Hereditary spherocytosis of man

Altered binding of cytoskeletal components to the erythrocyte membrane

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(Received 22 June 1981/Accepted 23 September 1981)

Human erythrocytes possess a lattice work of extrinsic proteins on the inner face of the membrane ('cytoskeleton') that maintains the shape and deformability of the cell. The major proteins of the cytoskeleton are spectrin and actin, which are attached to the membrane by protein bands 2.1 ('ankyrin') and 4.1. The interactions of spectrin/actin with erythrocyte membranes from normal subjects and from patients with hereditary spherocytosis (HS) have been studied by using an air-driven ultracentrifuge, which can rapidly separate membranes from soluble proteins (150000 g for 30 s). The total amount of spectrin/actin in HS and normal ghosts is similar. However, the rate of dissociation of spectrin and actin from HS erythrocyte membranes at low ionic strength is significantly lower than that observed for normal membranes. Spectrin and actin isolated from either HS or normal membranes re-associated in a similar manner to spectrin/actin-depleted vesicles prepared from normal cells. Scatchard analysis showed an average binding capacity of 278 µg/mg of membrane protein. However, spectrin/actin-depleted vesicles prepared from HS cells bound significantly less spectrin/actin prepared from either the normal or abnormal cells (average binding capacity 158 µg/mg of membrane protein). The defect was defined further by studying the cytoskeleton obtained by Triton X-100 extraction of membranes. Under conditions of low ionic strength cytoskeletons prepared from HS membranes dissociated more slowly than those prepared from normal membranes, and only 80% of the protein from HS cytoskeletons could be solubilized after 180 min compared with 100% for normal cytoskeletons. The difference between HS and normal membranes, which persists in isolated cytoskeletons, suggests that alterations in either the primary structure or the degree of phosphorylation of protein bands 2.1 or 4.1 may be central to the molecular basis of hereditary spherocytosis.

The shape and deformability of the erythrocyte membrane appears to be maintained by a network of cytoskeletal proteins on the inner face of the membrane. A major component of this network, namely spectrin, exists as an \( \alpha_2\beta_2 \)-type tetramer that is bound to the membrane by protein band 2.1 near one end of the elongated \( \alpha\beta \) heterodimer, and by band 4.1 at the other end. There is evidence to suggest that band 2.1 itself is associated with band 3, a major intrinsic protein of the membrane, so providing a physical link between the cytoskeleton and the membrane proper (Hargreaves et al., 1980). In addition, the lateral extension of the cytoskeletal network may be generated by the ability of actin to cross-link the spectrin–band 2.1–band 4.1 complexes (for reviews, see Lux, 1979; Branton et al., 1980).

The membrane nature of the defect in HS was first suspected from the finding of an increased \( \text{Na}^+ \) permeability in intact erythrocytes, an observation that has been shown consistently (Bertles, 1957; Jacob & Jandl, 1964; Wiley, 1972). Other membrane abnormalities have been described, such as an increased \( ^{32}\text{P} \) turnover in the phospholipids (Reed, 1968), a reduction in the amount of membrane lipid per cell (Cooper & Jandl, 1969a) and decreased drug-induced endocytosis (Schrier et al., 1974). Despite these defects in membrane function, there have been no gross abnormalities detected with respect to membrane phospholipid distribution or fatty acid composition (de Gier et al., 1964;
Robertson & Lands, 1964), although minor differences in the amounts of long-chain fatty acids in some phospholipid types have been claimed but not substantiated (Kuiper & Livne, 1972; Zail & Pickering, 1979). Such differences do not result in any change in the microviscosity of the membrane (Cooper et al., 1980). Several workers have reported small differences in SDS/polyacrylamide-gel electrophoresis patterns of HS and normal membranes but these have not been substantiated in other laboratories (Robinson et al., 1979; for review, see Zail, 1977). Differences in phosphorylation of protein in normal and HS membranes are equally uncertain (Greenquist & Shohet, 1976; Matsumoto et al., 1977; Wolfe & Lux, 1978; Boivin et al., 1979).

