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The dissociation of peripheral proteins from erythrocyte membranes brought about by *p*-mercuribenzenesulfonate

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The organic mercurial *p*-mercuribenzenesulfonate in 5 mM phosphate buffer (pH 8.0) solubilized ankyrin, bands 4.1 and 4.2, and glyceraldehyde-3-phosphate dehydrogenase from spectrin-depleted erythrocyte membranes. Glyceraldehyde-3-phosphate dehydrogenase was the protein most readily solubilized, being almost completely extracted by 0.5 mM reagent. The solubilization of ankyrin was similar to that of band 4.2, both showing maximal solubilization with 1.0 mM reagent. Band 4.1 was not appreciably solubilized below 2.5 mM *p*-mercuribenzenesulfonate. *N*-Ethylmaleimide did not itself solubilize proteins from ghosts or spectrin-depleted vesicles, and pretreatment at low temperature by 4 mM *N*-ethylmaleimide did not prevent subsequent solubilization by the mercurial. However, pretreatment at 37°C with *N*-ethylmaleimide inhibited subsequent solubilization of ankyrin and band 4.2 by the mercurial and also resulted in the loss of binding of 1 mol mercurial per mol band 3. These data suggest that release of ankyrin and band 4.2 from the membrane by mercurial is linked to modification of band 3 by the reagent. After incubation of intact erythrocyte membranes with 0.1 M NaCl, treatment with *p*-mercuribenzenesulfonate selectively solubilized actin from the membranes. The resulting actin-depleted membranes did not vesiculate, but became spherical and lost their biconcave shape. Fragmentation was observed after subsequent removal of spectrin at low ionic strength.

Introduction

The organic mercurial, *p*-mercuribenzenesulfonate, has been reported to solubilize the peripheral proteins spectrin, actin, ankyrin, band 4.1 and band 4.2 proteins from erythrocyte membranes [1–3]. Part of the action of the mercurial in solubilizing these proteins can be explained in terms of the disruption of the erythrocyte cytoskeleton through the depolymerizing effects of the mercurial on the actin protofilaments and on spectrin oligomers [4–6]. However, this does not explain how *p*-mercuribenzenesulfonate brings about actual solubilization of the peripheral proteins other than actin, since these proteins are also bound to sites on the membrane. Instead, there appear to be other effects of *p*-mercuribenzenesulfonate that lead to dissociation of the cytoskeletal fragments from the membrane.

It is possible to study the *p*-mercuribenzenesulfonate-dependent dissociation of peripheral proteins from the membrane in isolation from the cytoskeleton disruption effect, through the use of spectrin-depleted vesicles. When red blood cell ghosts are incubated in a low ionic strength medium, almost all of the spectrin, actin and band 4.9 are solubilized from the membrane and a small amount of band 4.1 protein is released. Vesiculation of the membrane results, with peripheral proteins ankyrin, and bands 4.1, 4.2 and 6 remaining associated with the membrane vesicles, which are predominantly in an inside-out orientation. Ankyrin and bands 4.2 and 6 are known to be linked to band 3 which is the major integral membrane protein [7,8]. Ankyrin mediates the attachment of spectrin to band 3 and is thus the main attachment site of spectrin to the membrane [9]. Band 4.1 also appears to act as a membrane attachment protein in the cytoskeleton [10,11].

Radiolabelling studies on erythrocyte membranes have shown that the major components binding *p*-[^{203}Hg]mercuribenzenesulfonate were band 3 and a component that migrates in polyacrylamide gels in the region between bands 4.2 and 5 [3]. Rao [12] has reported that reaction of band 3 with *N*-ethylmaleimide for 1 h at 37°C inhibits the subsequent reaction of *p*-mercuribenzenesulfonate with this protein. Since it is likely that reaction of *p*-mercuribenzenesulfonate with band 3 results in the dissociation of some of the peripheral proteins from erythrocyte membranes, the dissoci-

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ation of these peripheral proteins may be prevented by blocking the reaction of *p*-mercuribenzenesulfonate with band 3 by *N*-ethylmaleimide.

In the present study both spectrin-depleted vesicles and erythrocyte ghosts were treated with *N*-ethylmaleimide for 1 h at 37° C to examine the effect of such treatment on the *p*-mercuribenzenesulfonate-dependent solubilization of proteins from erythrocyte membranes.

