SELECTIVE LABELING OF HUMAN ERYTHROCYTE MEMBRANE COMPONENTS WITH TRITIATED TRINITROBENZENESULFONIC ACID AND PICRYL CHLORIDE

John J. Arrotti and James E. Garvin

Department of Biochemistry, Northwestern University Medical School
Chicago, Illinois 60611

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SUMMARY: Treatment of intact human erythrocytes with tritiated trinitrobenzenesulfonic acid (TNBS) and picryl chloride (PC) resulted in labeling of their membrane components. Separation of these components by polyacrylamide gel electrophoresis in 1% SDS yielded three significant results: 1) in the protein region significant TNBS label was confined to a single sharp high peak centering on band 3 of Fairbanks et al. (1), 2) protein components of bands 1 and 2 contained significant amounts of PC label and were free of TNBS label, 3) the low molecular weight lipid components were heavily labeled by both reagents. Chemical separation of the protein and lipid components showed about 70% of the TNBS label and about 53% of the PC label to be in the protein fraction with the balance of the activity appearing in the lipid fraction. The selectivity of these membrane-penetrating reagents for different membrane components may be a function of their differing polarities.

In a recent report (2) we have described some aspects of the kinetics of the entry of ^{3}H-TNBS and ^{3}H-PC into the intact human erythrocyte. These reagents are similar in size, shape, and reaction specificities but differ greatly in polarity. We found that the more polar TNBS entered the erythrocytes more slowly than the apolar PC. This is consistent with the general finding that the more apolar compounds during passive permeation, presumably traveling through apolar portions of the membrane, enter the cell more rapidly (3,4). We now report on the distribution of these two reagents among the membrane components as revealed by SDS polyacrylamide gel electrophoresis and chemical extractions. The data reveal a selectivity of the reagents for different components of the membrane which may be related to different paths of permeation.

MATERIALS AND METHODS: Blood was drawn from healthy donors without regard to type, using 0.11 M EDTA as an anticoagulant. After removal of the plasma and buffy coat, the cells were washed four times with modified Krebs-Ringer

phosphate (KRP-G) (2). In aspirating the plasma supernatants many erythrocytes were sacrificed to ensure removal of most of the leucocytes and platelets. A 0.5 ml portion of packed erythrocytes were incubated with 4.0 ml of either ^{3}H-TNBS (0.1 mM, 9.5 mCi/mmole) or ^{3}H-PC (0.1 mM, 14.0 mCi/mmole) in KRP-G, pH 7.4 for 60 min with stirring at 37°C, followed by the addition of 5 ml of KRP-G to each tube. The erythrocytes were centrifuged at 2000 x g for 2 min and the supernatants discarded. This procedure was repeated 3 times using 5 ml of KRP-G in each wash. These modified erythrocytes were stored overnight at 4°C, then lysed with 15 mM Tris (pH 7.5) in a ratio of packed cells to Tris of 1/40, v/v, and centrifuged at 25,000 x g for 30 min. This procedure was repeated three times with centrifugation for 15 min. The washed ghosts were dialyzed for 18 hrs. at 4°C against 5 mM EDTA, 5 mM 2-mercaptoethanol, pH 7.5. The dialyzed ghost suspension was brought to a volume of 1.2 ml with distilled water and aliquots were removed for radioactive counting and protein assay. To the remaining 1.0 ml of ghost suspension was added 0.35 ml of 10% SDS, 10 mM 2-mercaptoethanol and 250 mg of sucrose and the solution was heated to 100°C for 3 min. These solubilized membranes were layered on 6.25% polyacrylamide gels at 30-50 µg protein per gel and electrophoresis carried out at 5 volts/cm. Bright yellow TNP-myoglobin was used as the tracking substance. The gel composition was 6.25% acrylamide, 0.13% N,N'-methylenebisacrylamide, 0.05% N,N,N',N'-tetramethylenediamine and 0.075% ammonium persulfate. One gel of each set was stained with Coomassie Blue according to Fairbanks et al. (1). Two (TNBS) or three (PC) identical unstained gels were sliced, corresponding slices were combined and oxidized in a Packard Model 300 tritium oxidizer and counted in a Mark I, Nuclear-Chicago liquid scintillation spectrometer using a dioxane-based cocktail (2). Ghosts were lipid-extracted with ethanol-ether, 3/1, v/v, according to Rosenberg and Guidotti (5). Protein analysis was by the method of Lowry (6) and lipid phosphorus according to Bartlett (7).