In the present paper we focus attention on the interaction of cytoskeletal proteins with the membrane proper and show that differences exist in the elution and re-association behaviour of the water-soluble proteins of HS and normal membranes.

Experimental
Reagents
Dithiothreitol was from Calbiochem. Adenosine, inosine, bovine serum albumin and phenylmethanesulphonyl fluoride were from Sigma. Triton X-100 was from Ajax Chemical Co. All reagents were of analytical grade or better.

Methods
Selection of HS patients. Patients from seven different families were diagnosed as having hereditary spherocytosis on the basis of elevated reticulocyte counts, increased osmotic fragility and autohaemolysis. Four of the seven patients were post-splenectomy and had normal reticulocyte counts. All patients had increased erythrocyte osmotic fragility with median fragilities between 0.470 and 0.485 g of NaCl/dl (normal range 0.40-0.44 g of NaCl/dl).

Preparation of erythrocyte ghosts. Fresh venous blood from normal or HS subjects was drawn into heparinized tubes and washed three times by centrifugation at 2500 g for 5 min, followed by aspiration of the Buffy coat and resuspension of the cells in a medium of composition 145 mM-NaCl/5 mM-KCl/20 mM-imidazole (pH 7.6). Packed cells (1 vol.) were haemolysed into 10 vol. of 5 mM-Tris/HCl buffer (pH 7.6, 5°C) containing 50 μg of phenylmethanesulphonyl fluoride/ml to minimize intrinsic protease activity. Stroma were separated by centrifugation at 40000 g for 20 min. After aspiration of the supernatant and removal of the underlying granulocyte button, the stroma were washed three times with the haemolysis buffer followed by a final wash in 6 mM-KCl/1 mM-imidazole (pH 7.6). The resulting ghosts were white.

Time-course of spectrin/actin dissociation from erythrocyte membranes. Erythrocyte membranes suspended in 6 mM-KCl/1 mM-imidazole (pH 7.6) (approx. 5 mg of protein/ml) were diluted 5-fold into 0.1 mM-EDTA. The suspensions were incubated at 23°C, and duplicate samples (150 μl each) were removed at successive time intervals. The soluble proteins, hereafter referred to as 'spectrin/actin', were separated from stroma by centrifugation at 150000 g for 30 s at 4°C using an air-driven ultracentrifuge (Beckman Airfuge), and 100 μl of the supernatant was removed for protein analysis. The centrifugation was completed within 4 min of sampling. Control experiments using the water-soluble protein dissociated from stroma at low ionic strength (see below) showed that there was no appreciable sedimentation of this protein under the conditions used, nor was there evidence of incomplete sedimentation of spectrin/actin-depleted vesicles.

Preparation of spectrin/actin. Spectrin/actin used in re-association experiments was prepared from erythrocyte ghosts suspended in 6 mM-KCl/1 mM-imidazole (pH 7.6). The suspension was diluted 20-fold into 0.1 mM-EDTA, incubated at 37°C for 30 min, and centrifuged at 40000 g for 20 min at 5°C to separate the soluble proteins. SDS/polyacrylamide-gel electrophoresis showed that the dissociated proteins were predominantly spectrin and actin. The supernatant (spectrin/actin) was centrifuged again under the same conditions to remove any residual cell debris, and was concentrated to a protein concentration of approx. 2.5 mg/ml for use in re-association studies.

Preparation of spectrin/actin-depleted vesicles. Water-soluble proteins were dissociated from ghosts at 37°C as described above. The pellet of membrane vesicles was washed twice with 0.1 mM-EDTA to remove any residual spectrin/actin and resuspended in the appropriate buffer for the re-association studies reported in Figs. 3 and 4. Full binding studies (Fig. 4) were carried out with inside-out and right-side-out vesicles (Steck, 1974) prepared as follows. Pelleted spectrin/actin-depleted vesicles were vortex-mixed in phosphate buffer (5 mM-phosphate, 0.5 mM-EDTA, pH 8.4) and homogenized by three passes through a 27-gauge syringe needle. Approx. 8 ml of the homogenate was layered on to 12 ml of dextran T70 (4.46 g/100 ml in the same phosphate buffer) and spun at 30000 g for 40 min. A fraction rich in inside-out vesicles remained at the barrier and was removed by a Pasteur pipette. Right-side-out vesicles were harvested from the pellet. Both fractions were washed and suspended in imidazole buffer in readiness for binding experiments. The sidedness of the vesicles
was determined by assay of the acetylcholinesterase activity (Ellman et al., 1961; Steck, 1974).

