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The Reaction of Chemical Probes with the Erythrocyte Membrane

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Summary. Trinitrobenzenesulfonate (TNBS), fluorodinitrobenzene (FDNB) and suberimidate have been reacted with intact human erythrocytes. TNBS does not penetrate the cell membrane significantly at 23 °C in bicarbonate-NaCl buffer, pH 8.6, as estimated by the labeling of the N-terminal valine of hemoglobin. Hence, under these conditions it can be used as a vectorial probe. However, at 37 °C, especially in phosphate buffer, at pH 8.6, TNBS does penetrate the cell membrane. FDNB and suberimidate both penetrate the erythrocyte membrane. The time course reaction of TNBS with intact erythrocytes over a 24-hr period at 23 °C is complex and shows transition zones for both membrane phosphatidylethanolamine (PE) and membrane proteins. No significant cell lysis occurs up to 10 hr. The fraction of total PE or phosphatidylserine (PS) which reacts with TNBS by this time period can be considered to be located on the outer surface of the cell membrane. Under these conditions it can be shown that 10 to 20% of the total PE and no PS is located on the outer surface of the membrane and hence these amino phospholipids are asymmetrically arranged. The pH gradient between the inside and outside of the cell in our system is 0.4 pH units. Nigericin has no effect on the extent of labeling of PE or PS by TNBS. Isotonic sucrose gives a slight enhancement of the labeling of PE by TNBS. Hence, the inability of PE and PS to react with the TNBS is considered not due to the inside of the cell having a lower pH. The extent of reaction of TNBS with PE is not influenced by changing the osmolarity of the medium or by treatment of cells with pronase, trypsin, phospholipase A or phospholipase D. However, bovine serum albumin (BSA) does protect some of the PE molecules from reacting with TNBS.

Cells treated with suberimidate were suspended in either isotonic NaCl or in distilled water. In both cases the suberimidate-treated cells became refractory to hypotonic lysis. Pretreatment of cells with TNBS did not prevent them from interacting with suberimidate and becoming refractory to lysis. However, pretreatment of cells with the penetrating probe FDNB abolished the suberimidate effect. Electron-microscopic analysis of the cells showed a continuous membrane in the case of cells suspended in isotonic saline. The cells suspended in water did not lyse but their membranes had many large holes, sufficient to let the hemoglobin leak out. Since the hemoglobin did not leak out we know that the hemoglobin is cross-linked into a large supramolecular aggregate.

Trinitrobenzenesulfonate (TNBS) has been used as an analytical reagent for the study of amino acids and polypeptides (Okuyama & Satake, 1960; Freedman & Radda, 1968), amines (Means, Congdon & Bender, 1972),

lipids (Siakotos, 1966) and as a probe for the erythrocyte membrane (Weiss, Bello & Cudney, 1968; Papahadjopoulos & Weiss, 1969; Bonsall & Hunt, 1971; Gitler, 1971; Knauf & Rothstein, 1971; Poensgen & Passow, 1971; Arrotti & Garvin, 1972; Godin & Ng, 1972; Gordesky, Marinetti & Segel, 1972; Gordesky & Marinetti, 1973; Gordesky, Marinetti & Segel, 1973). There is some controversy regarding the extent of penetration of the erythrocyte membrane by TNBS. Bonsall and Hunt (1971) and Gordesky and Marinetti (1973) report very little penetration of the intact erythrocyte membrane by TNBS whereas Arrotti and Garvin (1972) report that radioactive TNBS does penetrate the erythrocyte membrane. These workers used different experimental conditions in their studies which can account for the apparent discrepancy. In this paper we report that the TNBS penetration of the erythrocyte membrane is markedly influenced by temperature and buffer composition.

The question of penetrability of TNBS is important in using this probe as a vectorial agent to study whether membrane proteins or phospholipids are located on the inside or outside surface of the membrane. The conditions of the reaction must be selected so that penetration of the probe is minimal or nonexistent if one is to use TNBS to probe only the outer surface of the cell membrane.

In contrast to TNBS which can be used as a nonpenetrating probe under proper conditions, fluorodinitrobenzene (FDNB) is a probe which readily penetrates the erythrocyte membrane (Krupka, 1972). We have previously shown (Gordesky *et al.*, 1972, 1973) that FDNB reacts to a different extent with phosphatidylethanolamine (PE) and phosphatidylserine (PS) in the intact erythrocyte but neither of these phospholipids reacts to completion. Moreover, the extent of reaction of PE and PS in ghosts is much greater than that of intact cells. Both FDNB (Knauf & Rothstein, 1971; Poensgen & Passow, 1971; Gordesky *et al.*, 1973) and TNBS (Knauf & Rothstein, 1971) influence ion fluxes in intact erythrocytes. These probes are believed to alter amino groups at specific sites on the membrane which are involved in ion transport.

Experimental Procedure

Reaction of Cells with TNBS or FDNB

The reaction of TNBS (Pierce Chemical Co.) or FDNB (Eastman Kodak) with human erythrocytes and erythrocyte ghosts and the isolation and identification of the lipids was carried out as previously described (Gordesky *et al.*, 1972, 1973; Gordesky & Marinetti, 1973). The blood (5 to 10 ml) obtained by venipuncture and collected in heparinized vacutainers, or Red Cross blood bank blood, was centrifuged at 2000 rpm

for 10 min. The plasma and buffy coat were removed and the cells were washed twice with isotonic saline. Aliquots of 0.4 ml of packed cells were added to 29.6 ml of a reaction medium containing 120 mM $NaHCO_3$, 40 mM NaCl and 1.5 mM TNBS or FDNB. The pH of the suspension remained at 8.5 or 8.6 throughout the incubation. The cells were incubated for different times at 22 to 23 °C and then centrifuged. The supernatants were discarded and the pellets washed twice with the isotonic bicarbonate buffer. Modifications of the above reaction are given where necessary in the Tables or legends to the Figures.

Ghosts were made from the cells by the method of Dodge, Mitchell and Hanahan (1963), by lysis of cells at 23 °C in 5 mM Tris buffer, pH 7.4, containing 1 mM ethylenediaminetetracetic acid (EDTA). The lipids were extracted with 20 volumes of $CHCl_3$/MeOH 1:1 (v/v). The lipid extracts were centrifuged and the supernatants 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 65:25:4 (v/v). The reacted lipids were detected by their yellow color. 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, centrifuged at 3000 rpm for 3 min and the absorbance of the supernatant was determined at 337 nm. Total lipid P was determined by the method of Harris and Popat (1954).

Determination of the Penetrability of TNBS

Erythrocytes were incubated with TNBS for different periods of time in two different buffers. An equal amount of cells was incubated for the same periods without TNBS and served as control. After the appropriate incubation the reacted and control cells were washed three times with 30 ml of the appropriate buffer and lysed with 15 ml of 20 mM Tris buffer, pH 7.4, containing 1 mM EDTA and centrifuged at 20,000 rpm for 25 min in a Spinco Model L centrifuge using a type 30 rotor. Ten ml of each supernatant were taken for analysis of TNP-hemoglobin. Globin was isolated by a modification of the method of Anson and Mirsky (1930). The isolated globin was dissolved in 10 ml of cold 0.1 N HCl and the globin precipitated with 200 ml of acetone. The supernatant was decanted off and the globin was washed once with 100 ml of acetone. The globin was obtained by centrifugation for 10 min at 2000 rpm and dried. Fifteen mg of each globin sample were put into 5 ml ampoules containing 1 ml of 6 N HCl. The ampoules were sealed and put into a 110 °C oven for 16 hr. To each digest were added 2 ml of H_2O. The digests were then extracted three times with 1 ml of diethyl ether and the extracts dried under nitrogen. The TNP-valine (plus picric acid produced by hydrolysis of TNP-valine) (N-terminal amino acid of the α and β chains of hemoglobin) was dissolved in 5 ml MeOH and the absorbance was read at 340 nm and compared to the fully labeled hemoglobin prepared as follows:

Two groups of 0.4 ml of packed cells were lysed in 15 ml of a 12 mM $NaHCO_3$ solution, pH 8.6. One lysate was incubated for 2 hr at 37 °C in the presence of 15 mM TNBS. The other lysate was incubated 10 hr at 22 °C in 15 mM TNBS. The globin of hemoglobin was isolated and hydrolyzed with 6 N HCl at 110 °C for 16 hr. The amount of hemoglobin labeled (measured as TNP-valine) in both experiments was similar and was used as the 100% reference value to measure the degree of penetration of TNBS into incubated erythrocytes.

