Biochimica et Biophysica Acta, 649 (1981) 98–104
Elsevier/North-Holland Biomedical Press

BBA 79428

THE ACTION OF ORGANIC MERCURIALS ON THE ERYTHROCYTE MEMBRANE

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(Received March 30th, 1981)
(Revised manuscript received June 30th, 1981)

Key words: p-Mercuribenzoate; p-Mercuribenzenesulfonate; Spectrin, Cytoskeleton; Organic mercurial

The solubilisation of proteins from erythrocyte membranes by treatment with organic mercurials has been studied with different species. The marked solubilisation previously reported for human membranes does not seem to be a general phenomenon. All of the other species examined showed less than 50% of the solubilisation shown by human membranes. The protein-solubilising effect seems to be dependent on hydrophobic mercury derivatives carrying a net negative charge. Uncharged compounds like phenylmercuric acetate blocked the effect, although *N*-ethylmaleimide and iodoacetamide did not. With the aid of radioactively labelled compounds, and of atomic absorption spectrophotometry, the proteins reactive towards the mercurials were identified. The major integral protein, band 3, was the major protein capable of binding the mercurial. Reaction with the mercurial appears to disrupt interaction of band 3 with bands 2.1 and 4.2, allowing dissociation of the cytoskeleton from the membrane. In addition, band 4.9 was also found to react with the mercurials, possibly resulting in disruption of the cytoskeleton.

Introduction

In the erythrocyte membrane, there is mounting evidence that the peripheral proteins spectrin and actin are intimately involved with the determination and maintenance of red cell shape and membrane integrity [1,2]. In 1973, the organomercurial compounds *p*-mercuribenzoate (PMB) and *p*-mercuribenzenesulphonate (PMBS) were shown to bring about the release of peripheral proteins from human erythrocyte membranes under conditions of ionic strength that normally inhibited such release [3,4]. Concurrently with the extraction of protein, the membranes underwent fragmentation to form small vesicles [4]. Similar treatment of camel erythrocyte membrane led to a partial loss of spectrin [5], and converted the membranes from their normal elliptical shape into spheres. However, unlike the human membranes the camel ghosts remained intact after this treatment.

A number of attempts have been made to relate particular classes of reactive sulphydryl groups to specific proteins in the erythrocyte [4,6]. Iodoacetate reacts selectively with glyceraldehyde phosphate dehydrogenase [7], while 5,5′-dithiobis(2-nitrobenzoic acid) has been reported to react specifically with band 3 [8]. However, PMB and PMBS are the only sulphydryl reagents so far reported to cause the solubilisation of proteins from the red cell membrane at moderate ionic strength.

The effects of the mercurials are not blocked by pretreatment of the membranes with *N*-ethylmaleimide [3,5], suggesting that the mercurials react with a class of sulphydryl groups different from those reactive towards *N*-ethylmaleimide. Electron paramagnetic resonance studies [9] also indicate that there may be classes of sulphydryl groups which differ in their reactivities towards various reagents.

The present report describes the action of some mercurial reagents on the erythrocyte membranes

Abbreviations: PMB, *p*-mercuribenzoate; PMBS, *p*-mercuribenzenesulphonate; SDS, sodium dodecyl sulphate.

from several species. By the use of radioactively labelled mercurial compounds, and of atomic absorption spectrophotometry, the sites of action of the mercury compounds have been determined.

Materials and Methods

Reagents. Sodium *p*-mercuribenzoate (PMB) and *N*-ethylmaleimide were obtained from Calbiochem; iodoacetamide, phenylmercuric acetate and Triton X-100 from B.D.H.; and sodium *p*-chloromercuribenzenesulphonate (PMBS) from Sigma. 2-Chloromercuri-4,6-dinitrophenol was prepared by the method of McMurray and Trentham [10]. ^{203}Hg-labelled PMB (63.1 mCi/g) and PMBS (28 mCi/g) were obtained from the Radiochemical Centre, Amersham. All other chemicals were of analytical reagent grade.

