENTAL PROCEDURE

Fresh human blood (10 ml) was obtained by venipuncture and

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* Abbreviations
FDNB is 1-fluoro 2,4-dinitrobenzene
PE is phosphatidyl ethanolamine
PS is phosphatidyl serine
RBC is red blood cell(s)

collected in a 13 ml heparinized tube. The blood was immediately centrifuged for 10 min. at 2000 rpm in an IEC table top centrifuge at room temperature and the plasma and buffy coat removed. The packed RBC were resuspended in 5 ml of isotonic saline and washed twice.

Preparation of Ghosts

RBC ghosts were prepared according to the method of Dodge et al (2) by suspending 0.6 ml of washed, packed RBC in a lysing buffer containing 5 mM Tris and 1 mM EDTA at a pH of 7.5.

FDNB Labeling

A stock solution of FDNB was prepared by dissolving 50 µl of FDNB in 50 ml of 5% $NaHCO_3$. 6 ml of this stock solution was added to 23.6 ml of a solution containing glucose, NaCl, and BSA, and then 0.4 ml of packed RBC or ghosts was added so that the final concentrations of the components were as follows: NaCl - 40 mM, $NaHCO_3$ - 120 mM, glucose - 5.5 mM, FDNB - 1.5 mM, and BSA - 0.5%. The tubes were then incubated at room temperature for various times from 1 to 3 hours.

To stop the reaction the tubes were centrifuged at room temperature for 15 min. at 2000 rpm in an IEC model PR-2 centrifuge. The supernatants were discarded and the pellets washed twice with 20 ml of the suspending medium, without FDNB. Ghosts were prepared from the incubations of intact cells for lipid extraction.

Lipid Analysis

Ghost lipids were extracted with 20 volumes of redistilled chloroform:methanol, 1:1 v/v. The lipid extracts were centrifuged and the supernatants concentrated under nitrogen for spotting on thin layer chromatography plates. The plates, SG5763, Merck & Co. Darmstadt, were developed in a solvent system of chloroform:methanol: water, 130:50:7 (v/v). The lipids which reacted with FDNB were identi-

fied by their yellow color, and the free PE and PS were located by their reaction with ninhydrin. The areas of silica gel containing these specific lipids were scraped into centrifuge tubes and the lipids were extracted three times with 2 ml of methanol. The methano extracts were combined and concentrated to a final volume of 5 ml. The absorbance of the yellow color was determined with a Gilford spectrophotometer at 345 nm. Total lipid P of these samples was determined (after digestion) by the elon method of Harris and Popat (3).

RESULTS

Sufficient amounts of FDNB were found to be soluble in the incubation medium. It was not necessary to add methanol to dissolve the FDNB. The RBC did not hemolyze in this medium during a 3 hour perioc

The yellow DNB-PE color is linearly proportional to phospholipid concentration, Figure 1. The absorption maximum of the DNB-lipids is 345 nm. No absorption by unreacted phospholipids present in the sample occurs in this region. Wheeldon and Collins (1) found that DNB-PS has the same extinction coefficient as DNB-PE.

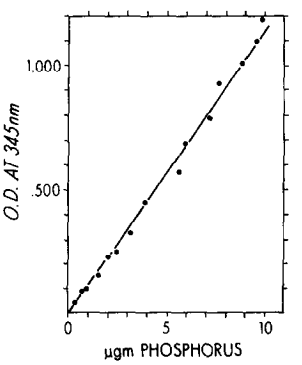

Figure 1 DNB-PE Absorption as a Function of PE Phosphorus

Ths slope of the line was computed by the method of least squares. This slope is 0.118 ± 0.003 (S.D.) and the intercept is -0.020 ± 0.020 (S.D.). The rat liver DNB-PE used in this experiment was purified by TLC. Synthetic dipalmityl-PE (Calbiochem) gave a slope identical to that of liver PE.

