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# A METHOD OF SOLUBILIZING HUMAN ERYTHROCYTE MEMBRANE PROTEINS USING MERSALYL

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## SUMMARY

Certain protein components of human erythrocyte membranes can be solubilized by a 0.1 M aqueous solution of the organic mercurial, mersalyl. These dissolved proteins were separated by polyacrylamide gel electrophoresis and the results compared with those obtained using sodium dodecyl sulfate. This method can be used to study a large number of polypeptides in the erythrocyte membrane in the absence of glycoproteins.

## INTRODUCTION

One of two alternative approaches exist for the study of membrane proteins. The first involves the isolation and characterization of one or more specific components of the membrane. This approach was used by Marchesi *et al.*[1] to prepare what was claimed to be a pure protein, spectrin, from erythrocyte ghost. Spectrin has subsequently been reported to be a heterogeneous mixture of polypeptides[2]. Glycoprotein components of the ghost also have been studied extensively[3–5]. The other approach is to solubilize as much of the membrane as possible and study the full spectrum of components released. Results thus obtained are useful in examining interactions between components and in the postulation of model membrane systems[6]. Recent reviews have evaluated the many methods available[7–10]. In most cases solubilization is via a single procedure but sequential steps are also used[11].

Of the methods available, sodium dodecyl sulfate is being used extensively in membrane studies. In addition to solubilizing membrane efficiently[7], its use allows for molecular weight estimations of the solubilized polypeptides on subsequent polyacrylamide gel electrophoresis[12,13]. Using this method on erythrocyte ghosts, electropherograms with a number of components have been demonstrated, certain of which stain as glycoprotein[14,15]. While the use of sodium dodecyl sulfate has been developed to a high degree of sophistication, the resolution of the higher molecular weight components so obtained is such that densitometry is not always satisfactory. The present study was prompted by a report by Dijkstra and Weide[16] that the organic mercurial, mersalyl, completely solubilized the refractory complex of rat liver chromatin prior to electrophoretic study. A method for solubilizing ghost proteins is presented and the

high degree of resolution obtained on polycrylamide electrophoresis is compared with that obtained using sodium dodecyl sulfate.

Erythrocyte ghosts were prepared by the method of Dodge *et al.*[17] from 2-week-old O-positive human blood preserved with acid–citrate–dextrose. The buffers used to prepare the membranes contained 0.001 M EDTA. Mersalyl acid (*o*-[3-hydroxymercury-2-methoxypropyl carbamoyl] phenoxyacetic acid) was obtained from Saphar Laboratories, Johannesburg; acrylamide and *N,N'*-methylene bisacrylamide from Pleuger and *N,N,N',N'*-tetramethylethylenediamine from Fluka. Sodium dodecyl sulfate was supplied by Merck and was purified before use (Albrecht, C. F., personal communication). A 5% solution of sodium dodecyl sulfate was made up in 95% ethanol at 60 °C. The fraction crystallizing out from 20 to 5 °C was recovered, dried over $P_2O_5$ and stored under vacuum.

