BBA 75757

# REACTIVITY OF THE HUMAN ERYTHROCYTE MEMBRANE TO SODIUM TRINITROBENZENESULPHONATE

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(Received June 15th, 1971)

SUMMARY

1. 58% of phospholipid amine groups in human erythrocyte ghosts reacted with the water-soluble reagent, sodium trinitrobenzenesulphonate (TNBS), though only one third of the protein amine groups were similarly accessible. When the ghosts were solubilised in 1% Triton X-100, all the lipid amines could react with TNBS but there was almost no change in the reactivity of the protein components.

2. Whole human erythrocytes reacted much more slowly with TNBS than did ghosts and the reagent failed to penetrate the membrane and react with haemoglobin.

3. The isoelectric point of the bulk of the membrane protein solubilised in Triton X-100 from ghosts or erythrocytes after reaction with TNBS was decreased, indicating that the protein particles all contained uncharged trinitrophenyl (TNP)-amine groups which had been accessible from outside the permeability barrier.

Previous attempts to label the outside of the erythrocyte membrane[1-4] have been mainly concerned with characterising components accessible from the exterior of the cell. The primary purpose of this investigation, on the other hand, has been to study changes in the accessibilities of membrane constituents of intact human erythrocytes when solubilised in the nonionic surfactant, Triton X-100. The trinitrophenylating agent, 2,3,5-trinitrobenzenesulphonate (TNBS) has been employed because its reaction products are easily detected spectrophotometrically and because it reacts at relatively low pH (8.0). It has been previously used for measuring primary amine concentrations but its reactivity with proteins has been shown to be dependent on the accessibility and the ionic environment of the amine groups[5]. Like other protein reagents which have been used to study the outside of the erythrocyte membrane[1-3], it is insoluble in lipophylic solvents and is, therefore, unlikely to penetrate the permeability barrier of the membrane.

Haemoglobin-free erythrocyte ghosts, prepared by the method of DODGE *et al.*[6] and at a concentration giving 0.375 mg protein/ml, were reacted with 0.1% TNBS n 100 mM, sodium phosphate buffer pH 8.0. Aliquots (2.5 ml) were removed at timed

Abbreviations: TNBS, 2,3,5-trinitrobenzenesulphonate; TNP, trinitrophenyl.

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intervals and mixed with 0.5 ml 1 M HCl, 0.5% sodium dodecyl sulphate to acidify and clarify the suspension. The absorbance at 344 nm was determined against a zero-time blank. The concentration of trinitrophenyl (TNP) derivatives was calculated using a molar extinction coefficient of $1.1 \cdot 10^4$ (see ref. 5). Protein concentrations were measured using the method of LOWRY *et al.*[7], modified, as described elsewhere[8], for use in the presence of Triton X-100.

The initial reaction of ghosts with TNBS was rapid but the rate decreased so that the TNP concentration after 20 min was only 6% less than the value of 0.252 μmole/mg protein attained in 50 min. When the experiment was repeated in the presence of 1% Triton X-100, a value of 0.351 μmole TNP/mg protein was reached after 50 min, but the shape of the time course of the reaction was the same as in the absence of surfactant. Ghosts and ghosts in 1% Triton X-100, which had been reacted for 50 min with TNBS, were extracted with 2 vol. of 1:1 chloroform–methanol. The lipid extracts were evaporated to dryness in nitrogen. The TNP contents of these extracts, determined in acid-surfactant, were 0.237 and 0.410 μmole/μmole total phosphate respectively, and, when the TNP-lipids were further reacted with TNBS, a TNP content of 0.413 μmole/μmole phosphate was obtained. Thus, all the lipid amines in the ghost-surfactant preparation were accessible to TNBS, a finding consistent with the observation that phospholipids are separated from proteins under these conditions[8], whilst only 58% of the lipid amines in the intact ghosts were accessible. The difference in the lipid extracts was equivalent to 0.092 μmole/mg protein and so accounted for almost all (93%) of the change in the reactivity of ghosts to TNBS when Triton X-100 was added. This shows that 1% Triton X-100 made almost no difference to the accessibility of the protein amines.