TNP-valine and picric acid were confirmed by chromatography on SG 81 silica gel loaded paper (Reeve-Angel) using isopropanol-conc. NH_4OH/H_2O 7:2:1 (v/v). Thin-layer chromatography was also carried out on silica gel plates using chloroform/methanol/water 65:25:4 (v/v). The ether extract from the hydrolyzed labeled hemoglobin con-

tained in addition to picric acid only one TNP derivative which had the same R_f value as an authentic sample of TNP-valine. The hydrolysis of TNP-amino acids by HCl has been noted previously by Okuyama and Satake (1960). They report that TNP-valine is hydrolyzed to the extent of 35 to 45% after 5 hr at 110 °C.

Results

Membrane Penetrability of TNBS

The effect of temperature on the penetration of TNBS into intact erythrocytes is shown in Table 1. After a 2-hr reaction at 23 °C with 1.5 mM TNBS in either phosphate buffer or bicarbonate buffer, pH 8.5 to 8.6, only 0.5% of the total N-terminal valine groups of hemoglobin which are accessible to TNBS become labeled. Thus, the penetration of the excess TNBS is very small. This small degree of penetration of TNBS is seen up to 10 hr at 23 °C in either phosphate or bicarbonate buffer. However, at 37 °C, the penetration of the probe is increased to 2.5% at 2 hr in bicarbonate buffer and 6.0% in phosphate buffer. After 3 hr or longer at 37 °C in either buffer the cells undergo extensive lysis.

Table 1. Effect of temperature on the penetration of TNBS into intact erythrocytes [a]

Reaction time (hr) and buffer	Percent labeling of hemoglobin	
	22 °C	37 °C
2 $NaHCO_3$ buffer	0.5	2.5
2 phosphate buffer	0.5	6.0
3 $NaHCO_3$ buffer	0.5	hemolysis [b]
3 phosphate buffer	0.5	hemolysis [b]
10 $NaHCO_3$ buffer	0.5	hemolysis [b]
10 phosphate buffer	0.5	hemolysis [b]

[a] Intact erythrocytes (0.4 ml packed cells) were reacted with 1.5 mM TNBS in 30 ml of 120 mM $NaHCO_3$ − 40 mM NaCl buffer, pH 8.6, or 100 mM phosphate buffer, pH 8.6, at either 23 or 37 °C for different time periods. The cells were centrifuged, washed and lysed in 5 mM Tris buffer, pH 7.4, containing 1.0 mM EDTA. The globin of hemoglobin was isolated as explained in the text and hydrolyzed in 1 ml 6 N HCl at 110 °C for 20 hr in a sealed ampoule. The TNP-valine and picric acid produced by hydrolysis of TNP-valine or other TNP-amino acids were extracted into ether, dried, dissolved in 5 ml of methanol and the absorbance read at 340 nm. In order to measure percent penetration, 0.4 ml aliquots of packed cells were completely lysed and reacted with 15 mM TNBS for 2 hr at 37 °C or for 10 hr at 23 °C. The globin was isolated, hydrolyzed, and TNP-valine and picric acid extracted into ether. The amount of TNP-valine (and picric acid) measures the extent of penetration of TNBS. The values represent the average of duplicate analyses.

[b] The cells underwent extensive hemolysis so that penetration measurements could not be done.

Table 2. Reaction of hemoglobin, membrane proteins and membrane amino-phospholipids of intact erythrocytes with FDNB and TNBS

	nmoles of labeling	
	FDNB	TNBS
Globin (from hemoglobin)	6700	174
Membrane proteins	98	25
Membrane PE	226	19
Membrane PS	41	0

Duplicate aliquots of 0.4 ml of packed erythrocytes were suspended in 20 ml of 120 mM $NaHCO_3$ – 40 mM NaCl buffer, pH 8.6, with either 1.5 mM FDNB or TNBS. Another control aliquot of 0.4 ml cells was suspended in 20 ml of the same buffer without probe. After reacting for 2 hr at 23 °C the cells were washed once with 15 ml of the same buffer containing 0.5% bovine serum albumin and once with buffer alone. The cells were lysed in 20 ml of 5 mM Tris buffer pH 7.4, containing 1 mM EDTA and centrifuged at 20,000 rpm for 20 min. Ten-ml aliquots of the red supernatant were used for preparation of globin as described in the text. The globin was washed twice with 15 ml of acetone-HCl and dissolved in 20 ml of 1% sodium dodecylsulfate by heating at 75 °C for 1 hr. The samples were cooled and the absorbance read at 337 nm for TNP-globin and at 345 nm for DNP-globin. The ghosts were washed twice with 15 ml of Tris-EDTA buffer and extracted once with 8 ml of chloroform/methanol and once with 2 ml of chloroform/methanol to remove lipid. The lipid extracts were concentrated to a small volume and chromatographed on silica gel plates as explained in the text. The PE and PS bands were scraped off and eluted twice with 2-ml aliquots of methanol. The methanol extracts were brought to 5.0 ml volume, centrifuged at 2000 rpm for 10 min and the absorbance read at 337 and 345 nm for the TNBS- and FDNB-labeled lipids, respectively. The protein residues from the lipid-extracted ghosts were dissolved in 3 ml of 1% sodium dodecylsulfate at 100 °C for 3 hr, centrifuged at 2000 rpm for 10 min and the absorbance read at 337 and 345 nm as specified above.

The data in Table 2 where the probes are used in excess demonstrate that of the 30,000 nmoles of FDNB or TNBS added to the cell system, 6700 nmoles of FDNB have reacted with hemoglobin in contrast to only 174 nmoles of TNBS. Thus, 22.3% of the added FDNB has reacted with hemoglogin as compared to only 0.058% of the added TNBS. The labeling of membrane proteins and membrane amino-phospholipids is small relative to the labeling of globin. The membrane proteins labeled by TNBS are considered to be on the external surface of the membrane whereas membrane proteins labeled with FDNB represent both external and internal proteins. The same situation pertains also to PE and PS. These results indicate that 8, 0 and 25% of PE, PS and protein in the membrane are on the external surface. The very small degree of hemoglobin labeling by TNBS may be due primarily to a small number of leaky cells. A very small amount of hemolysis (1 to 2%) is usually observed in the 2-hr reaction time with the two probes. It is important to

Table 3. Susceptibility of erythrocytes to hemolysis after treatment with TNBS and suberimidate [a]

System	Percent hemolysis [b]
I Control	100
II TNBS	100
III Suberimidate	0.16±0.14
IV TNBS then suberimidate	0.32±0.19
V FDNB	100
VI FDNB then suberimidate	100