Re-association studies. The kinetics of re-association were studied by adding spectrin/actin to depleted vesicles and incubating at 23°C. The final amounts were: spectrin/actin, 0.9 mg; depleted vesicles, 2.1 mg of membrane protein in a total volume of 3 ml. The final salt concentration was 30 mM-KCl and 0.1 mM-EDTA. Portions (150 µl) were withdrawn at successive time intervals, and the unbound protein was separated by centrifugation in the air-driven ultracentrifuge at 150000g for 30 s at 4°C. The supernatants (100 µl) were withdrawn for protein estimation. All samples were taken in duplicate.

Binding studies were carried out by mixing inside-out vesicles (200 µg of membrane protein) with spectrin/actin (0–400 µg) in a total volume of 0.3 ml of 30 mM-KCl plus 0.1 mM-EDTA and incubating for 90 min at 4°C. Unbound protein was separated and estimated as described above. All samples were in duplicate and results were reproducible to within 3%. Corrections for non-specific binding were made by subtracting the amounts of protein bound to normal or HS right-side-out vesicles (Bennett & Branton, 1977; Anderson & Tyler, 1980). This correction factor was small (<20%) and was found to be similar using either normal or HS right-side-out vesicles.

Dissociation of cytoskeletons. The time course of the disaggregation of cytoskeletons at low ionic strength was followed by using a procedure similar to that described for the dissociation of spectrin/actin from ghosts. In both assays the final concentrations were 1.2 mM-KCl, 0.2 mM-imidazole and 0.1 mM-EDTA. Cytoskeletons (approx. 5 mg of protein/ml) prepared from ghosts by extraction with 2% Triton X-100 (Sheetz & Sawyer, 1978) were washed and resuspended by several passes through a pasteur pipette into a buffer containing 24 mM-KCl/4 mM-imidazole (pH 7.6). SDS/polyacrylamide-gel-electrophoresis patterns of this material showed the presence of spectrin, actin, bands 2.1 and 4.1, and small amounts of bands 3, 4.2 and 4.9. The dissociation of the cytoskeleton was induced by diluting this preparation 20-fold into 0.1 mM-EDTA. The kinetics of this process were followed by separating soluble protein from insoluble residue by centrifugation in the air-driven ultracentrifuge at 150000g for 30 s at 4°C.

Other procedures. Protein concentrations were determined by the method of Lowry et al. (1951) or by the dye-binding procedure of Bradford (1976). Bovine serum albumin was used as the standard. The phospholipid content of cytoskeletons was determined by analysis of phospholipid phosphorus using the modified Allen procedure described by Kates (1972). SDS/polyacrylamide-gel electrophoresis was carried out in composite gels of 2.5% acrylamide and 0.3% agarose (Peacock & Dingman, 1968; Palek & Liu, 1979). Gels were stained with Coomassie Blue and densitometric traces were made at 525 nm with a Gelman scanner.

Results

Dissociation of spectrin/actin from normal membranes

Extrinsic proteins are released rapidly from erythocyte membranes suspended in low-ionic-strength media (approx. 0.1 mM-imidazole + 0.1 mM-EDTA, pH 7.6) at 37°C, the reaction having a half-time of 8 min (Fig. 1). Approx. 30% of the membrane protein dissociates under these conditions. SDS/polyacrylamide-gel-electrophoretic analysis showed that the dissociated proteins were predominantly spectrin and actin. The rate of dissociation was decreased by adjusting the temperature to 23°C and the ionic strength to 1.4 mM (+0.1 mM-EDTA), whereupon the half-time of the reaction increased to approx. 60 min (Fig. 1). No more than 3% of the membrane protein was in the supernatant of material sampled at 2 min, but 20% became soluble after 180 min. In many experiments there was a short lag phase during the early part of the reaction (<10 min). The addition of 0.2 mM-Ca²⁺ completely prevented the dissociation of spectrin/actin from the membrane.