Materials and Methods

Fresh human blood was supplied as packed cells in acid-citrate-dextrose anticoagulant by the Red Cross Transfusion Service, Sydney. Solutions of the sulfhydryl reagents *p*-mercuribenzenesulfonate (sodium salt) and *N*-ethylmaleimide were prepared in 5 mM sodium phosphate buffer (pH 8.0) and the pH was adjusted if necessary to pH 8.0. These solutions were used within 24 h.

Preparation of erythrocyte ghosts

Red cells were washed three times in cold (2–4° C) 5 mM sodium phosphate buffer (pH 7.5) containing 0.95% (w/v) NaCl, by repeated centrifugation at $800 \times g$ for 15 min. The 'buffy coat' was removed after each wash. Membranes were prepared by hemolysis at 2 to 4° C in 5 mM sodium phosphate buffer (pH 8) followed by repeated washing in the hemolysis buffer by centrifugation at $35\,000 \times g$ for 30 min until the ghosts were pale cream coloured [13]. After each centrifugation step the supernatant and a tightly packed pink pellet containing the remaining leucocytes and platelets underlying the membranes were removed. Ghost preparations were used as soon as possible after preparation.

Preparation of spectrin-depleted vesicles

Vesicles were prepared by incubating erythrocyte ghosts (1 vol) in distilled water (5 vol) at 37° C for 15 min. The resulting membrane fragments were sedimented by centrifugation at $35\,000 \times g$ for 40 min at 2–4° C and were then washed twice in 5 mM sodium phosphate buffer (pH 8.0) by centrifugation. The pellet of spectrin-depleted vesicles was resuspended in 5 mM sodium phosphate buffer (pH 8.0) to the original undiluted erythrocyte ghost volume. The vesicles were stored at 2–4° C and used within two days.

The solubilization of proteins from spectrin-depleted vesicles by p-mercuribenzenesulfonate

Separate aliquots (1 ml) of spectrin-depleted vesicles suspended in 5 mM sodium phosphate buffer (pH 8.0) were treated with solutions of different *p*-mercuribenzenesulfonate concentration (5 ml, 0–5 mM), in the presence and absence of added NaCl in the range 5–100 mM. The mixtures were allowed to react for 45 min on ice and were then centrifuged at $35\,000 \times g$ for 40 min at 2–4° C. The supernatants were retained and the pellets were washed twice in 5 mM sodium phosphate buffer (pH 8.0) at 2–4° C by centrifugation. The pellets were finally resuspended in sodium phosphate buffer (pH 8.0) (2 ml, 5 mM). The proteins present in the supernatant and those remaining with the membrane pellet were identified by polyacrylamide gel electrophoresis in dodecyl sulfate as described by Fairbanks et al. [13].

The effect of N-ethylmaleimide on the solubilization of proteins by p-mercuribenzenesulfonate

Aliquots (1 ml) of spectrin-depleted vesicles were suspended in *N*-ethylmaleimide solution (5 ml, 5 mM) and were held either at 37° C or on ice for 1 h. Control samples of spectrin-depleted vesicles were suspended in the relevant buffer (5 ml) and were held for 1 h either at 37° C or on ice. All of the samples were subsequently centrifuged at $35\,000 \times g$ for 30 min at 2–4° C.

Vesicle pellets were resuspended in 5 ml of either 1 mM or 5 mM *p*-mercuribenzenesulfonate in 5 mM sodium phosphate (pH 8.0). All of these samples were kept on ice for 30 min and were then centrifuged at $35\,000 \times g$ for 30 min at 2–4° C. The supernatants from each sample were retained and the pellets were washed twice in cold sodium phosphate buffer (5 mM) (pH 8.0). The proteins of the pellets and supernatants were analysed by polyacrylamide gel electrophoresis in dodecyl sulfate [13].

The above experiments were also performed with 0.1 M NaCl included in the pre-incubation solutions. After the pre-incubation in either the presence or absence of *N*-ethylmaleimide, the vesicles were collected by centrifugation at $35\,000 \times g$ for 30 min at 2–4° C. The pellets were then resuspended in the relevant concentration of *p*-mercuribenzenesulfonate in 5 mM sodium phosphate buffer (pH 8.0).