Solutions of the sulphydryl reagents were normally prepared in 5 mM phosphate buffer, pH 8.0. At this pH value, the low solubility of PMB did not allow the use of concentrations above 1 mM. Higher concentrations could only be prepared in more alkaline solutions. These were avoided in the present study because of the extraction of peripheral proteins brought about by high pH alone. Solutions of PMBS were adjusted back to pH 8.0 with 0.1 M NaOH as the dissolving of this compound brought about a lowering of the pH of the buffer by up to one unit.

Preparation of erythrocyte membranes. Fresh human packed cells in citrate anticoagulant were obtained from the Red Cross Transfusion Service, Sydney. Bovine blood was collected into citrate anticoagulant at the Homebush Abattoir and canine blood in heparin anticoagulant was obtained from the School of Veterinary Physiology, Sydney University. Blood from the Tammar Wallaby was collected into citrate anticoagulant at the CSIRO Wildlife Research Station, Canberra, transported to the laboratory on ice, and used within 48 h. Rat blood was collected by heart puncture, treated with citrate anticoagulant and used immediately.

Red cell membranes, prepared by haemolysis in 5 mM phosphate buffer, pH 8.0, as previously described [5], were used on the day of preparation.

Treatment of membranes with sulphydryl reagents. The membranes were mixed rapidly with 5 vol. of the sulphydryl reagent in ice-cold 5 mM sodium phosphate buffer adjusted to pH 8.0. At this buffer concentration, extraction of peripheral proteins by the buffer alone was negligible, yet maximum solubilisation was achieved with mercurials. The suspension was mixed thoroughly, and allowed to stand in ice for up to 1 h. Soluble protein was isolated by centrifuging the suspension at 17 750 × *g* for 30 min at 2–4°C. Protein concentrations of the supernatant and pellet fractions were examined by means of electrophoresis in acrylamide gels containing SDS, and by means of the method of Lowry et al.

Estimation of bound mercurial. In studies aimed at locating the proteins responsible for the mercurial-induced solubilisation effect, nonspecific thiol groups were blocked by pretreatment with 5 mM *N*-ethylmaleimide for 1 h at 0°C prior to reaction with mercurial. This pretreatment was found to block completely the subsequent reaction of spectrin with mercurial, yet did not prevent the solubilisation of proteins by PMBS.

Mercury bound to membrane proteins was estimated either by atomic absorption spectrophotometry or by scintillation counting of ^{203}Hg-labelled reagents. Unbound reagent was removed from the membranes by dialysis against 5 mM phosphate buffer, pH 8.0 or by means of gel filtration on Sephadex G-25 in 50 mM sodium phosphate buffer, pH 8.0. Protein solutions were concentrated by dialysis against dry Aquacide I.

Electrophoresis. Samples were examined by electrophoresis in 5.6% acrylamide gels containing 1% SDS, essentially by the method of Fairbanks et al. [11]. Disulphide-reducing agents were omitted from the samples to be used for mercury quantitation, to avoid displacing the mercurial. For experiments involving scintillation counting of gel slices, the cleavable cross-linking agent diallyltartaridiamide was used in place of methylenebis-acrylamide. Proteins were stained with Coomassie blue, and glycoproteins were detected with the periodic acid-Schiff stain [11].

Atomic absorption spectrophotometry. Slices from four replicate electrophoresis gels were digested with 5 ml concentrated nitric acid at 100°C for several hours. Samples of membrane suspensions or of soluble protein fractions were digested in a similar manner.

The digested samples were diluted to 50 ml with 1.0 M HCl; tin(II) chloride (1 M; 1.0 ml) was added

to reduce the mercury(II) ions to elemental mercury, and the solution was stirred vigorously for exactly 90 s. Mercury concentrations were determined in a Varian Techtron Atomic Absorption Spectrophotometer, model 1200, fitted with a cold vapour mercury attachment. Mercury(II) chloride solutions were used as standards.