Dissociation of spectrin/actin from HS membranes

The kinetics of spectrin/actin release showed marked differences between HS and normal membranes. Fig. 1 compares the average dissociation behaviour of membranes from seven HS patients and 14 normal subjects. The amount of spectrin/actin released is significantly less for HS membranes compared with normal membranes (Fig. 1) between 40 and 180 min. Densitometric tracings of SDS/polyacrylamide-gel-electrophoresis patterns of HS and normal membranes indicated that the amounts of spectrin and actin relative to other proteins were similar (Figs. 2a and 2b). This was confirmed by the finding that the amount of protein dissociated by dialysis against 0.1 mM-EDTA for 24 h at 4°C was similar for normal and HS membranes (32 ± 1% and 30 ± 1% respectively).

Re-association studies

The proteins dissociated from membrane preparations at low ionic strength re-associated with spectrin/actin depleted vesicles simply by adjustment of ionic strength to higher levels (30 mM). The kinetics of this reassociation are depicted in Fig. 3, which shows that approx. 80% of the water-soluble proteins re-associated under these conditions, the reaction being complete within 20 min.
Fig. 1. Kinetics of the dissociation of protein from erythrocyte cell membranes at low ionic strength

The percentage of total protein solubilized represents the fraction that remains in the supernatant after centrifugation at 150,000 g for 30 s (total centrifugation time including acceleration and deceleration, 4 min). Results for normal ghosts in 0.1 mM-imidazole + 0.1 mM-EDTA, pH 7.6 at 37°C are indicated by △. Results for normal ghosts (○) in 1.2 mM-KCl/0.1 mM-EDTA/0.2 mM-imidazole (pH 7.6) at 23°C are also shown. The error bars represent means (±1 s.d.) for membranes obtained from seven different HS patients and from paired normal subjects. The filled symbols represent control experiments in which membranes were maintained in a solution containing 6 mM-KCl, 0.1 mM-imidazole (pH 7.6) at 23°C.

Fig. 2. Densitometric scans of SDS/polyacrylamide-gel electrophoresis

(a) Normal ghosts; (b) HS ghosts; (c) Normal inside-out vesicles; (d) HS inside-out vesicles. The amount of protein applied per well was 75 μg.

This re-association assay was utilized to explore further the differences between normal and HS membranes. The behaviour of HS membranes depicted in Fig. 1 suggests that the molecular defect responsible for HS resides either in the spectrin/actin fraction or in those proteins responsible for their binding to the membrane. To distinguish between these possibilities a 'cross-over' or 'hybridization' experiment was designed in which spectrin/actin from HS membranes was induced to re-associate with spectrin/actin-depleted vesicles from normal subjects. Conversely, spectrin/actin from normal membranes was induced to re-associate with spectrin/actin-depleted vesicles from HS patients. The results are presented in Fig. 3, together with the control experiments, which examined the homologous re-associations (normal spectrin/actin to normal depleted vesicles, and HS spectrin/actin to HS depleted vesicles). Spectrin/actin isolated from normal and HS cells re-associated equally well to normal spectrin/actin-depleted vesicles (Fig. 3). On the other hand, when HS depleted vesicles were used, only approx. 60% of the water-soluble protein re-associated, regardless of whether that protein was derived from normal or HS cells (Fig. 3). The results therefore suggest that the HS defect resides not in a structurally altered spectrin or actin but in an abnormality in the spectrin/actin-depleted vesicles. It is important to note that the sidedness of the spectrin-depleted vesicles used in these experiments was the same (44–46% inside-out vesicles) for HS and normal preparations (see below).