[a] Erythrocytes (50 μliters of packed cells) were suspended at 23 °C in 10 ml of 120 mM $NaHCO_3$ − 40 mM NaCl buffer, pH 8.6, and treated as follows: I incubated 1.5 hr; II incubated 0.5 hr in buffer then reacted for 1 hr with 1.5 mM TNBS; III incubated 1 hr in buffer then reacted for 0.5 hr with 10 mM suberimidate (Pierce Chemical Co.); IV reacted for 1 hr with 1.5 mM TNBS then for 0.5 hr with 10 mM suberimidate; V incubated 0.5 hr in buffer then reacted for 1 hr with 1.5 mM FDNB; VI reacted for 1 hr with 1.5 mM FDNB then for 0.5 hr with 10 mM suberimidate. The cells were centrifuged, washed with buffer and lysed in distilled water. Percent hemolysis was determined by measuring the absorbance of liberated hemoglobin at 540 nm.
[b] Values represent the mean ± SD of 4 experiments.

note that when hemoglobin isolated from 0.4 ml of packed cells is reacted with 1.5 mM FDNB or TNBS, both probes label hemoglobin to about the same extent (about 14,000 nmoles of labeling occurs).

Further support of the very small degree of TNBS penetration into intact erythrocytes is shown in Table 3. When cells are treated with the cross-linking agent suberimidate they are rendered refractory to lysis by water, whereas untreated cells or cells treated with TNBS or FDNB are lysed completely by water. When cells are first treated with FDNB and then treated with suberimidate, the cells are no longer protected from lysis by water. However, if cells are first treated with TNBS, washed and then treated with suberimidate, the cells still remain refractory to lysis.

To determine whether cells are nonlytic because the cell membrane is cross-linked or because the hemoglobin is cross-linked, an electron-microscopic study was performed on cells treated with suberimidate and kept in isotonic medium (*A*) and cells treated with suberimidate and then treated with distilled water (*B*). In both cases the cells were refractory to lysis. The cells were examined by electron-microscopy (Fig. 1). The *A* cells show a smooth membrane border with no holes. However, the *B* cells show many large holes in the membrane and a different granularity inside the cell. The size of the membrane holes is about 0.09 μ, sufficient to allow noncross-linked hemoglobin to diffuse. Since these membrane holes do exist and since

the hemoglobin does not leak out, it is evident that these cells do not lyse because the hemoglobin has been extensively cross-linked into a large aggregate. Niehaus and Wold (1970) have postulated that the increased resistance of adipimidate-treated erythrocytes to lysis is due to cross-linking of the membrane and to cross-linking of hemoglobin. Our studies indicate that cross-linking of hemoglobin is the major reason for the resistance of erythrocytes to lysis. The above studies lead to the conclusion that TNBS does not penetrate the intact erythrocyte to any appreciable extent because if it did so it would allow lysis of the cell by blocking the reaction of hemoglobin with suberimidate which does penetrate the cell.

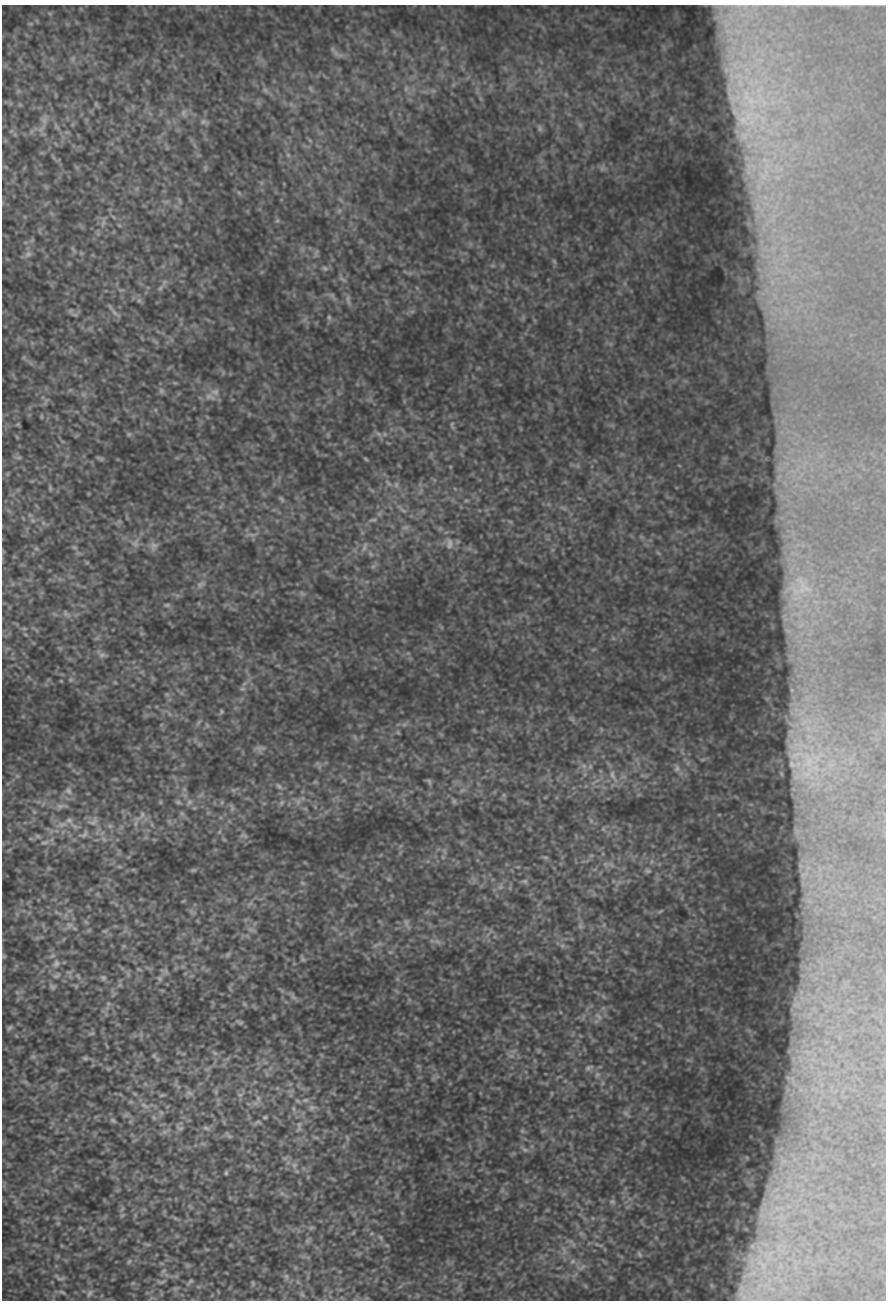
The effect of temperature on the extent of reaction of PE in bicarbonate and phosphate buffers is seen in Fig. 2. In both buffers there is a much greater reaction of PE with TNBS at 37 °C. This is probably due in part to the increased penetration of TNBS into the cell and in part to alteration of the membrane at 37 °C. The values at 3 hr or longer are high due in large part to cell lysis. The extent of reaction of PE is greater in phosphate buffer than in bicarbonate buffer.

The influence of osmolarity on the reaction of PE of intact cells with TNBS is shown in Table 4. The extent of reaction of PE was the same in isotonic, hypotonic and hypertonic buffers at pH 8.6 and 23 °C.

In order to rule out noncovalently bound TNBS as a contaminant in our experiments, we studied the effect of washing cells with buffer containing 0.3% albumin after the TNBS reaction. The results showed that the percent of labeling of PE was the same in control cells (7.1 ± 0.8) as in albumin-washed cells (6.8 ± 0.2).