Solubilization of peripheral proteins from intact membranes

A similar procedure was followed using erythrocyte ghosts instead of spectrin-depleted vesicles except that the 37° C incubation solutions were only performed in the presence of 0.1 M NaCl, since incubation of erythrocyte membranes at 37° C in low ionic strength alone, in the absence of mercurial, leads to the solubilization of spectrin, actin and 4.1.

Phase contrast microscopy

The morphology of unfixed erythrocyte membrane samples was examined under phase-contrast illumination using a Zeiss Photomicroscope. Aliquots of samples were pipetted onto glass slides, covered with glass cover-slips and were examined immediately. Photographs of representative fields of the samples were taken using Copex Rapid film.

Radiolabeling of proteins from spectrin-depleted vesicles

The general approach to this section of the work was to quantify the radiolabel associated with individual membrane proteins as a result of reaction with *p*-[^{203}Hg]mercuribenzenesulfonate after various conditions of *N*-ethylmaleimide pretreatment.

Spectrin-depleted vesicles (1 ml) were suspended in *N*-ethylmaleimide solution (5 ml, 5 mM) and were held for 1 h either on ice or at 37 °C. Each suspension was then centrifuted at 35 000 × *g* for 30 min at 2–4 °C. The resulting pellets were suspended in *p*-[^{203}Hg]mercuribenzenesulfonate (2.5 ml, 5 mM, 50 μCi) and the samples were kept on ice for 30 min. Both samples were then centrifuged at 35 000 × *g* for 30 min at 2–4 °C. The supernatants were removed and the membrane pellets were washed twice by centrifugation in 5 mM sodium phosphate buffer (pH 8.0).

To minimize radiolabel exchange reactions, a method was developed to separate the radiolabelled proteins. Each membrane pellet was resuspended in *N*-ethylmaleimide solution (1 ml, 5 mM) and to each suspension sodium dodecyl sulfate (50 μl, 20% w/v) was added. The presence of the *N*-ethylmaleimide was found to block previously unreactive sulfhydryl groups that become exposed in the presence of the detergent and that can lead to exchange reactions and migration of the radiolabel. The solutions were applied to a 6% agarose column (50 cm × 1.5 cm), and the proteins were eluted in 1-ml fractions using a buffer comprising 10 mM sodium phosphate, (pH 8.0), 50 mM NaCl and 1% sodium dodecyl sulfate (w/v) at 22 °C. Fractions were monitored for protein by means of absorbance at 280 nm and aliquots (0.5 ml) of fractions were analysed for radioactivity by use of a Searle Automatic Gamma Counter, model 1197. The individual proteins in each fraction were analysed by means of electrophoresis in acrylamide gels containing dodecyl sulfate [13].

Results

The solubilization of proteins from spectrin-depleted vesicles by p-mercuribenzenesulfonate

When vesicles depleted of spectrin, actin and band 4.9 were subsequently treated with *p*-mercuribenzenesulfonate in 5 mM phosphate buffer, ankyrin (band 2.1), its proteolytically trimmed derivatives, and bands 4.1, 4.2 and 6 were then released from the membrane in increasing amounts with increasing reagent concentration (Fig. 1). There appeared to be no extraction of proteins with 0.1 mM reagent, with the exception of trace amounts of residual spectrin. Band 6 was the protein most easily removed from the membrane by *p*-mercuribenzenesulfonate; nearly all of band 6 was extracted with a reagent concentration of 0.5 mM. Ankyrin and band 4.2 appeared to share a similar sensitivity to *p*-mercuribenzenesulfonate concentration;