Binding equilibria

The air-driven ultracentrifuge was used to separate vesicle-bound from unbound protein over a range of spectrin/actin concentrations. The results in Figs. 4(a) and 4(b) show that the binding of the water-soluble proteins to spectrin/actin-depleted inside-out vesicles (approx. 70% inside-out) is saturable and that the Scatchard binding plots are linear. The derived association constants and bind-
normal inside-out vesicles) was repeated using vesicles prepared from three different donors to determine the error associated with the binding parameters and the reproducibility of the procedure for preparing fractions enriched with inside-out vesicles.

The data confirm that the HS defect resides in the depleted vesicles rather than in the spectrin/actin itself. For example, the binding parameters for normal depleted vesicles are similar regardless of whether the spectrin/actin is derived from normal or HS cells, and a similar case exists for the HS depleted vesicles. Of particular note is the 40% reduction in binding capacity of HS vesicles compared with normal vesicles. Moreover, spectrin/actin is bound more tightly to HS vesicles than to normal vesicles.

Dissociation of spectrin and actin from the cytoplasmic side of erythrocyte ghosts results in breakdown of the membrane into a mixture of inside-out and right-side-out vesicles (Lange et al., 1980). Thus the difference in binding capacity of normal and HS vesicles described above could be explained by a decrease in the ratio of inside-out to right-side-out vesicles in HS compared with normal membrane preparations. The values for the sidedness of vesicle preparations (Table 1) indicate that this is not the case. That is, preparations of inside-out vesicles had the same degree of sidedness. Control experiments indicated that the degree of sidedness did not change on the addition of spectrin/actin.

The reduced binding capacity of HS compared with normal inside-out vesicles could be due to incomplete dissociation of spectrin/actin from the membrane vesicles (a) or HS membranes (■).

The final amount of spectrin and actin was 0.9 mg, and depleted vesicles, 2.1 mg, in a total volume of 3 ml. The final solution conditions were 30 mM-KCl, 0.1 mM-EDTA, pH 7.6, 23°C (see the Experimental section). The ordinate represents the fraction of protein remaining in the supernatant after centrifugation in the air-driven ultracentrifuge (30s, 150000g). The continuous curve refers to results obtained with vesicles prepared from normal membranes, and spectrin and actin from either normal (□) or HS membranes (■). The broken curve refers to results obtained using vesicles prepared from HS membranes, and spectrin and actin from either normal (○) or HS membranes (■).

**Fig. 3.** Re-association of the protein solubilized at low ionic strength (predominantly spectrin and actin) with depleted membrane vesicles

**Fig. 4.** Binding curves for the re-association of spectrin and actin with depleted inside-out vesicles (a) and the corresponding Scatchard plot of the data (b)

The conditions of the experiment were similar to those described in the legend to Fig. 3. Vesicles (approx. 70% inside-out vesicles: 200 μg of protein) were mixed with various amounts of spectrin and actin (0–400 μg) in a total volume of 0.3 ml. Results are presented for the binding of HS spectrin and actin to either normal (□) or HS (○) depleted inside-out vesicles. Binding points were obtained in duplicate and corrected for the non-specific binding of spectrin and actin to right-side-out normal (■) depleted vesicles.
Table 1. Binding parameters for the association of water-soluble proteins with spectrin/actin-depleted vesicles

<table>
<thead>
<tr>
<th>Water-soluble protein</th>
<th>Acceptor vesicles</th>
<th>$10^3 \times K_A$ (ml/µg)</th>
<th>Binding capacity* (µg/mg of membrane protein)</th>
<th>Proportion of inside-out vesicles (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal spectrin/actin</td>
<td>Normal</td>
<td>13.8 ± 1.5†</td>
<td>277 ± 18†</td>
<td>69 ± 2†</td>
</tr>
<tr>
<td>HS spectrin/actin</td>
<td>Normal</td>
<td>13.7</td>
<td>279</td>
<td>72</td>
</tr>
<tr>
<td>Normal spectrin/actin</td>
<td>HS</td>
<td>41.0</td>
<td>174</td>
<td>71</td>
</tr>
<tr>
<td>HS spectrin/actin</td>
<td>HS</td>
<td>43.4</td>
<td>142</td>
<td>67</td>
</tr>
</tbody>
</table>

* Abscissa intercept of Scatchard plot.  
† Determined by assay of acetylcholinesterase activity.  
‡ S.D. (n = 3).