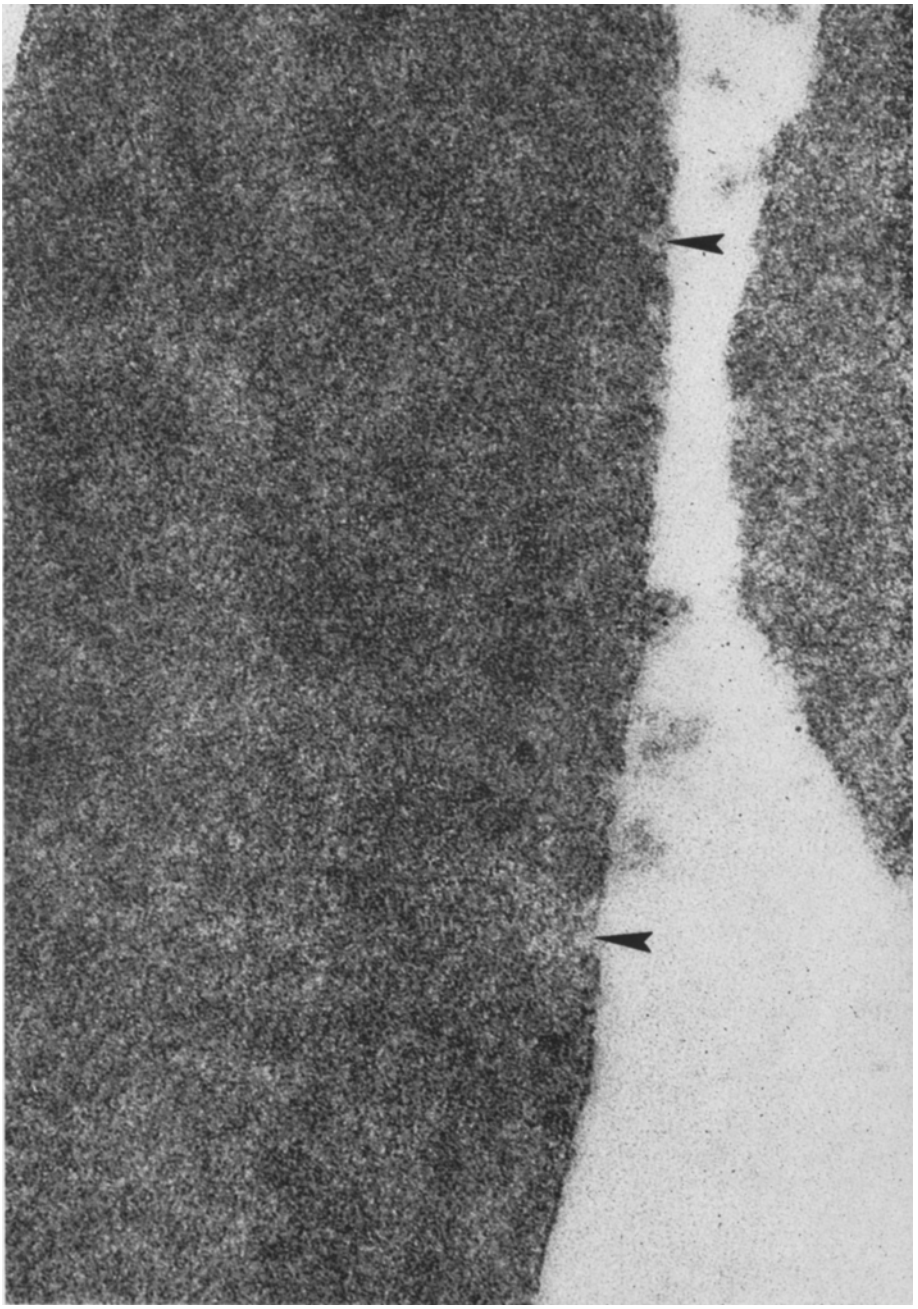
Time-Course of Reaction of Membrane PE, Total Membrane Amino-Phospholipids and Proteins with TNBS

In a previous paper (Gordesky & Marinetti, 1973) we showed the time course of reaction of PE and PS with TNBS in intact erythrocytes and erythrocyte ghosts. With intact cells PS did not react during a 24-hr period at 23 °C. The reaction profile of PE was complex and required about 22 to 24 hr to reach a plateau. The time course indicated two or possibly three regions between 1 and 16 hr where the experimental points did not fit a linear function but rather showed possible transition zones. The profile for ghosts was markedly different. The PE reacted up to 95% in 90 min whereas no PS reacted for 40 min and then the PS reacted to the extent of 50% after 90 min. In the present study we examined the time course of the reaction of TNBS with total amino-phospholipids and total membrane proteins of



A

Fig. 1. Electron-micrographs of intact erythrocytes which have been treated with suberimidate. Erythrocytes (0.4 ml packed cells) were treated with 15 mM suberimidate in $NaHCO_3$ – NaCl buffer, pH 8.5, for 30 min at 23 °C. The cells were washed twice with buffer and centrifuged. Half the cells were suspended in 10 ml of isotonic NaCl (cells *A*) and the other half suspended in 10 ml of distilled water (cells *B*). No significant cell lysis occurred in either sample. After 30 min the cells were spun into a pellet which was used for electron-microscopic analysis. The erythrocyte pellets were fixed in paraformaldehyde-glutaraldehyde, rinsed in 0.1 M sodium cacodylate buffer, pH 7.2, post-



B

fixed in 1% OsO_4 for 1 hr, dehydrated in increasing concentrations of ethanol, and embedded in epoxy resin. For each above step of tissue preparation, the pellets were resuspended and centrifuged. Thick sections (1 μ) of epoxy-embedded erythrocytes were stained with a 1% aqueous solution of toluidine blue. Thin sections (400 to 800 Å) were cut on an LKB Ultratome, stained with uranyl acetate and lead citrate, and observed and photographed in an RCA EMU 3-H electron-microscope. The final magnification was 113,000. Plates *A* and *B* were made from cells *A* and *B*, respectively. Arrows show membrane holes