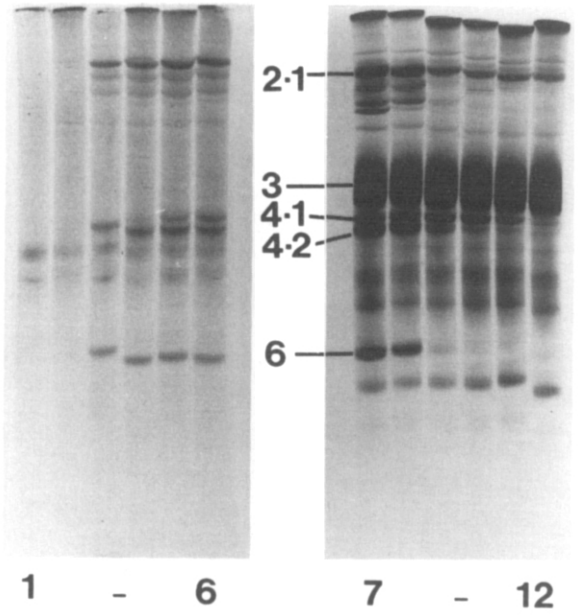

Fig. 1. Electrophoresis of the proteins solubilized from spectrin-depleted vesicles by *p*-mercuribenzenesulfonate. Gels 1–6: proteins solubilized by 0, 0.1, 0.5, 1, 2.5 and 5 mM reagent, respectively; gels 7–12: vesicle pellets remaining after treatment with 0, 0.1, 0.5, 1, 2.5 and 5 mM reagent, respectively.

more than 50% of the ankyrin and band 4.2 were extracted with a reagent concentration of 0.5 mM, and both proteins were maximally extracted with a reagent concentration of 2.5 mM. Band 4.1, however, was not extracted appreciably at reagent concentrations below 2.5 mM. The integral membrane proteins, bands 3 and 7, several minor proteins migrating between bands 4.2 and 5, and the glycophorins were not extracted even at a reagent concentration of 5 mM.

The residual ankyrin, bands 4.1, 4.2 and 6 seen in the pellets at reagent concentrations above 5 mM probably reflects the presence of a small population of right-side-out vesicles, to which access of the reagent would be blocked and in which any soluble proteins would in any case be trapped.

The presence of added NaCl above 20 mM decreased the effectiveness of the mercurial in solubilizing the proteins; in the presence of 0.1 M NaCl the release of ankyrin, band 4.1 and band 4.2 was effectively abolished, while band 6 extraction was unaffected (data not shown).

The effect of N-ethylmaleimide on the solubilization of proteins by p-mercuribenzenesulfonate

When spectrin-depleted vesicles were treated with 4 mM *N*-ethylmaleimide on ice for 1 h, no protein was solubilized, and the subsequent solubilization of the peripheral proteins by *p*-mercuribenzenesulfonate appeared unaffected by this pretreatment. *N*-Ethylmaleimide treatment of spectrin-depleted vesicles at 37 °C for 1 h also did not result in protein solubilization. However, this treatment inhibited the subsequent solu-

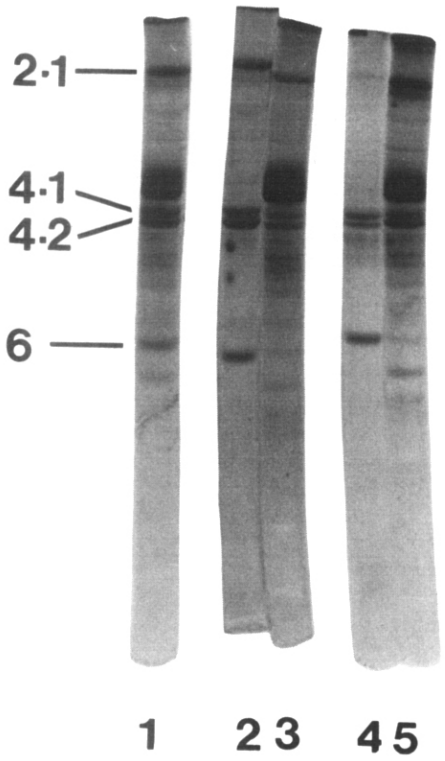

Fig. 2. The effect of 4 mM *N*-ethylmaleimide in 5 mM phosphate at 37 °C for 1 h on the subsequent solubilization of proteins from spectrin-depleted vesicles by 5 mM *p*-mercuribenzenesulfonate. Gel 1: spectrin-depleted vesicles. Gels 2 and 3: without *N*-ethylmaleimide pre-treatment. 2, Supernatant; 3, pellet. Gels 4 and 5: with *N*-ethylmaleimide pre-treatment. 4, Supernatant; 5, pellet.

bilization by *p*-mercuribenzenesulfonate of ankyrin and band 4.2; only trace amounts of ankyrin and band 4.2 were extracted by 1 mM *p*-mercuribenzenesulfonate. In contrast, approx. 80% of both ankyrin and band 4.2 were extracted by this concentration of mercurial, either from spectrin-depleted vesicles treated with *N*-ethylmaleimide on ice or from the control vesicles incubated in 5 mM phosphate buffer at 37 °C.