Using published data[9] on the proportional composition of primary amine groups in erythrocyte phospholipids, a value of 0.402 μmole amine/μmole phosphate is obtained and this compares well with the TNP content found experimentally. On the other hand, amino acid analysis of total ghost protein[10] gives a primary amine content of 0.384 μmole/mg protein which contrasts with the values of 0.126 and 0.133 μmole/mg obtained for the protein of ghosts and ghosts in 1% Triton X-100 respectively. This result indicates that a large proportion of protein amines remain inaccessible and is consistent with a finding that only 57% of the total amino acid side groups which should titrate in the pH 8–11 region, do so in ghosts in 1% Triton X-100.

The reactivity of whole erythrocytes was studied by suspending the cells, at about 10% haematocrit, in a medium containing 0.1% TNBS, 100 mM sodium phosphate and 1% NaCl, pH 8.0. Aliquots were removed at timed intervals and haemoglobin-free ghosts were prepared from the reacted erythrocytes. The TNP content of these ghosts was determined in acid-surfactant. Haemoglobin from the red cells was also analysed for TNP. After 50 min the ghosts contained only 0.073 μmole TNP/mg protein. Thus, the intact erythrocytes were much less reactive than were ghosts and this is in substantial agreement with the results of experiments using other reagents[2]. After 20 h, ghosts made from red cells reacted with TNBS contained 0.229 μmole TNP/mg protein, but the haemoglobin obtained from them had reacted with only 5% of the TNBS which haemoglobin in free solution combined with in only 50 min. It must be concluded, then, that the red cell membrane is almost impermeable to TNBS.

Since trinitrophenylated primary amines are uncharged, the isoelectric point

of proteins reacting with TNBS is expected to progressively decrease. When TNP-ghosts or ghosts obtained from TNP-erythrocytes were subjected to isoelectric focusing in 100 ml, pH 3–6 gradients containing 0.25% Triton X-100, and the results were compared with those obtained with unreacted ghosts[8], it was found that both the phospholipid peak and the major protein peak had shifted to a more acid pH (Table I). The alkaline protein band, forming beyond the linear region of the gradient, also contained TNP. These results indicate that most of the protein particles existing in 0.25% Triton X-100 contained amine groups which had been accessible to TNBS from the outside of the cell, and this is consistent with very few amine groups being exposed on solubilisation. The acidic tail of the protein band from the unreacted ghost overlapped with the band of TNP-protein, and it would be necessary to use a reagent which reacts with carboxyl groups to confirm that these more acidic proteins are also accessible from the outside of the membrane.

TABLE I

ISOELECTRIC FOCUSING OF SOLUBILISED ERYTHROCYTE GHOSTS AFTER REACTION WITH TNBS

The positions and spreads of the major bands of membrane protein and phospholipid were measured when ghosts, TNP-ghosts and ghosts made from intact erythrocytes after reaction with TNBS were subjected to isoelectric focusing on gradients of pH 3–6 containing 0.25% Triton X 100.

| | *Position of protein* | | *Position of phospholipid* | |
|---|---|---|---|---|
| | *Peak max.* | *Spread* | *Peak max.* | *Spread* |
| Ghosts | 4.78 | 3.00–5.25 | 4.50 | 4.15–5.25 |
| TNP ghosts | 3.95 | 3.00–4.50 | 3.75 | 2.40–4.25 |
| Ghosts from TNP erythrocytes | 3.73 | 3.00–4.55 | 3.55 | 1.50–4.00 |

Nevertheless, the characteristics of the reactivity of the erythrocyte membrane with TNBS cannot be easily reconciled with the classical protein–lipid–protein 'sandwich' model and, that solubilisation of erythrocyte ghosts in Triton X-100 hardly changes the accessibility of protein amine groups, suggests that these solubilisates are worthy of further physical study which might better illuminate the structure of the membrane.

ACKNOWLEDGEMENTS

The authors wish to thank the Medical Research Council of Great Britain for financial support and Professor G. R. Barker for the provision of facilities in the Department of Biological Chemistry.

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