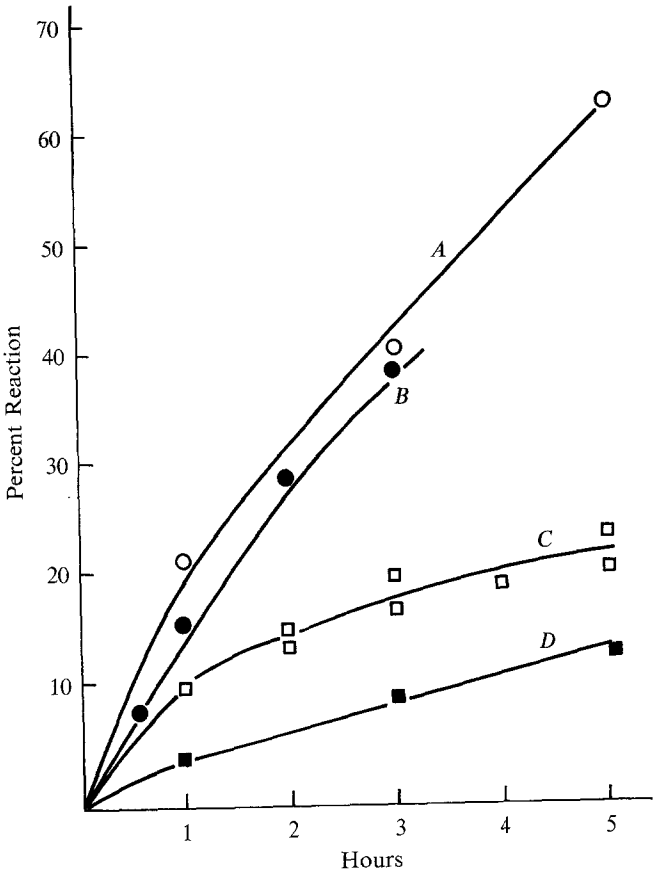


Fig. 2. The effect of temperature and buffer on the time-course of reaction of PE of erythrocytes with TNBS. Erythrocytes (0.4 ml packed cells) were reacted at 23 or 37 °C with 1.5 mM TNBS in 30 ml of either 100 mM phosphate buffer, pH 8.6, or 120 mM $NaHCO_3$ − 40 mM NaCl buffer, pH 8.6, for different time periods. The extraction of the lipids and analysis of TNP-PE were done as explained in the text. *A* and *C* represent the reaction in 100 mM phosphate buffer at 37 and 23 °C, respectively; *B* and *D* represent the reaction in 120 mM bicarbonate-40 mM NaCl buffer at 37 and 23 °C, respectively. Each point represents the average of two experiments analyzed in duplicate

intact cells. The labeling of both the phospholipids and proteins show a complex profile with transition zones (Fig. 3). These transition zones may in part represent lipid phase changes or protein conformational changes of membrane components with the exposing of new reactive sites. A more extensive time course in two different buffers at pH 8.5 is shown in Fig. 4. Only in bicarbonate buffer is the profile complex with the indicated transition zones.

Table 4. Effect of osmolarity on the reaction of PE of intact erythrocytes with TNBS [a]

System	Percent reaction [b]
Isotonic, 300 mosm	6.1 ± 0.6 (3)
Hypotonic, 160 mosm	7.4 ± 2.5 (3)
Hypertonic, 460 mosm	6.2 ± 1.3 (3)

[a] Erythrocytes (0.4 ml packed cells) were reacted with 1.5 mM TNBS at 23 °C for 1.5 hr in 30 ml of different buffers. The isotonic medium contained 80 mM $NaHCO_3$ and 70 mM NaCl; the hypertonic medium contained 80 mM $NaHCO_3$ and 150 mM NaCl; the hypotonic medium contained 80 mM $NaHCO_3$. All buffers were at pH 8.6. The cells were washed two times with 10 ml of the same buffer, and the ghosts prepared as explained in the text. The total lipids were extracted with chloroform/methanol, evaporated, dissolved in 3 ml of methanol and the absorbance determined at 337 nm. Aliquots of the lipid extracts were analyzed for total lipid P. Since 24.6% of the total lipid P is due to PE, from the calibration curve of TNP-PE the percent of total PE which has reacted with TNBS was calculated.

[b] Values represent the percent of total membrane PE which reacts with TNBS (mean ± SD) of triplicate analyses of three experiments).

The reaction of TNBS with amino groups is dependent on the pH of the medium and on the pK_a of the amino group (Means *et al.*, 1972). The effect of pH on the reaction of TNBS with proteins and with PE of intact erythrocytes is shown in Table 5. The extent of reaction for a 2-hr period at 23 °C is appreciably greater for both PE and proteins at pH 8.5 than at pH 7.4.

This raises the question whether the pH inside the cell is sufficiently different from the pH outside the cell to influence the rate and extent to which components inside the cell can react with TNBS. This point has been raised by Bretscher (1973). We examined this problem in two ways. First, we measured the pH difference between the inside of the cell and the external medium when cells were incubated in a medium of 120 mM $NaHCO_3$ – 40 mM NaCl buffer, pH 8.5. The pH difference was measured by the freeze-lysis technique (Lichtman, Murphy, Whitbeck & Kearney, 1974) and was found to be 0.4. Therefore, there is a small pH gradient between the inside and outside of the cell under our experimental conditions, the internal pH being lower. This pH gradient may be significant for short time studies or in studies where TNBS is not used in excess. In our system, TNBS is used in excess and we have examined the reaction over a long time period (up to 24 hr) under conditions where equilibrium has been reached and hence the effect of pH is no longer a factor. In the second method, we added nigericin to the incubation medium. This agent acts to enhance cation transport and eliminate the pH gradient between the inside and outside of mitochondria

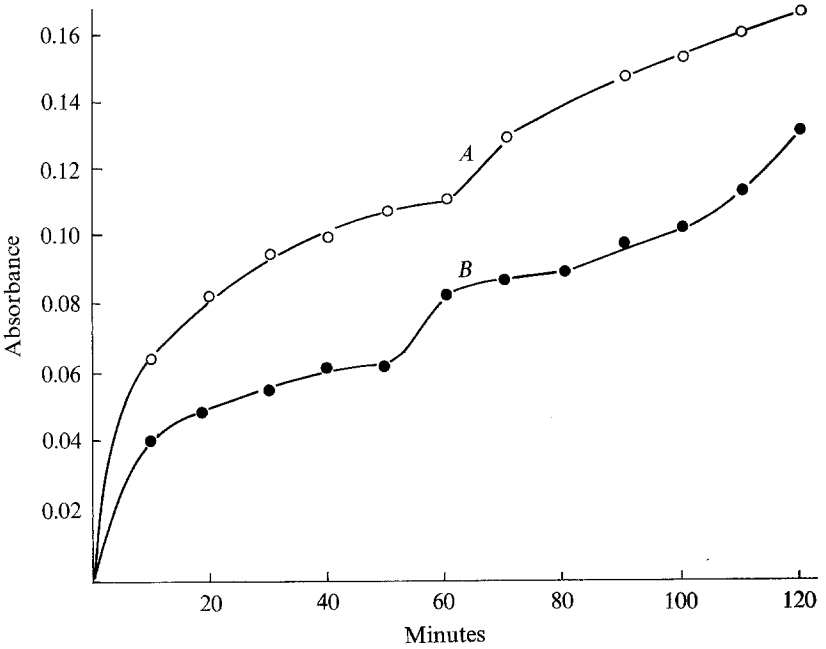


Fig. 3. Time course reaction of proteins and amino-phospholipids of erythrocytes with TNBS. 4.8 ml of fresh erythrocytes were suspended in 360 ml of 120 mM $NaHCO_3$ – 40 mM NaCl buffer, pH 8.6, containing 125 mg of TNBS (1.5 mM). The cells were reacted at 23 °C. At 10-min intervals, 30-ml aliquots were removed and centrifuged at 2000 rpm for 5 min. The cells were washed twice with 30 ml of bicarbonate-NaCl buffer and then lysed in 5 mM Tris buffer, pH 7.4, containing 1 mM EDTA. The lipids of the ghosts were extracted twice with 8 ml of chloroform/methanol 1:1. The extracts were evaporated under nitrogen and the yellow lipid residue dissolved in 5 ml of methanol and centrifuged at 2000 rpm for 5 min. The absorbance of the clear methanol extract was measured at 337 nm. The protein residues were dissolved in 5 ml of 1% sodium dodecyl sulfate at 85 °C for 1 hr, centrifuged at 2000 rpm for 5 min, and the absorbance measured at 337 nm. Curves *A* and *B* represent the labeling of proteins and lipids, respectively. Each point represents the mean of duplicate analyses

and cells (Lardy, Graven & Estrada-O, 1967; Pressman, 1968, 1973; Tosteson, 1968). The data in Table 5 show that the extent of labeling of the membrane PE and proteins was not influenced by nigericin.