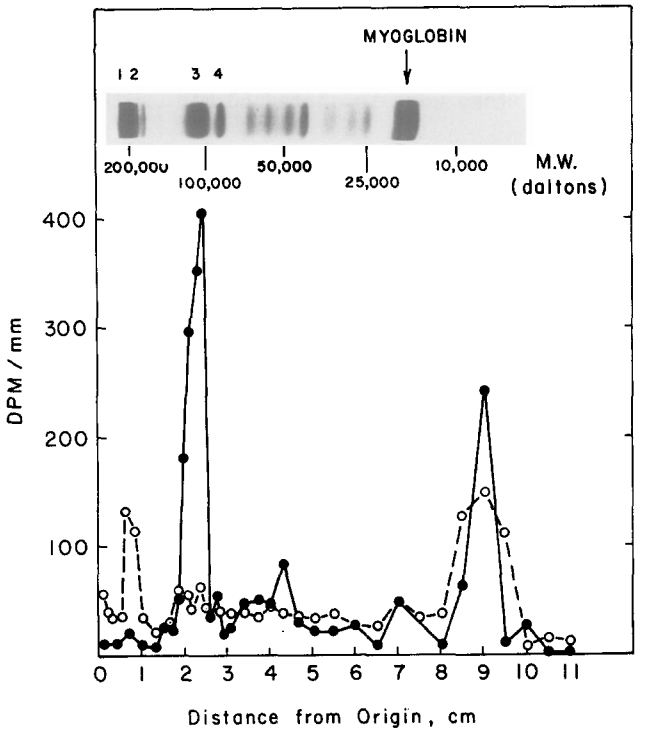

Fig. 1 Comparison of radioactivity versus distance plots obtained by treating intact erythrocytes with TNBS (●—●), or PC (o--o) and fractionating the isolated membranes by electrophoresis on SDS polyacrylamide gels.

RESULTS: Fig. 1 shows the profiles of radioactivity obtained with TNBS and PC plotted below a photograph of a typical polyacrylamide gel of solubilized erythrocyte membranes stained for protein with Coomassie Blue. The TNBS curve shows a narrow peak centering on band 3 which contains about 49% of the protein label and a substantial but smaller peak, centering on about 8000 daltons in the lipid region which contains 30% of the total label. An important feature of the TNBS curve is the sparsity of label in the region of bands 1 and 2. The PC curve shows only a small amount of radioactivity in band 3 but has a distinct narrow peak centering on bands 1 and 2 and a broader large peak centering on 8000 daltons in the lipid region which contains about 50% of the total label.

In order to determine whether our incubation conditions permitted satura-

tion of reactive groups in any membrane component, intact erythrocytes were doubly-pulsed, that is, they were incubated 60 min with TNBS or PC, washed and reincubated for another 60 min with fresh reagent. Electrophoresis of these membranes showed a doubling of specific activity in each band, indicating that the amount of reagent was the limiting factor in all cases and that the relative labeling differences between bands was not due to the absolute number of reactive groups. The arrow indicates the location to which a myoglobin or hemoglobin standard would migrate. Very little label appears in this position, confirming our previous conclusion that the total radioactivity in the whole membrane was not due to adsorption of labeled hemoglobin of very high specific activity (2).