Even at a reagent concentration of 5 mM, almost all of the ankyrin and more than 80% of band 4.2 remained associated with the spectrin depleted vesicles that had been pretreated with *N*-ethylmaleimide at 37 °C (Fig. 2). The solubilization of band 6 by *p*-mercuribenzenesulfonate, however, appeared unaffected, and that of band 4.1 appeared only slightly reduced, by this pretreatment with *N*-ethylmaleimide.

Solubilization from intact membranes

Incubation of intact erythrocyte ghosts suspended in 5 mM sodium phosphate buffer with 1 mM *p*-mercuribenzenesulfonate on ice resulted in the solubilization of almost all of the actin and band 6, most of the spectrin, ankyrin, and band 4.2, but very little band 4.1. At a concentration of 5 mM most of band 4.1 was also solubilized and only traces of the other peripheral proteins remained with the membrane, consistent with previous reports [1,3].

The effect of salt on the extraction of peripheral proteins by p-mercuribenzenesulfonate

Incubation of erythrocyte ghosts at 37 °C in 0.1 M NaCl alone resulted in the extraction of band 6 from the membranes, as has previously been reported [13]. These conditions also resulted in an overall decrease in the subsequent solubilization of all of the peripheral proteins by *p*-mercuribenzenesulfonate in 5 mM phosphate buffer, except, interestingly, for actin. The subsequent treatment of salt-incubated erythrocyte ghosts with 1 mM *p*-mercuribenzenesulfonate in 5 mM phosphate resulted in solubilization of nearly all of the actin. Traces of spectrin and ankyrin and only barely detectable amounts of other components were released (Fig. 3, gels 1 and 2). With 5 mM reagent, almost all of the actin and most of bands 4.1 and 4.2 were solubilized. However, most of the spectrin and ankyrin remained associated with the membrane (Fig. 3, gels 3 and 4).

This effect does not seem to be due to residual NaCl in the extraction medium. The concentration of residual NaCl was calculated to be less than 20 mM, sufficient only to cause partial inhibition of the solubilization of ankyrin and band 4.2. Furthermore, after subsequent washing of the membrane pellet three times with 5 mM phosphate buffer (pH 8.0) a second incubation with mercurial released only traces of spectrin, ankyrin and band 4.2.

Treatment of erythrocyte ghosts with *N*-ethylmaleimide in 0.1 M NaCl at 37 °C for 1 h resulted in almost complete inhibition of the subsequent *p*-mercuribenzenesulfonate-dependent solubilization of spectrin and ankyrin. The solubilization of band 4.2 was also markedly reduced, while that of band 4.1 and actin was slightly reduced (Fig. 3, gels 5 and 6).

Incubation of spectrin-depleted vesicles with 0.1 M NaCl at 37 °C for 1 h was also found to prevent the subsequent release of ankyrin from the vesicles by *p*-mercuribenzenesulfonate, suggesting that the effect primarily concerns the release of ankyrin, and that it is not simply due to resealing of the intact membranes in the NaCl solution.

Under phase contrast, the erythrocyte membranes in 5 mM phosphate buffer in the absence of *p*-mercuribenzenesulfonate exhibited a variety of shapes, ranging from discocyte through stomatocyte (Fig. 4a). After addition of 1 mM *p*-mercuribenzenesulfonate to erythrocyte ghosts in 5 mM phosphate buffer, the membranes appeared grossly vesiculated (Fig. 4b). In contrast, erythrocyte membranes that had been pre-incubated in 0.1 M NaCl at 37 °C, either in the presence or absence of *N*-ethylmaleimide, did not vesiculate after subsequent treatment with either 1 mM or 5 mM *p*-mercuribenzenesulfonate. However, the membranes be-