Isotonic sucrose has been shown to eliminate the pH gradient between the inside of the erythrocyte and the bulk solution[1]. As seen in Table 5, 0.15 M sucrose gave a small enhancement of the extent of labeling of PE with TNBS. Whether this small effect is due to a pH effect or to a separate effect of sucrose on the membrane is not known.

1 Lichtman, M. A., Whitbeck, A. A., Murphy, M. Factitious changes in binding of oxygen to hemoglobin when based on extracellular pH in the presence of certain blood additives like radiographic contrast media. *Invest Radiol. (submitted for publication).*

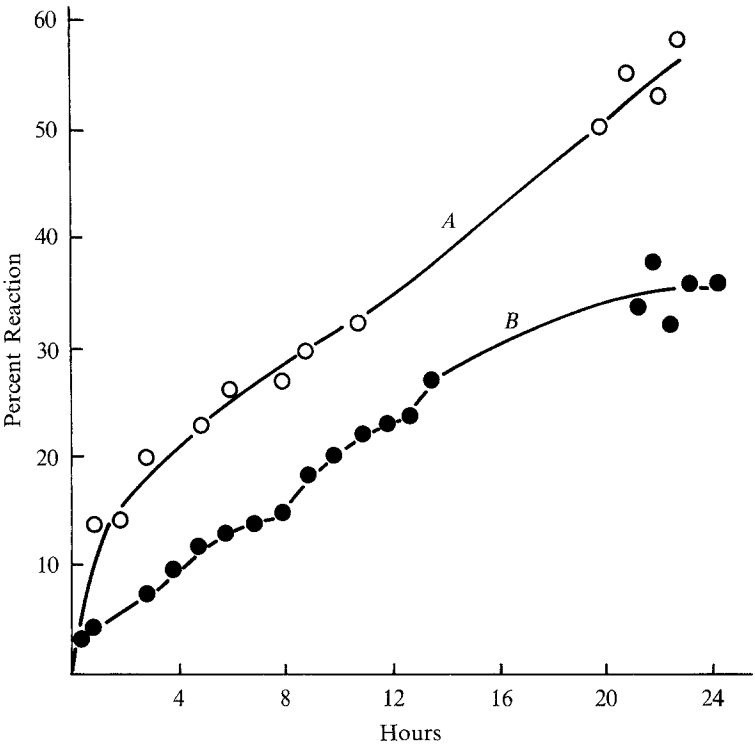


Fig. 4. Time course of reaction of PE of erythrocytes with TNBS in bicarbonate and phosphate buffer. Erythrocytes (0.4 ml packed cells) were reacted at 23 °C in 30 ml of 120 mM $NaHCO_3$ – 40 mM NaCl buffer, pH 8.6, or 100 mM phosphate buffer, pH 8.6, for different time periods. The analysis of TNP-PE was done as explained in the text. Curve *A*, phosphate buffer; curve *B*, bicarbonate buffer. Each point represents the mean of 4 experiments done in duplicate

The effects of proteases and phospholipases on the extent of reaction of PE with TNBS are shown in Table 6. None of these agents had any significant effect on the reaction of PE with TNBS in intact cells. The control values in these experiments and other experiments reported here show a variation which is due to different samples of erythrocytes obtained from the blood bank. The age of the cells may be the most important factor in this variability.

We previously have reported the protective effect of bovine serum albumin (BSA) on the reaction of PE and PS with FDNB both in intact erythrocytes and erythrocyte ghosts (Gordesky *et al.*, 1973). BSA also protects the membrane PE from TNBS in intact erythrocytes as shown in Table 7. The protection is seen in the presence of excess TNBS and thus is not due to the added BSA acting as a sink for the probe. With FDNB the effect of BSA is

Table 5. Effect of pH, nigericin and sucrose on the reaction of TNBS with membrane PE and proteins of intact erythrocytes

Exp. No.	Absorbance	
	PE	Protein
Exp. I		
Bicarbonate buffer, pH 8.6	0.152±0.022 (6)	0.363±0.01 (6)
Krebs-Henseleit buffer, pH 7.4	0.095±0.014 (6)	0.207±0.02 (6)
Exp. II		
Control	0.140±0.003 (4)	0.272±0.037 (4)
Nigericin (100 μg)	0.146±0.005 (4)	0.275±0.007 (3)
Exp. III		
Control	0.055±0.01 (5)	
0.15 M sucrose	0.074±0.01 (5)	

In Exp. I, erythrocytes (0.4 ml packed cells) were suspended in 30 ml of either Krebs-Henseleit buffer, pH 7.4, or 30 ml of 120 mM $NaHCO_3$ − 40 mM NaCl buffer, pH 8.6. To each were added 100 μliters of TNBS (10.4 mg) in methanol to make a final concentration of TNBS of 1.5 mM. The cells were reacted at 23 °C for 2 hr, washed with the respective buffers containing 0.3% albumin, and ghosts were prepared. The lipids were extracted and TNP-PE was isolated by TLC as explained in the text. The protein residues were dissolved in 3 ml of 1% sodium dodecyl sulfate, spun at 3000 rpm and the absorbance determined at 337 nm. The absorbance at 337 nm in 3 ml of methanol was used to measure the TNP-PE. The Krebs-Henseleit buffer contained (in mM) 118 NaCl, 25 $NaHCO_3$, 4.7 KCl, 4.3 glucose, 1.3 $CaCl_2$, 1.2 mM $MgSO_4$ and 1.2 mM KH_2PO_4.

In Exp. II, erythrocytes (0.4 ml packed cells) were suspended in 30 ml of $NaHCO_3$ − NaCl buffer, pH 8.6, with and without 100 μg of nigericin. The cells were incubated for 15 min at 23 °C. TNBS was then added to both cells to make a final TNBS concentration of 1.5 mM. The cells were reacted for 2 hr at 23 °C, and washed with buffer. The analyses of labeled PE and proteins were done as described in Exp. I. In Exp. III, 0.4 ml of packed cells from a different batch of outdated blood was used. The reaction was carried out in 30 ml of $NaHCO_3$ − NaCl buffer, pH 8.6, with and without 0.15 M sucrose. The values represent the mean±SD of 3 to 6 experiments. The lower values in Exp. III are due to the age of the blood giving a lower yield of lipid.

maximal at 40 to 70 μM BSA. It was suggested (Gordesky *et al.*, 1973) that the BSA effect is due to its interaction with and saturation of specific binding sites on the red cell membrane with concomitant protection of certain PE molecules. When dinitrophenylated BSA is used in place of BSA, the protective action of this protein is abolished. This finding indicates that certain amino groups on BSA are important in its interaction with the erythrocyte membrane. The data in Table 7 also show that any monoamine oxidase contamination of commercial BSA is not involved in the protective effect since purified BSA in which monoamine oxidase activity was unmeasurable,

Table 6. The effect of proteases and phospholipases on the reaction of TNBS with membrane PE of intact erythrocytes [a]

Exp. No.	Absorbance [b]
Exp. I	
Control	0.150 ± 0.018 (6)
Trypsin-treated	0.163 ± 0.018 (6)
Exp. II	
Control	0.102 ± 0.001 (3)
Pronase-treated	0.096 ± 0.007 (3)
Exp. III	
Control	0.079 ± 0.014 (6)
Phospholipase A-treated	0.078 ± 0.003 (6)
Phospholipase D-treated	0.081 ± 0.021 (6)

[a] In Exps. I and II different batches of erythrocytes (0.4 ml packed cells) were treated with 200 μg of trypsin (Sigma) or 100 μg of pronase (Sigma) in 30 ml of Krebs-Henseleit buffer, pH 7.4, for 1 hr at 37 °C. The cells were washed two times with buffer, resuspended in 30 ml of $NaHCO_3 - NaCl$ buffer, pH 8.6, containing 1.5 mM TNBS and reacted at 23 °C for 2 hr. The preparation of ghosts and lipid extracts were done as given in the text. The TNP-PE was isolated by TLC, eluted with 3 ml of methanol and the absorbance was measured at 337 nm. (*See* Table 5 for the composition of the Krebs-Henseleit buffer.) In Exp. III, a different batch of erythrocytes (0.4 ml packed cells) was reacted as explained above except that 100 μg of phospholipase D (Boehringer-Soehne) or 100 μg of phospholipase A_2 (*Naia naia*) were used in place of pronase or trypsin.
[b] The values represent the mean ± SD of 3 or 6 experiments.