The relative labeling of proteins and lipids was also determined by chemical means as described in MATERIALS AND METHODS. In three determinations of the partition of the TNBS label, proteins contained 67 ± 1%, and 33 ± 1%; in three determinations of the partition of the PC label, protein contained 54 ± 1% and lipid 46 ± 1%. Table 1 shows the degree of cross-contamination of the chemically extracted protein and lipid fractions. In all cases less than 11% of activity is assignable to contamination. The levels of contamination are similar to those reported by Rosenberg and Guidotti (5). Finally, an analysis of the total radioactivity incorporated into the whole ghosts by the two reagents, corrected for their specific activities, showed that under our conditions anywhere from 3.4 to 4.4 times as many TNBS molecules as PC molecules were incorporated. It can be calculated that after labeling with ^{3}H-TNBS, approximately one TNP group is associated with every fifth polypeptide chain of band 3 (1).

DISCUSSION: The contrast previously shown (2) between the behavior of the polar TNBS and the apolar PC are emphasized by the data described in the present paper. The greater total labeling of the membrane by the TNBS under identical initial incubation conditions seems most easily accounted for by its

TABLE 1

CROSS CONTAMINATION OF LIPID AND PROTEIN FRACTIONS*

Lipid Contamination in Protein Fractions

Derivatizing agent	μM P / mg. protein	DPM equivalent	DPM / mg. of protein	Per cent contamination
TNBS	0.056	785	16,567	4.7%
PC	0.057	266	2,493	10.7%

Protein Contamination in Lipid Fractions

Derivatizing agent	mg. protein / μM of P	DPM equivalent	DPM / μM of P	Per cent contamination
TNBS	0.093	1540	14,067	11.0%
PC	0.103	257	4,667	5.5%

*Ghosts were lipid extracted, and the lipid and protein fractions counted as described in Methods. Values are the means of three experiments.

slower disappearance from the medium (2). The rapid disappearance of the PC from the medium can be most readily explained by the more rapid permeation of the membrane usually found for apolar compounds (3,4), although a more rapid reaction of the PC with the hemoglobin sink inside the erythrocyte cannot be excluded as a contributing factor. The greater proportion of PC label in the lipid fraction likewise is consistent with its entering by, and reacting within the bilayer. Knauf and Rothstein (8) have used similar arguments to explain the more rapid permeation of the relatively apolar p-chloromercuribenzoate over the more polar p-chloromercuribenzenesulfonate. The selective labeling of band 3 by the TNBS and bands 1 and 2 by PC are the most striking results of the present investigation. An interpretation of this selectivity in terms of either access governed by partition coefficient or specificity of reac-

tion is desirable but the data available do not permit an unambiguous decision. Both reagents react primarily with amino and sulfhydryl groups under our conditions (2,9,10). It is possible that the selectivity is due primarily to reaction specificity, that is, that bands 1 and 2 contain proteins whose reactive groups are uniquely vulnerable to PC, and band 3 contains proteins especially reactive with TNBS. Further experiments using 10% acrylamide gels have shown that the glycoprotein, glycophorin (11), is not being labeled by the TNBS (12). Cohen (13) has described comparable selectivity among soluble proteins. In previous experiments (2) we have reported that for the first 30 min of reaction with human serum albumin, TNBS and PC were taken up at exactly the same rate. However, reaction in homogeneous solution with a single protein cannot be taken as a guide to reaction in a heterogeneous system with many different polypeptides. Phillips and Morrison (14,15) have found that band 3 proteins and glycophorin (11) are iodinated on the surface of the intact erythrocyte. Both Steck et al. (16) and Bretscher (17) using a variety of techniques have interpreted their data to imply that the polypeptides of band 3 span the thickness of the membrane and are exposed on both sides. Recently Avruch and Fairbanks (18) have reported that a radioactively labeled reactive intermediate in the operation of the sodium pump is also found in band 3. All of these data are consistent with the possibility that band 3 protein(s) make up some or all of a hydrophilic protein channel through the membrane. TNBS being highly polar with a unit charge could react at either surface or at some point in transit through the channel. On the other hand PC being quite apolar would transit primarily through hydrophobic channels and would be more likely to label in hydrophobic regions.

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