Measurement of radioactivity. Proteins, labelled with ^{203}Hg, were fractionated by means of electrophoresis in cleavable acrylamide gels. The gels were sliced, and the slices dissolved in 1 ml of 1% periodic acid. A toluene/Triton X-100 scintillant was added, and radioactivity measured in a Packard TriCarb Scintillation Spectrometer model 2002. Small, but significant, loss of label during electrophoresis, fixing and staining of the gels prevented absolute quantitation of the incorporation of mercury.

Results

Protein release from human erythrocyte membranes

Treatment of human erythrocyte membranes with PMB or PMBS cause vesiculation and fragmentation of the membranes and a concentration-dependent solubilisation of protein (Fig. 1).

Electrophoresis showed that both PMB and PMBS released spectrin (components 1 and 2), and actin (component 5), at low concentrations of the reagents. At concentrations above 0.5 mM, however, components 2.1, 4.2 and 6, together with increasing amounts of band 4.1, were also released (Fig. 2). Although not prominent, band 4.9 (moving just behind band 5) was also completely extracted by 0.5 mM PMBS. The protein released by mercurial treatment was in a highly associated state, emerging in the void volume of 4% agarose columns. Very little spectrin tetramer and dimer could be detected in gel filtration experiments or on gradipore electrophoresis.

N-Ethylmaleimide or iodoacetamide extracted no more than 5% of the membrane protein, an amount similar to that extracted by the phosphate buffer alone (Figs. 1 and 2). Pretreatment with *N*-ethyl-

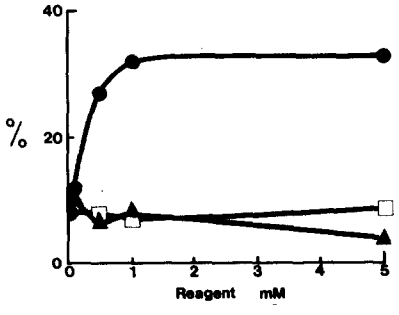

Fig. 1. Solubilisation of protein from human erythrocyte membranes by thiol reagents. The percentage of total membrane protein extracted as determined by the method of Lowry et al. is plotted against reagent concentration. •, *p*-mercuribenzene sulphonate; □, *N*-ethylmaleimide; ▲, phenylmercuric acetate. Results are the means of duplicate determinations.