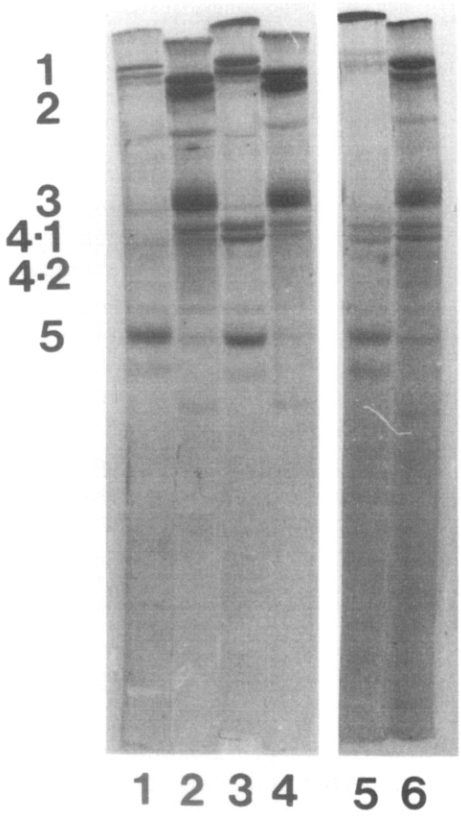

Fig. 3. Solubilization of proteins from intact erythrocyte membranes by *p*-mercuribenzenesulfonate. Gels 1, 2: membranes pre-incubated in 0.1 M NaCl, 37 °C, then treated with 1 mM *p*-mercuribenzenesulfonate. 1, Supernatant; 2, pellet. Gels 3, 4: membranes pre-incubated in 0.1 M NaCl, 37 °C, then treated with 5 mM *p*-mercuribenzenesulfonate. 3, Supernatant; 4, pellet, Gels 5, 6: membranes pre-incubated with 4 mM *N*-ethylmaleimide in 0.1 M NaCl at 37 °C, then treated with 5 mM *p*-mercuribenzenesulfonate. 5, Supernatant; 6, pellet.

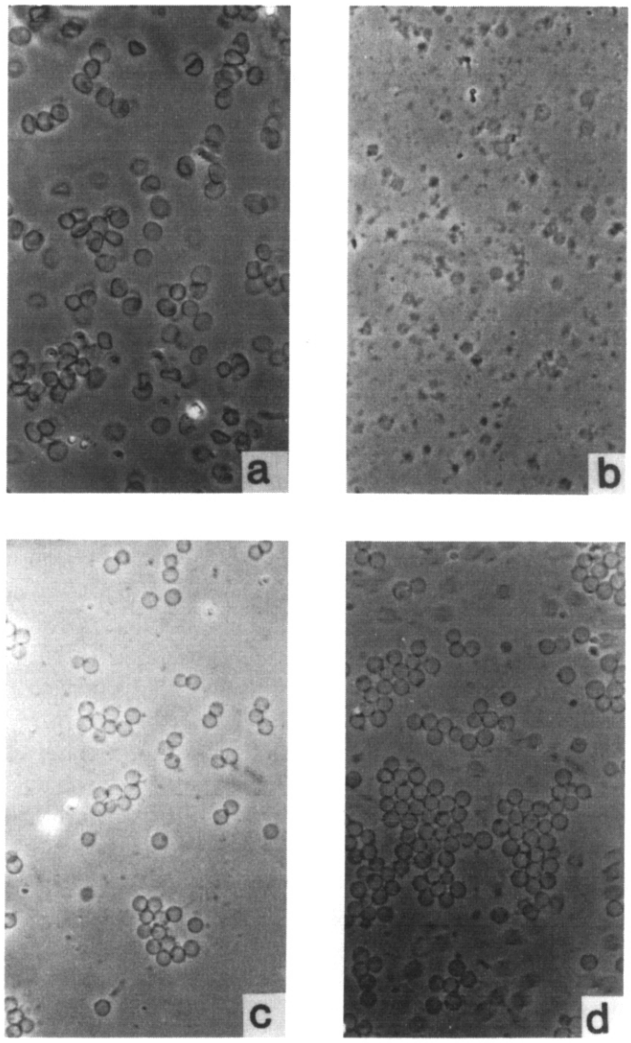

Fig. 4. Morphology of erythrocytes under phase contrast illumination. (a) Erythrocyte membranes in 5 mM phosphate; (b) erythrocyte membranes treated with 1 mM *p*-mercuribenzenesulfonate; (c) erythrocyte membranes treated with 5 mM *p*-mercuribenzenesulfonate after incubation with 0.1 M NaCl for 1 h at 37 °C; (d) erythrocyte membranes treated with 5 mM *p*-mercuribenzenesulfonate after incubation with 5 mM *N*-ethylmaleimide in 0.1 M NaCl for 1 h at 37 °C. (Magnification: 500×).

came spherical after this treatment (Figs. 4c, d). Thus it appears that, even when almost all of the actin and most of band 4.1 and band 4.2 are solubilized, the erythrocyte membrane is able to remain intact, though it loses the discoid morphology.