Table 7. The effect of albumin on the reaction of TNBS with membrane PE of intact erythrocytes

	Absorbance
Control – no BSA	0.138 (0.128 – 0.148)
BSA, 70 μM	0.079 (0.071 – 0.087)
Purified BSA, 70 μM	0.081 (0.081 – 0.083)

Erythrocytes (0.4 ml packed cells) were reacted for 2 hr at 23 °C with 1.5 mM TNBS in 30 ml of $NaHCO_3 - NaCl$ buffer, pH 8.6, with and without albumin. The analysis of TNP-PE was carried out as explained in the text. The absorbance of TNP-PE in methanol was measured at 337 nm. The values represent the average of two experiments run in duplicate. The range of values is given in parentheses.

gave the same amount of protection as did commercial BSA. This experiment rules out the possibility that monoamine oxidase destruction of amino groups of PE led to the observed effect.

FDNB, TNBS, and other amino-reacting probes are known to influence cation leak and hemolysis of erythrocytes (Knauf & Rothstein, 1971;

Table 8. Effect of FDNB and TNBS on the susceptibility of erythrocytes to hemolysis by Triton X-100 [a]

System	Concentration of Triton to obtain 50% hemolysis (μM) [d]					
	1 min	Δ	15 min	Δ	90 min	Δ
Controls [b]	123 ± 8	21	115 ± 13	30	105 ± 3	54
FDNB-treated [b]	102 ± 11		85 ± 9		51 ± 16	
Controls [c]	121 ± 10	17	114 ± 8	11	111 ± 9	19
TNBS-treated [c]	104 ± 10		103 ± 9		92 ± 8	

[a] Intact erythrocytes (50 μliters of packed cells) were incubated in 10 ml of 120 mM $NaHCO_3$ − 40 mM NaCl buffer, pH 8.6, with and without TNBS (1.5 mM) or FDNB (1.5 mM). Incubations were carried out for 1, 15 and 90 min. The cells were centrifuged, washed and resuspended in buffer containing varying amounts of Triton X-100 (20 to 300 μM). The extent of lysis was determined by measuring the absorption at 540 nm in the supernatant after centrifuging nonlysed cells for 5 min at 3000 rpm.

[b] System contained 0.4% methanol (the FDNB was added as a solution in methanol).

[c] System contained no methanol.

[d] Values represent the mean ± SD of 5 experiments. The difference between the FDNB- and TNBS-treated cells and their respective controls were significant to $p < 0.02$.

Poensgen & Passow, 1971; Gordesky *et al.*, 1973). This effect is markedly enhanced if these probes are added in alcohol solution to red cells suspended in buffer, the final alcohol concentration being from 0.4 to 2.0%. When FDNB or TNBS is used without alcohol, cell lysis is below 2% over 10 hr. However, cation leak (Na^+ or K^+) does occur without lysis during FDNB treatment of cells (Gordesky *et al.*, 1973).

Next we examined the perturbation of the red cell membrane by TNBS and FDNB by measuring the susceptibility of erythrocytes to hemolysis by various concentrations of Triton X-100 (Table 8). FDNB was added in methanol solution to enhance its perturbation of the membrane. The final concentration of methanol was 0.4%. The control cells were exposed to the same buffer and alcohol concentration but without probe. TNBS was used without methanol. The control cells for TNBS were incubated in the same buffer without methanol and TNBS. The cells were treated for 1, 15 and 90 min with FDNB or TNBS. The cells were washed and aliquots were added to the same buffer containing different concentrations of Triton X-100. After 15 min at 23 °C the cells were centrifuged at 3000 rpm for 5 min and lysis determined by measuring the released hemoglobin at 540 nm. From the titration curves, the concentration of Triton X-100 giving 50% hemolysis was calculated. The results show that both FDNB and TNBS produce a quantitatively similar effect on the erythrocyte within one minute. The

reacted cells become slightly more susceptible to hemolysis by detergent than controls. This rapid effect of the probe we believe is due to a subtle perturbation on the outer surface of the membrane which may enhance the interaction of the membrane with Triton X-100.

When cells are treated for 15 min with each probe, the magnitude of the TNBS effect is unchanged whereas the FDNB effect is greater. We interpret this effect of FDNB to be caused by an action of FDNB on the inside surface of the cell membrane since FDNB has penetrated the cell membrane to an appreciable extent in 15 min whereas TNBS penetration of the membrane is negligible. The control cells also become slightly more susceptible to hemolysis with time. The increased susceptibility to hemolysis is not due to methanol since control cells for the TNBS experiment where methanol was not used gave the same titration curve as did control cells for FDNB where methanol was used.

After a 90 min exposure of cells to the probes, the FDNB perturbation is very pronounced when compared to the TNBS effect. When the effect of FDNB relative to its control is compared to that of TNBS relative to its control, it can be seen that these Δ values for FDNB with time of exposure increase from 21 to 30 to 54 whereas the Δ values for TNBS change with time from 17 to 11 to 19. These observations show that TNBS and FDNB, although they both react with the same functional groups of protein and lipids, must act at different domains of the cell membrane, this difference being due in major part to their different penetration into the cell. FDNB penetrates the erythrocyte membrane very rapidly (Krupka, 1972) whereas TNBS penetration of the membrane is negligible. Under appropriately controlled conditions these probes can be used in combination to examine the outer and inner surfaces of the erythrocyte membrane.

Discussion

We have concluded in a previous study (Gordesky & Marinetti, 1973) that the amino-phospholipids are asymmetrically distributed in the erythrocyte membrane; i.e., these amino-phospholipids are localized primarily on the inner surface of the membrane. This finding was later confirmed by an independent method, using phospholipases, by Verkleij, Zwaal, Roelofsen, Comfurius, Kastelijn & van Deenen (1973).

Knauf and Rothstein (1971) have studied the effect of FDNB, TNBS and other probes on anion and cation permeability in erythrocytes. They found that exposure of red cells to FDNB, SITS, pCMBS and TNBS, for 2 hr at 37 °C in phosphate NaCl buffer, pH 7.4, gave the following effects:

(a) FDNB and TNBS and SITS decreased anion (sulfate ion) permeability; (b) FDNB increased cation permeability the same as pCMBS whereas TNBS gave a smaller increase in cation permeability; (c) pCMBS did not increase anion permeability; (d) SITS did not increase cation permeability. These authors concluded that two ion permeability barriers exist in the red cell membrane; an outer barrier in which amino groups control anion permeability and an internal barrier in which both amino groups and SH groups control cation permeability. Only a small number of the total NH_2 and SH groups in the membrane are involved in the control of ion permeation and the active amino groups are presumed to be in the NH_3^+ form. Had these workers used the lower temperature of 23 °C rather than 37 °C and had they employed a bicarbonate-NaCl buffer system in order to minimize TNBS penetration into the cell, we predict their results with TNBS would have been similar to or identical with the results with SITS which is considered to be a nonpenetrating probe for the erythrocyte membrane (Maddy, 1964).