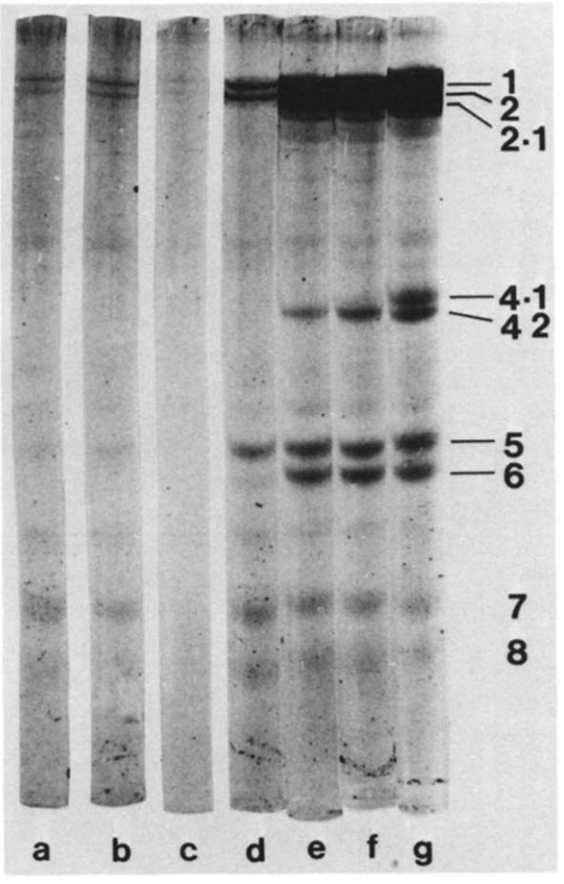

Fig. 2. Polyacrylamide gel electrophoresis of protein solubilised from human erythrocyte membranes. Supernatant solutions were examined from membranes incubated on ice for 1 h with the following reagents: Gel a, phosphate buffer, pH 8.0; gel b, 5 mM *N*-ethylmaleimide; gel c, 5 mM phenylmercuric acetate; gels d–g, *p*-mercuribenzene sulphonate, 0.1, 0.5, 1 and 5 mM, respectively.

maleimide did not block the effect of the mercurials (Fig. 3).

At concentrations up to 5 mM, phenylmercuric acetate and 2-mercuri-4,6-dinitrophenol (both uncharged mercurials) caused no extraction of protein beyond that extracted by the buffer alone (Figs. 1 and 2).

Unlike *N*-ethylmaleimide and iodoacetamide, phenylmercuric acetate substantially blocked the subsequent solubilisation effects of PMBS (Fig. 3), and also prevented the subsequent fragmentation by PMBS.

Other species

Protein solubilisation after incubation with increasing concentrations of PMBS for human, bovine, rat, canine and wallaby erythrocyte membranes are shown in Fig. 4. In each case, *N*-ethylmaleimide neither solubilised significant protein itself, nor blocked the effects of the mercurial. While spectrin, actin, component 2.1 and a component apparently corresponding to band 4.2 were the predominant proteins extracted from the membranes of all species, the amounts of these proteins extracted were considerably reduced, and proportionally more minor components were present. Lack of protein extraction was not due to proteolysis, as examination of the residue showed a normal electrophoretic pattern.

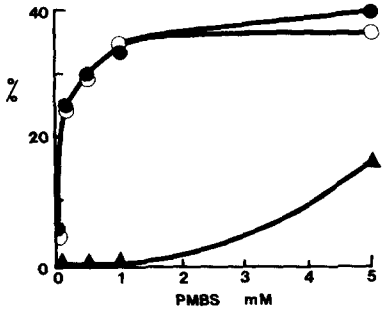

Fig. 3. The effect of pretreatment on the percentage protein solubilised from human erythrocyte membranes by *p*-mercuribenzene sulphonate. Membranes were incubated for 15 min on ice with the following reagents, collected by centrifugation, and then treated with different concentrations of *p*-mercuribenzene sulphonate. Points represent means of duplicate determinations. •, No pretreatment; ○, pretreated with 5 mM *N*-ethylmaleimide; ▲, pretreated with 5 mM phenylmercuric acetate.

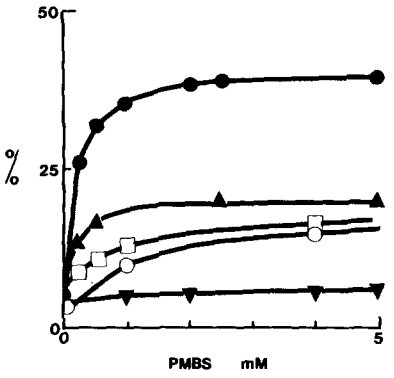

Fig. 4. Effect of species difference on percentage solubilisation of proteins from erythrocyte membranes by treatment with *p*-mercuribenzene sulphonate. The points represent the means of five replicate measurements. Absolute S.D. was less than 2% for all points. •, human; ▲, canine; □, bovine; ○, rat; ▲, wallaby.

Distribution of the mercurial

Extractibility of the label. Human red cell ghosts were pretreated with 5 mM *N*-ethylmaleimide to block the sulphydryl groups reactive to this reagent. After reaction of the membranes with 1 mM PMBS, the soluble proteins showed only small amounts of mercury on analysis by atomic absorption. However, the residue remaining after extraction of the soluble protein showed the presence of substantial amounts of mercury.