A 0.1 M solution of mersalyl (mol. wt 484) was made up at pH 8.8 (ref. 16). A pellet of washed membranes was suspended in 40 volumes of this solution and stirred for 18 h at 4 °C with a magnetic stirrer. The resulting solution was slightly opaque and did not yield a pellet after centrifugation at $45000 \times g$ for 60 min. A slight pellet was observed after spinning at $105000 \times g$ for an hour. Using the method of Lees and Paxman[18] to assay protein in the $105000 \times g$ supernatant and pellet, it was found that mersalyl had solubilized 66.1% of the ghost protein. Free mersalyl was removed from the supernatant by dialysis against two changes of 0.01 M Tris–HCl buffer at pH 8.0, and the proteins characterised on disc gel electrophoresis by a modification of the method of Fambrough and Bonner[19]. Urea was added to 4.8 M and the resulting solution (pH 8.3) was adjusted to pH 3.2 with 0.1 M HCl. On reaching the isoelectric point of the proteins at approx. pH 5.0 turbidity of the preparation increased but reverted to its original clarity at pH 3.2. The urea concentration of the final solution was 4 M and was required to prevent aggregation of the proteins. Electrophoresis was carried out at pH 3.2 in 7.5% acrylamide gels (5 mm × 7 cm) in the presence of 6 M urea. The reservoir buffer was 0.2 M glycine·HCl at pH 3.2. The sample contained 5% 2-mercaptoethanol and a load of 25 $\mu$g protein was layered onto each gel. This was overlaid with reservoir buffer and electrophoresis carried out at 5 mA per gel for 3 h, with migration towards the cathode. Reduction of the sample with 2-mercaptoethanol was essential for the proper resolution of the slower-moving bands which otherwise formed an ill-defined smudge near the origin. It was found that if the supernatant was electrophoresed at pH 9.4, also in the presence of urea, the resolution obtained was not as good as at pH 3.2.

Further investigation of the dialyzed mersalyl preparation after centrifugation at $105000 \times g$ was carried out. The proteins in the supernatant were precipitated with 10% trichloracetic acid, washed with buffer and centrifuged at $5000 \times g$. This pellet and the $105000 \times g$-pellet were then treated with 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol and 8 M urea at 94 °C for 1 min to give a clear solution. Disc electrophoresis of the samples was carried out at pH 7.2 in 7.5% acrylamide gel containing 0.1% sodium dodecyl sulfate (ref. 12). Sample aliquots (25 $\mu$g protein) were layered onto the gel and overlaid with reservoir buffer. Migration was towards the anode with 4 mA per gel being applied for 30 min followed by 5 mA per gel for a further 2.5 h. A pellet of whole, untreated, erythrocyte ghosts was also treated with sodium dodecyl sulfate as described above and electrophoresed at pH 7.2.

All gels were fixed in 20% trichloroacetic acid for 1 h and then stained for protein

and glycoprotein. Protein bands were visualized by staining overnight with Coomassie Brilliant Blue R-250. Glycoprotein was demonstrated using periodic acid–Schiff staining[20]. The gels were then photographed[21] and are presented in Fig. 1. In addition the photographic negatives were scanned using a Spinco Analytrol densitometer.

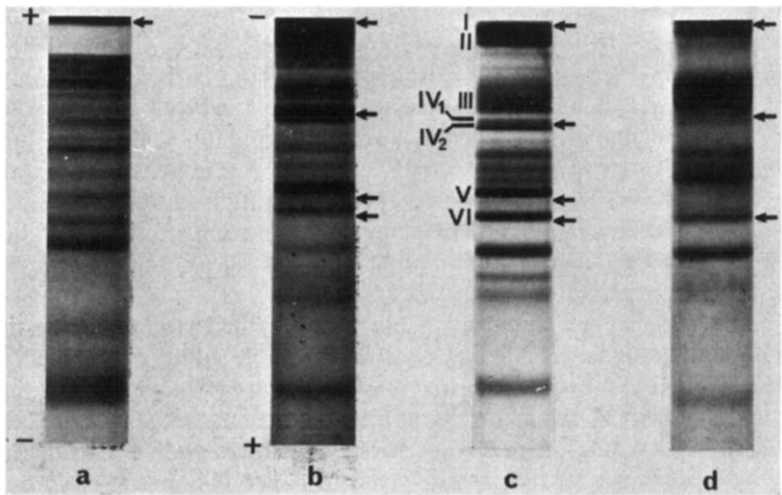


Fig. 1. Fractionation of human erythrocyte ghost proteins on 7.5% polyacrylamide disc gels. (a) Proteins solubilized with 0.1 M mersalyl at pH 8.8 and electrophoresed at pH 3.2 with 6 M urea. (b) Proteins solubilized with mersalyl then treated with 1% sodium dodecyl sulfate and run at pH 7.2 with gels containing 0.1% sodium dodecyl sulfate[12]. (c) Whole ghosts solubilized with sodium dodecyl sulfate and electrophoresed as in (b). (d) Proteins not solubilized by mersalyl, then treated with 1% sodium dodecyl sulfate and electrophoresed as in (b). Arrows indicate periodate–Schiff-positive bands. Roman numerals designate the fractions described by Wallach for sodium dodecyl sulfate gels[23].