HS and normal cytoskeletons

Cytoskeletons prepared by extraction of normal ghosts with 2% Triton X-100 were completely disaggregated when diluted 20-fold into low-ionic-strength EDTA buffer (Fig. 5). The time course of this reaction was similar to that observed for the dissociation of spectrin/actin from membranes at low ionic strength (Fig. 1), suggesting that the rate-determining steps might be similar in each case. It was therefore of interest to determine if the rate of disaggregation of HS cytoskeletons showed a similar abnormality to that observed in Fig. 1. The results in Fig. 5 indicate that the HS cytoskeletons dissociate more slowly and that only 80% of the protein can be solubilized after 180 min compared with 100% for normal cytoskeletons. The results were similar for four experiments involving four different HS donors. Separate experiments showed that the phospholipid contents of the HS and normal cytoskeletons were equivalent (0.56 ± 0.01 µmol of P/mg of cytoskeletal protein) and similar to values reported in the literature (Sheetz & Sawyer, 1978).

Discussion

Several features of hereditary spherocytosis suggest that the cytoskeleton of these cells may be defective. Deformability of hereditary spherocytes is impaired when assessed either by filtration times of cell suspensions through a Millipore filter or by the pressure required to suck cells into a micropipette (Murphy, 1967; La Celle, 1970), and there is evidence that the shape and deformability of erythrocytes is influenced by the organization of the cytoskeleton (Palek & Liu, 1979). An animal model of hereditary spherocytosis is provided by the spherocytic anaemia of mice (sph/sph), which is characterized by an almost total absence of spectrin from the erythrocyte membrane (Shohet, 1979). These mutant erythrocytes thus lack a cytoskeleton.

HS membrane. The densitometric traces of SDS/polyacrylamide-gel-electrophoresis experiments shown in Figs. 2(c) and 2(d) indicate that the inside-out vesicles prepared from normal and HS membranes were both significantly depleted of spectrin and actin (>90% depleted). Thus the differences in binding capacity cannot be attributed to differences in the occupation of spectrin-binding sites in the vesicle preparations.
Abnormal cytoskeletal binding in hereditary spherocytosis

and exhibit a marked increase in lipid loss from the cell membrane, which contracts cell surface area and leads to spherocytosis. Although hereditary spherocytes of man also show an increased loss of membrane lipid on prolonged incubation in vitro (Cooper & Jandl, 1969a) and a lower total amount of membrane lipid (Cooper & Jandl, 1969b), our results (Fig. 2), as well as those of others (Sheehy & Ralston, 1978), do not show any quantitative deficiency of either spectrin or actin. However, some other defect, either in a minor component or in the interactions between the multiple components of the erythrocyte cytoskeleton, could give rise to the observed instability of membrane lipid and the reduced deformability of hereditary spherocytes.

This study reveals major differences between normal and HS erythrocytes in the kinetics of spectrin/actin dissociation from membranes. Conditions of ionic strength and temperature were chosen to slow the dissociation reaction sufficiently to allow the use of the air-driven ultracentrifuge to follow the kinetics. All seven patients studied showed a similar slowing in the rate of spectrin/actin dissociation and this defect was seen both in patients with intact spleens and high reticulocytes as well as post-splenectomy when the reticulocyte count had returned to normal. Sheehy & Ralston (1978) reported an abnormally tight spectrin binding to membranes in two out of 12 cases of HS examined. This conclusion was based on assay after 72h of low-ionic-strength dialysis of membranes and the rate of spectrin release was not studied. The dissociation reaction appears complex, since an early lag phase was found before dissociation commenced, suggestive of more than one reaction step. Exposure of membranes to low-ionic-strength media may not only dissociate spectrin/actin, but also cause irreversible damage to the spectrin/actin-binding sites. Thus the re-association of spectrin/actin to depleted vesicles occurs with an association constant that is far lower than that which must exist in the intact erythrocyte. Fig. 4(a) shows that saturation of binding sites in the re-association assay would occur with a concentration of unbound spectrin/actin of about 0.5 mg/ml. No unbound protein is detectable before low-ionic-strength treatment indicating very tight binding. Binding studies at pH 6.6 have revealed a second high-affinity site (Bennett & Branton, 1977). Recently, Golan & Veatch (1980) have identified structural changes that occur at moderate ionic strength before the onset of spectrin/actin dissociation. The first was completely reversible and involved an increase in the lateral diffusion coefficient of band 3, whereas the second was only partially reversible and involved an increase in the fraction of mobile band 3 molecules.