Approx. 70% of the mercury in the treated membrane residue was solubilised with the nonionic detergent Triton X-100, both in 5 mM and in 50 mM sodium phosphate solutions, while only 14% was solubilised by water at 37°C. Glycoproteins, solubilised in chloroform/methanol [12], did not appear to bind the mercurial reagent. More than 90% of the label remained with the integral proteins that are not extracted with the chloroform/methanol solvent.

Electrophoretic analysis of the labelled protein. Quantitation of bound mercury, either by atomic absorption, or as ^{203}Hg, showed that the major proteins to bind mercury were components 3, and 4.9. Traces of mercury were detected on components

4.1 and 4.2, while binding to other components was negligible (Fig. 5).

Similar experiments made over a range of PMBS concentrations showed that labelling of band 4.9 reached a plateau near 2 mM reagent. Labelling of all the other components, however, showed a gradual rise in amount of labelling between 2 and 5 mM reagent.

In canine ghosts, the major labelled proteins appeared to be those of 4.9 region, with some labelling of components 5 and 7. Much less mercury was detected in components 3, 4.1 and 4.2 than was the case with the human ghosts. Bovine membranes revealed much less labelling in all proteins, with components 1, 2, 3 and the entire 4 region being labelled poorly and almost equally. Therefore, it appears that solubilisation of the membrane proteins by the mercurials is species-dependent.

Extraction from spectrin-depleted membranes. Spectrin-depleted vesicles were treated with 2 mM PMBS in 5 mM phosphate buffer, pH 8.0. Bands 2.1, 4.2, 6 and traces of band 4.1 were solubilised (Fig. 6).

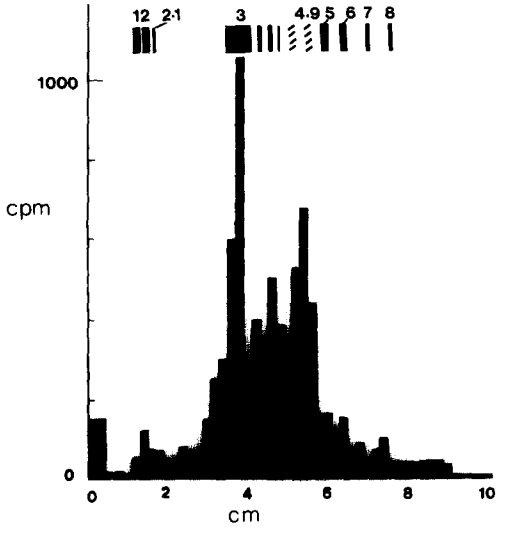

Fig. 5. ^{203}Hg incorporation into proteins of the human erythrocyte membrane after treatment with 1 mM *p*[^{203}Hg]mercuribenzene sulphonate. Membranes were dialysed against unlabelled PMBS to prevent nonspecific labelling in SDS. Radioactivity, corrected for background, was measured in 2 mm gel slices after electrophoresis of labelled membranes in cleavable polyacrylamide gels containing 1% dodecyl sulphate. The corresponding electrophoresis pattern is shown diagrammatically. Disulphide-reducing compounds were omitted from the samples for electrophoresis.