An important feature of the mersalyl system at pH 3.2 is the fine banding obtained, especially with the slower-moving components. Resolution is such that the densitometer records even the finest bands visible to the naked eye in the original gel. In this way a total of 27 protein bands is obtained. This compares favourably with the standard sodium dodecyl sulfate system where 18 bands are shown (Fig. 1c) while 14–30 bands have been reported[14,15]. Furthermore, migration of protein bands in the mersalyl system is determined by the charge carried by the various species. This is unlike the sodium dodecyl sulfate system where migration is in order of molecular weights and the mersalyl system thus offers a different criterion for separation.

It appears that the proteins separated in the mersalyl system are indicative of ghost membrane components. Mersalyl has been shown to be a specific thiol reagent[22] and should not degrade proteins during solubilization. In the present study two proteins, bovine serum albumin (N.B.C.) and cytochrome *c* (N.B.C.), were treated with 0.1 M mersalyl and electrophoresed at pH 3.2 with 6 M urea. No differences were observed between these and the untreated proteins. Further confirmation that mersalyl does not degrade membrane material is illustrated in the figure. A ghost preparation treated with mersalyl was centrifuged at $105000 \times g$. The proteins in both supernatant (Fig. 1b) and pellet (Fig. 1d) were treated with sodium dodecyl sulfate and

compared with sodium dodecyl sulfate solubilized ghost (Fig. 1c). The value of using a reference pattern can be seen in that all the bands appearing in the soluble and insoluble mersalyl fractions are represented in the total pattern and together account for all its bands. From the figure it is seen that mersalyl solubilized ghost proteins selectively. Bands I–VI in the sodium dodecyl sulfate system were designated by Wallach[23] and illustrate this selectivity. For example, Bands II, $IV_1$, $IV_2$ and V are recovered almost exclusively in the mersalyl-soluble fraction; Bands I and VI are partitioned between the soluble and insoluble fractions and Band III is found mainly in the latter fraction. Mersalyl can thus be used to separate selectively some of the major membrane components. Bands positive for the periodate–Schiff stain are found in all the gels. In the mersalyl system at pH 3.2, however, the only band is one staining strongly at the origin. The conclusion is therefore that glycoproteins do not migrate in a 7.5% polyacrylamide gel at low pH and that this system affords a method to separate and study a large number of the component polypeptides of the erythrocyte membrane in the absence of glycoproteins.

The exact mechanism by which mersalyl solubilizes erythrocyte membranes is not certain. Boyer first suggested using mersalyl as a water-soluble sulfhydryl reagent[24]. It is effective in dissolving the mitotic apparatus of sea urchin eggs[25] and disrupts the rigid arrangement of the electron transport chain in rat microsomes at very low concentrations[26]. Dijkstra and Weide[16] assume that mersalyl dissolves rat liver chromatin by reacting with sulfhydryl groups that are involved in either H-bonding or in covalent thiolester bonds. This solubilization is reversed by adding sulfhydryl compounds such as mercaptoethanol[16]. In the present study the binding of mersalyl to erythrocyte proteins was studied. Using atomic absorption analysis it was found that after centrifugation at $105000 \times g$ more mercury was bound in the soluble fraction (1.49 mg Hg/mg protein) than in the insoluble fraction (0.58 mg Hg/mg protein). As selective solubilization occurs, this suggests that the proteins not solubilized bind less mersalyl. Subsequent dialysis of the soluble fraction against buffer containing 0.1% 2-mercaptoethanol resulted in the displacement of mersalyl from the protein. Under these conditions urea was essential in the following electrophoresis.

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