The ‘cross-over’ experiments described in Figs. 3 and 4 establish that these differences between HS and normal membranes do not arise from an altered spectrin or actin but more probably from the structures responsible for the binding of these extrinsic proteins to the membrane. Scatchard analysis of spectrin/actin binding showed that HS vesicles possessed only about half the maximal binding capacity of normal vesicles (Table 1). This result cannot be explained by differences in the sidedness of the vesicle preparations or by differences in their content of residual spectrin. However, the result is in agreement with the known genetics of HS, which is inherited as a Mendelian dominant trait (i.e. one abnormal gene is sufficient to produce spherocytosis) so that only 50% of the protein copies from the affected gene locus would be abnormal. Although the decreased binding capacity of HS vesicles for spectrin/actin is consistent with the known membrane instability of HS cells, this result should be interpreted with caution, when applied to the native membrane. Perhaps the type and extent of the irreversible membrane changes occurring during low ionic strength treatment may give rise to the observed differences.

We note that the differences in the rate of spectrin/actin dissociation from HS and normal ghosts (Fig. 1) are paralleled by similar differences in the rate of solubilization of their respective cytoskeletons (Fig. 5). It is therefore unlikely that the differences observed arise from alterations in membrane lipid composition or in the intrinsic proteins, although it must be noted that cytoskeletons also contain some residual lipid and 10% of band 3 from the original membrane as well as the cytoskeletal proteins (spectrin, actin, bands 2.1, 4.1 and 4.9) (Yu et al., 1973). The lipid content of normal and HS cytoskeletons is the same, although it is possible that small differences in the lipid or protein composition exist. The two spectrin-binding proteins, 2.1 and 4.1, are known to bind to opposite ends of the spectrin heterodimer (Ungewickell et al., 1979; Branton et al., 1980; Tyler et al., 1980). Thus the differences observed between HS and normal membranes may be due to changes in one or both of these proteins. Although bands 2.1 and 4.1 appear normal on SDS/polyacrylamide-gel electrophoresis of HS membranes, the defect may reside in a molecular charge difference brought about by abnormal phosphorylation. Bands 2.1 and 4.1 are phosphorylated by a cyclic AMP-dependent protein kinase (Plut et al., 1978). It is also possible that the HS defect might reside in an altered protein kinase or phosphatase and that bands 2.1 and 4.1 act only as intermediaries in the total effect. To date, the evidence for altered phosphorylation in HS membranes has been somewhat inconsistent. However, it is known that spectrin phosphorylation does not affect the binding of spectrin to the membrane (Anderson & Tyler, 1980), and cannot account for
the differences we have observed. Alternatively, bands 2.1 and/or 4.1 in spherocytic cells may be defective in primary structure. One or more amino acid substitutions in these proteins may alter their interaction with spectrin, or may modify a phosphorylating site either by direct substitution of a serine or threonine residue or by substitution of amino acid residues adjacent to them.

These studies provide direct support for the involvement of the erythrocyte cytoskeleton in the molecular defect of HS. Although the spectrin/actin of these cells is both qualitatively and quantitatively normal, the above results suggest that the binding of this major structural element to the membrane is abnormal. Many of the abnormalities described in HS, such as instability and deficiency of membrane lipids, could result from an abnormal cytoskeletal support of the erythrocyte membrane.

This work was assisted by grants from the Australian National Health and Medical Research Council and the Australian Research Grants Committee. The technical assistance of Victor Wong and Ylva Sahlestrom is gratefully acknowledged. We thank the di Carlo, Freame, Gillies, McMullen, Mieschel, Pidoto and van Vliet family members for their generous co-operation.

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