Our studies have shown that under appropriate conditions TNBS can be employed as a vectorial probe to sense certain functional groups (primarily amino groups) of proteins and phospholipids on the outer surface of the erythrocyte membrane. To minimize penetration of TNBS into the cell the temperature must be kept around 23 °C and bicarbonate buffer free of phosphate should be used. TNBS is a stable water-soluble probe (Means *et al.*, 1972) and can be used for long reaction times allowing saturation of available sites. In bicarbonate buffer, pH 8.6, it undergoes only 8 to 10% hydrolysis in 20 hr at 23 °C. In contrast, other chemical probes such as dansyl chloride, formyl methionine methyl phosphate, diazosulfanilic acid are much less stable in water and are limited to short reaction times.

Arrotti and Garvin (1972) used phosphate buffer, pH 7.4 at 37 °C, in their studies with labeled TNBS. Therefore, their finding that TNBS penetrated the erythrocyte membrane is not unexpected. Schmidt-Ullrich, Knufermann and Wallach (1973) did not consider the importance of the experimental conditions involving temperature and buffer composition on the penetrability of TNBS. This led them to give preference to the work of Arrotti and Garvin (1972) over that of Bonsall and Hunt (1971). This also led them to question the use of TNBS and other small chemical reagents as vectorial probes for the cell membrane. The important practical question in the use of chemical probes is under what conditions and to what degree they penetrate the cell membrane and whether this is a significant deterrent to their being used as vectorial probes. Our present work shows that TNBS can be used as a vectorial probe under certain specified conditions and that

temperature and the length of time of exposure of cells to TNBS are important since TNBS itself and elevated temperature may eventually alter the cell membrane and render the cells gradually susceptible to lysis, or that incubated cells gradually become sufficiently unstable so that TNBS slowly begins to penetrate into the cell.

The complex nature of the time course of reaction of TNBS with intact erythrocytes indicates transition zones involving both lipids and proteins. These results are consistent with alterations occurring in the cell membrane. Very little hemolysis occurs in bicarbonate buffer pH 8.6 at 23 °C when cells are exposed to 1.5 mM TNBS for periods up to 12 hr. However, after 12 hr hemolysis begins to occur. Thus, the percent of total PE and PS which reacts in the period up to 12 hr can be considered as that PE and PS which is on the outer surface of the cell membrane. In this present study we conclude that 10 to 20% of the total PE and essentially none of the PS is located on the outer surface of the erythrocyte membrane. These values are in agreement with those obtained by an independent method by Verkleij *et al.* (1973).

In phosphate buffer, pH 8.6 at 23 °C, a plateau in the reaction of PE with TNBS is not reached after 24 hr since cell hemolysis is appreciable at this long time interval and hence the TNBS is continuing to react with leaky cells. With bicarbonate buffer, pH 8.6 at 23 °C, a plateau is reached at 22 to 24 hr even though some cells have undergone lysis. We suggest that the increase in the fraction of PE which reacts with TNBS in the 12- to 24-hr period in bicarbonate buffer represents a certain fixed population of erythrocytes which is unstable and either lyse or become leaky to TNBS. This represents about 10% of the total red cell population. In phosphate buffer a much greater fraction of the cells becomes leaky and undergoes lysis.

The small degree of penetration of TNBS into the erythrocyte over a 10 hr period at 23 °C in bicarbonate buffer is supported by the following experimental observations: (a) the cells do not lyse as they do when FDNB is used, (b) very little hemoglobin becomes labeled, (c) TNBS does not inhibit the ability of suberimidate to make cells refractory to lysis, (d) cells treated for 1, 15 and 90 min with TNBS are slightly more susceptible to lysis by Triton X-100 whereas cells treated with FDNB (a penetrating probe) become increasingly susceptible to lysis by Triton X-100, and (e) Knauf and Rothstein (1971) found a much smaller effect of TNBS on cation permeability compared to FDNB.

Changing the osmolarity of the reaction medium from 160 to 460 mosm had no significant effect on the extent of reaction of PE with TNBS. Treatment of erythrocytes with trypsin, pronase, phospholipase A and phospho-

lipase D also had no significant effect on the extent of reaction of PE with TNBS. However, the reaction of PE with TNBS was pH dependent. Considerably more PE reacted at pH 8.6 than at pH 7.4 during a 2-hr period. The reaction of membrane proteins was also greater at the higher pH. This is due in part to the inherently greater chemical reactivity of the nonprotonated amino group as compared to the protonated amino group. At pH 8.6 the population of proteins and amino-phospholipids having unprotonated amino groups is greater than at pH 7.4. It is also possible that the conformation of the membrane may be different at the higher pH and expose more amino groups.

In order to test whether the failure of the amino-phospholipids on the inner membrane surface to react with TNBS might be due to a lower pH in the inside of the cell, we measured the pH of the inside of the cell when it was exposed to 120 mM $NaHCO_3$ – 40 mM NaCl buffer, pH 8.6. The internal pH was found to be lower than the external pH by 0.4 units. Since nigericin had no effect on the extent of reaction of PE with TNBS and since the pH gradient is expected to be abolished by nigericin, we conclude that the amino-phospholipids on the inner membrane surface do not react with TNBS because the probe does not penetrate into the cell to any appreciable extent.

Added BSA (commercial or highly purified) decreased the reaction of PE with TNBS in intact cells. However, if the BSA is first dinitrophenylated it no longer produces its effects. Since the reaction time was limited to 2 hr and has not reached a plateau we cannot state whether this effect of BSA is mediated by its lowering the TNBS concentration or by its binding to the membrane and protecting certain PE molecules from reacting with the probe. In an earlier study with FDNB we were able to show that BSA protects a certain fraction of PE molecules from reacting with FDNB, by binding to the cell membrane. This effect of BSA was observed since the FDNB was used in excess and the reaction was allowed to proceed until all of the available PE amino groups had reacted with FDNB.

The present studies demonstrate that TNBS can be used as a vectorial probe for the outer surface of the erythrocyte membrane and confirms and extends our earlier conclusion that PE and PS are asymmetrically arranged in the membrane with nearly all of the PS and at least 80 to 90% of the PE being localized on the inner membrane surface or partially buried in the membrane. We have rebutted two possible criticisms raised by others (Bretscher, 1973; Schmidt-Ullrich *et al.*, 1973; Wallach & Schmidt-Ullrich, 1974) on the interpretation of our results by showing that under certain defined conditions TNBS does not penetrate the cell membrane significantly so that it does in fact act as a vectorial probe and that the pH gradient across the

cell membrane is not a significant factor in preventing TNBS from reacting with PE or PS on the inside surface of the cell membrane.

The pH gradient between the inside of the erythrocyte and the buffer was determined by the freeze-lysis technique by Dr. M. Lichtman, Departments of Medicine and Biophysics and Radiation Biology, University of Rochester. Nigericin was a gift sample from Dr. S. Estrada-O, Biochemistry Department, Polytechnic Institute, Mexico City. Highly purified BSA free from monamine oxidase was kindly furnished by Dr. L. Miller, Departments of Biochemistry and Biophysics and Radiation Biology, University of Rochester. The electron-micrographs were done by Dr. D. Penney, Department of Anatomy, University of Rochester.

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