In spite of the failure of *p*-mercuribenzenesulfonate to solubilize spectrin and ankyrin from membranes incubated in 0.1 M NaCl at 37 °C, most of the remaining spectrin and ankyrin were solubilized from the membranes by a subsequent incubation in water at 37 °C. A small amount of band 4.1 and only traces of the other peripheral proteins remained bound to these membranes. Incubation in water at 37 °C also resulted in most of the spectrin being released from the membranes that had been pretreated with *N*-ethylmaleimide in 0.1 M NaCl before mercurial treatment. Some of the remaining band 4.1 and actin were also solubilized. However, some of the spectrin, band 4.1 and a trace amount of actin remained membrane-bound. Substantial amounts of ankyrin and band 4.2 also remained bound to the membrane.

Phase contrast microscopy showed that incubation of the mercurial-treated samples in water at 37 °C resulted in gross vesiculation of both the *N*-ethylmaleimide-pretreated membranes and the membranes that had not been treated with *N*-ethylmaleimide. Thus, vesiculation appears to occur concomitantly with spectrin solubilization but does not seem to depend upon the extraction of any other peripheral proteins.

Radiolabelling of proteins from spectrin-depleted vesicles

Band 3 and a component that migrates between bands 4.2 and 5 on polyacrylamide gels in dodecyl sulfate were the major membrane-bound components radiolabelled after *p*-[^{203}Hg]mercuribenzenesulfonate

treatment of spectrin-depleted vesicles. Approximately 2.5 ± 1 mol of ^{203}Hg appeared to be bound per mol of band 3 in spectrin-depleted vesicles that had been pretreated with *N*-ethylmaleimide for 1 h on ice, although this estimate must be considered with caution for two reasons. Bands 4.1 and 4.2 are not completely resolved from band 3 on the agarose gel column and any residual band 4.1 or 4.2 not solubilized by *p*-mercuribenzenesulfonate would partially coelute with band 3 contributing to the estimate of the concentration of band 3. Further, radiolabel bound to residual proteins coeluting with band 3 may contribute to the amount of radiolabel associated with band 3.

After pretreatment of spectrin-depleted vesicles with *N*-ethylmaleimide for 1 h at 37 °C the radiolabel subsequently bound to band 3 by treatment with labeled mercurial decreased by approximately 1 mol of ^{203}Hg per mol of band 3, coincident with a marked reduction in the extent of mercurial-dependent solubilization of ankyrin and 4.2 from spectrin-depleted vesicles. The amount of radiolabel subsequently associated with the component that migrates in polyacrylamide gels between bands 4.2 and 5 was also reduced by approximately 14%. Although precise and accurate estimates of the concentration of this protein could not be made, based on a molecular weight of 60000 and an absorption coefficient of $1.0\ l \cdot g^{-1} \cdot cm^{-1}$, the extent of labelling in the absence of *N*-ethylmaleimide treatment was approx. 2.0 mol per mol, decreasing to 1.7 mol per mol after blocking with *N*-ethylmaleimide.

Discussion

The results of the present study showed that *p*-mercuribenzenesulfonate is able to dissociate the peripheral proteins from the membranes of spectrin-depleted vesicles. The extent of extraction of the proteins was dependent on mercurial concentration and there was a degree of selectivity in the release of proteins. The proteins that were released most readily were those known to be associated with band 3 (i.e., ankyrin and bands 4.2 and 6). The sensitivity of the solubilization of ankyrin to mercurial concentration was similar to that of band 4.2.

N-Ethylmaleimide pretreatment of spectrin-depleted vesicles at 37 °C reduced the extent of subsequent radiolabelling of band 3 with *p*-[^{203}Hg]mercuribenzenesulfonate by approx. 1 mol/mol and also reduced the extent of solubilization of both ankyrin and band 4.2. *p*-Mercuribenzenesulfonate, therefore, appears to react with band 3, presumably at a sulfhydryl group, to cause the release of ankyrin and band 4.2; the site of this reaction appears to be blocked by *N*-ethylmaleimide at 37 °C but not at low temperature and thus is not the 'cryptic' cysteine 479 [14]. The stoichiometry of approx. 1.5 mol mercurial/mol band 3 following *N*-ethylmaleimide treatment at 37 °C is similar to that obtained by Werner et al. [14] under similar conditions.