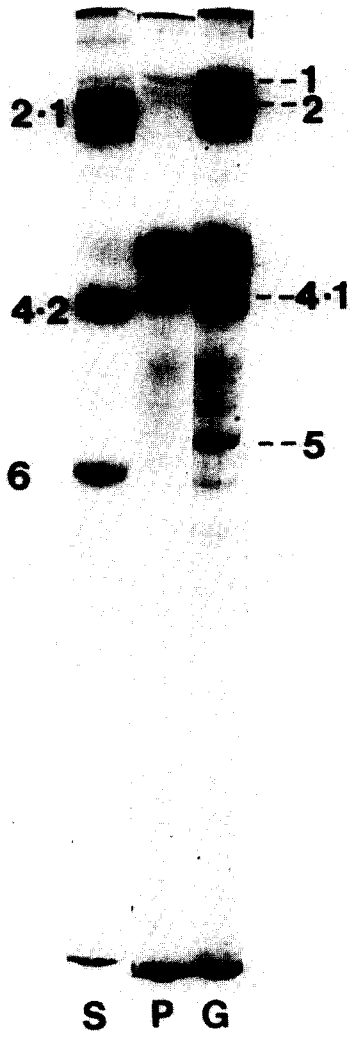

Fig. 6. Extraction of proteins from spectrin-depleted human erythrocyte membranes by 2 mM *p*-mercuribenzene sulphonate. Membranes were depleted of spectrin and actin by incubation in 0.5 mM phosphate buffer, pH 8.0, at 37°C for 15 min, followed by centrifugation at 30 000 × *g*. After incubation of spectrin-depleted membranes with mercurial, the soluble proteins (gel S) and residual membrane pellet (gel P) were examined by means of electrophoresis. Gel G is a whole ghost preparation for comparison.

Discussion

The present study has shown that fragmentation of the human erythrocyte membrane and solubilisa-

tion of the peripheral proteins by PMB and PMBS is apparently due to modification of sulphydryl groups of specific proteins. Covalently labelled proteins were isolated after [^{203}Hg]PMB treatment, and the label could be displaced by treatment with mercaptoethanol. The sensitivity of the peripheral proteins of the human erythrocyte membrane to anionic organic mercurial compounds is not a general feature of all species. The wallaby erythrocyte membranes showed little extractability towards this class of reagent, while other species displayed intermediate reactivity.

Our studies on human erythrocyte membranes showed that phenylmercuric acetate (an uncharged mercurial) blocked the effects of PMB and PMBS, but did not solubilise the proteins. This suggests that the net negative charge on PMB and PMBS plays an important role in the dissociation and solubilisation of cytoskeleton.

The nonmercurial reagents, such as *N*-ethylmaleimide and iodoacetamide, neither led to extraction of protein in any of the species studied, nor blocked the action of the anionic mercurials. Presumably, these nonmercurial reagents are not reactive towards the thiol residues involved in the dissociation of the cytoskeleton by organic mercurials. As the second-order rate constant for reaction of PMB with simple thiols (approx. $6 \cdot 10^6\ M^{-1} \cdot s^{-1}$ [13]) is several orders of magnitude greater than that for *N*-ethylmaleimide (approx. $1.5 \cdot 10^3\ M^{-1} \cdot s^{-1}$ [14]), it is not surprising that the mercurials react with residues unreactive towards *N*-ethylmaleimide.

The thiol residues of spectrin and actin appear to be blocked almost completely by pretreatment with *N*-ethylmaleimide, as these proteins subsequently showed little binding of labelled mercurial. Most of the labelled mercurial appeared to be associated with the membrane residue and not the solubilised proteins. Thus, the solubilisation of the peripheral proteins appears to be the result of the modification, not of the spectrin and actin themselves, but rather of other accessory proteins that may be responsible for stabilising the cytoskeleton or its interaction with the membrane. Several proteins, rather than a single component, appeared to react with the mercurials, even after pretreatment with *N*-ethylmaleimide. The major mercurial-binding proteins were bands 3 and 4.9, while substantially less label was found associated with bands 4.1 and 4.2. All of these proteins have been implicated to some extent in the interaction of spectrin with the membrane. The solubilisation effect of PMBS appears to be at least biphasic. At low reagent concentrations, spectrin and actin are solubilised, while only at higher concentrations are components 2.1, 4.2 and 6 extracted. A possible third stage may be represented by the solubilisation of band 4.1.