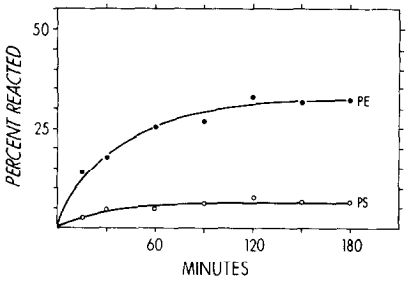

Figure 2 FDNB Reaction with PE and PS in Normal RBC

RBC were reacted and phospholipids were isolated and quantitated as described in "EXPERIMENTAL PROCEDURE".

The reaction, with excess FDNB, of PE and PS in intact RBC, reached a plateau after 120 min. as shown in Figure 2. Because the number of protein and lipid sites free to react with FDNB in the incubation medium was not known and in order to demonstrate that excess FDNB was present, RBC were reacted for 120 min. and then centrifuged. The supernatant then was used as the reaction medium for a second 120 min. incubation with fresh RBC. The membrane lipids from the second incubation were isolated and found to be labeled with FDNB, thus demonstrating that FDNB was not limiting. FDNB penetrated the RBC membrane, since the N-terminal valines of the α and β chains of hemoglobin, when isolated, were labeled.

The FDNB reactivity of PE and PS in normal RBC, in sickle cells, and in ghosts was measured. The availability of the phospholipids in ghosts and sickle cells was markedly different from that of normal RBC, as shown in Table I. 32% of the PE in the normal cells reacted with FDNB as compared to 21% in the sickle cells and 56% in the ghosts. 7% of the PS in normal cells reacted with FDNB compared to 5% in the sickle cells and 15% in the ghosts.

TABLE I

Reaction of RBC, RBC Ghost and Sickle Cell Phospholipids with FDNB.

	Percent of Total PE or PS Reacted		
	Normal Cells	Ghosts	Sickle Cells
PE	32.5 ± 1.4 (12)	56.2 ± 1.5 (7)	21.4 ± 2.1 (4)
PS	7.1 ± 0.6 (8)	14.5 ± 0.9 (7)	5.0 ± 0.6 (4)

The values represent the mean ± S.E. of the number of duplicate experiments given in parentheses. The reactions were carried out for 2 hours as described in "EXPERIMENTAL PROCEDURE".

DISCUSSION

These studies demonstrate that FDNB can be used to probe lipid amino groups of the RBC membrane in a completely aqueous system. FDNB is sufficiently soluble in the reaction medium so that FDNB can be present in excess and therefore not become a limiting reactant. Under the above conditions negligible hemolysis occurred during the reaction, allowing time to label all of the available phospholipid amino groups. Incubated cells, washed free of FDNB and resuspended in Krebs buffer, did not hemolyze for at least 10 hours after resuspension. This is in contrast to Berg et al (4) who found 80% hemolysis 4.5 hours after FDNB treated RBC were resuspended in Kreb's buffer, and also differs from the work of Poensgen and Passow (5) who found hemolysis during the incubation of RBC with FDNB.

During the preparation of ghosts, the RBC membrane must be presumed to be altered such that more lipid polar groups are exposed Similar observations have been made by Roelofsen et al (6) and Laster et al (7) using phospholipase C and Carraway et al (8) by acetylating RBC. Phospholipases do not react with intact RBC membranes (6,7) perhaps because the phospholipid polar ends are

inaccessible to this enzyme. FDNB, in contrast, permeates the membrane and provides a probe which is more sensitive. 68% of the polar groups of PE and 93% of the polar groups of PS in intact RBC are inaccessible to FDNB but many of these groups become available to both FDNB and phospholipase C when ghosts are made. Since FDNB is lipid soluble and penetrates the RBC membrane, it is possible that part of the lipid polar groups which react with FDNB but not with phospholipase C are on the inner membrane surface. It is noteworthy that both PE and PS are major lipids in the erythrocyte membrane, but that the more acidic PS is less exposed than is PE and can be presumed to be more tightly bound to protein. The decrease in availability of PE in the sickle cell may indicate that the lipids are more tightly bound to protein, or that some PE which reacts in the normal RBC has been lost or altered in the sickle cell. Separation of the reticulocyte rich portion of sickle blood by ultracentrifugation did not alter this finding.

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