One of the sulfhydryl groups on band 3 with which *p*-mercuribenzenesulfonate reacts may be in the vicinity of the binding sites of both proteins. Exposure of this sulfhydryl group to mercurial attack may then be linked with the dissociation of one or both of the proteins from band 3. Alternatively, the mercurial may react with a sulfhydryl group distant from the binding sites of ankyrin and band 4.2; such reaction may result in changes in the structure of band 3 which cause dissociation of both ankyrin and band 4.2. *p*-Mercuribenzenesulfonate-dependent solubilization of band 6 was not inhibited by *N*-ethylmaleimide pretreatment, which suggests that the mechanism of release of band 6 is different from that of ankyrin and band 4.2. Band 6 is probably bound at a site on band 3 different from the binding sites of ankyrin or band 4.2. The sulfhydryl group involved may not be as reactive towards *N*-ethylmaleimide.

The release of band 4.1 from spectrin-depleted vesicles and erythrocyte ghosts required higher mercurial concentrations than for the release of other peripheral proteins. *N*-Ethylmaleimide pretreatment did not prevent the mercurial-dependent solubilization of band 4.1. These observations confirm previous findings that band 4.1 is attached to the membrane via interactions distinct from those linking the other peripheral proteins [10,11].

Actin was selectively solubilized by 1 mM *p*-mercuribenzenesulfonate from intact erythrocyte membranes that had been incubated at 37 °C in a high ionic strength medium. *N*-Ethylmaleimide did not prevent this phenomenon, and the selective solubilization of actin, therefore, does not appear to be dependent upon the solubilization of any other peripheral protein; *p*-mercuribenzenesulfonate action on actin itself may result in its dissociation and release from the membrane, probably through the depolymerization of the short actin protofilaments brought about by mercurials [6].

With higher mercurial concentration, most of band 4.1 and band 4.2 were also solubilized from erythrocyte membranes that had been incubated at 37 °C in 0.1 M NaCl, whereas most of the spectrin and ankyrin remained with the membrane. Solubilization of spectrin by the mercurial was further inhibited by *N*-ethylmaleimide pretreatment of erythrocyte ghosts at 37 °C. This, presumably, was due to inhibition of the solubilization of ankyrin, the protein that anchors spectrin to the membrane. The mercurial, therefore, does not appear to disrupt the interaction between spectrin and ankyrin.

Incubation of erythrocyte ghosts in 0.1 M NaCl appears to have an irreversible effect on the extractability of the peripheral proteins, other than actin, brought about by mercurial. The effect is not reversed by repeated washing with low ionic strength buffer, and does not seem to be due to resealing. The 37 °C incubation

in salt seems to bring about changes in the organization of the cytoskeleton and/or membrane such that after reaction with mercurial, the ankyrin and its associated spectrin remains firmly attached to the membrane. Subsequent incubation at 37°C in water then leads to the solubilization not only of the spectrin, but also of the ankyrin. This unusual low ionic strength solubilization of ankyrin suggests that, as a result of reaction with mercurial, changes have taken place either in ankyrin itself, or in band 3, that weaken the ankyrin-band 3 interaction at very low ionic strength.

Examination of the morphology of the erythrocyte membranes during selective solubilization revealed that, of the cytoskeleton proteins, only spectrin appears to be necessary to maintain the integrity of the membrane. Removal of spectrin, either by incubation of erythrocyte ghosts in low ionic strength media or by reaction of erythrocyte ghosts with *p*-mercuribenzenesulfonate, resulted in membrane vesiculation. Solubilization of actin and most of bands 4.1 and 4.2 did not result in membrane vesiculation, even though the integrity of the cytoskeleton is thereby completely disrupted, but did result in the formation of spherical erythrocyte ghosts. Consistent with this result, it has been noted that 1 mM *p*-mercuribenzenesulfonate selectively solubilizes actin from the red cell ghosts of the guanaco (Ralston, G.B., unpublished observations). The guanaco erythrocyte membranes do not vesiculate after this treatment, but do change shape from an ellipsoid to a sphere. Thus, while spectrin alone seems to maintain the integrity of the membrane, actin appears to be essential, with spectrin, in controlling the shape of erythrocyte membranes.

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