The existence of several stages in the extraction process suggests that there may be more than one locus for the effect of PMBS. One of the likely sites for the solubilisation effect is component 3. PMBS treatment of membrane vesicles depleted of spectrin, actin, component 4.9 and part of component 4.1 resulted in solubilisation of components 2.1, 4.2 and 6. The major mercurial-binding protein left in these vesicles is component 3.

Recently, Bennett and Stenbuck [15] have shown that bands 2.1 and 4.2 interact with part of the band 3 population. The band 3-band 2.1 complex is capable of anchoring the spectrin-actin cytoskeleton to the membrane through specific interactions of band 2.1 with spectrin. A complex between bands 3 and 4.2 in Triton X-100 extracts has also been reported [16]. This complex can be dissociated by treatment with PMB [16]. We have shown that band 4.2 binds only trace amounts of mercurial, while band 3 is the major site of labelling. Thus, it appears that on treatment of the membrane with charged organic mercurials, band 3 is modified, resulting in disruption of its affinity for both components 2.1 and 4.2. As a result, both of these proteins appeared in the soluble protein fraction. The spectrin-actin cytoskeleton, formerly attached to the membrane via band 2.1, was presumably also dislodged from the membrane as an indirect result. Unequivocal verification of this mechanism must await direct studies of the effect of mercurials on the interaction between components 2.1, 3 and 4.2.

It is unlikely that component 3 is the only protein involved in the protein-solubilising effect. There is growing evidence that a spectrin-actin cytoskeletal network is attached to the cytoplasmic surface of the membrane in the intact erythrocyte. Tsukita et al. [17] have shown such structures in electron micrographs of thin sections of intact erythrocytes fixed with tannic acid and glutaraldehyde. Recently, band 4.9 has been found in association with the spectrin-

actin complex [18,19], with an apparently constant stoicheiometry of one molecule of band 4.9 per tetramer of spectrin (Dunbar, J.C. and Ralston, G.B., unpublished results). Our results have shown that the mercurials do not react appreciably with spectrin or actin in whole membranes pretreated with *N*-ethylmaleimide. There is, however, substantial reaction with band 4.9, and labelling of component 4.9 at low PMBS concentrations correlates with the early phase in the extraction of spectrin and actin.

We suggest that reaction of band 4.9 with mercurial results in the partial fragmentation of the cytoskeleton.

Triton shells of erythrocyte membranes, depleted of bands 3 and 4.2, can be disrupted into soluble fragments after treatment with PMBS [20], and it is tempting to ascribe this effect to band 4.9, although the possibility that other components contribute to the effect cannot at this stage be eliminated.

Exchange of label in our studies was minimised by blocking the freely exposed thiol groups with *N*-ethylmaleimide in the pretreatment step. Those thiol groups that showed decreased reactivity towards *N*-ethylmaleimide were subsequently reacted with PMBS. There is a possibility that some buried thiol groups, not capable of reacting with either reagent in the absence of detergent, may undergo some exchange on adding SDS. This may have been shown to some extent by bands 4.1 and 4.2.

Although it is possible that bands 4.1 and 4.2 play a role in the mercurial effect, such a role is probably minor. Band 4.1 is solubilised only at mercurial concentrations much higher than that required for solubilisation of the spectrin and actin. While a direct modification of band 4.2 may explain its dissociation from band 3, it is insufficient to explain the dissociation of band 2.1 from band 3. The most likely mechanism, therefore, is one in which solubilisation of both bands 4.2 and 2.1 arise from modification of band 3.

In conclusion, we have found that organic mercurials cause dissociation of proteins of human erythrocyte membranes by reacting with thiol groups of specific proteins. Concomitant with the solubilisation of the peripheral proteins, two major proteins in the residue are labelled with mercurial. These are components 4.9 and 3. We propose that these two proteins are important in stabilising the structure of the human erythrocyte membrane.

Acknowledgements

This work was supported by grants from the Australian Research Grants Committee, the University of Sydney and the University of Sydney Medical Research Committee.

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