Spectrin Tetramer–Dimer Equilibrium in Hereditary Elliptocytosis

By T. Coetzer and S. Zail

The proportion of spectrin tetramers and dimers in 4°C low ionic strength extracts of red cell membranes of 9 subjects with 4 different variants of hereditary elliptocytosis (HE) and 2 subjects with hereditary spherocytosis (HS) was determined by nondenaturing gel electrophoresis. Such extracts reflect the native oligomeric state of spectrin in the red cell membrane. In two hemolytic HE variants (an unclassified adult with increased thermal sensitivity of red cells and an infant also showing increased thermal sensitivity of red cells), the proportion of dimers was increased, whereas the remaining subjects had values within the control range. Conversion of spectrin tetramers to dimers under isotonic conditions at 37°C, or spectrin dimers to tetramers at 30°C, resulted in a high proportion of dimers in the above two HE variants, as well as in a third variant with probable mild HE and sporadic hemolysis. The mother of the infant with elliptocytosis and increased thermal sensitivity of red cells, although hematologically normal, had an increased proportion of dimers in 4°C low ionic strength extracts of her red cell membranes. These findings reflect an underlying primary or secondary abnormality of spectrin in these subjects that affects the association state of spectrin in the red cell membrane. Their exact relationship to the pathogenesis of the elliptical shape of the red cell, or to the presence of hemolysis, is at present unclear.

Hereditary Elliptocytosis (HE) and a closely related disorder, hereditary pyropoikilocytosis (HPP), appear to be due to a disorder of the red cell membrane skeleton, since the HE and HPP ghost, as well as the membrane skeleton, replicate the red cell morphology.1,2 A recent model of this network postulates that spectrin tetramers, formed by head-to-head association of heterodimers (bands 1 and 2) are linked to oligomers of actin and to band 4.1 The spectrin is linked to the major integral membrane protein band 3, via an interaction with ankyrin or syndeyin (band 2.1).4,5

In some families with HE, spectrin has been shown to be abnormally heat sensitive,1,2 a finding thought to reflect the diminished conformational stability of the molecule. Recently, spectrin obtained from the red cells of a patient with HE was found to generate a qualitatively abnormal pattern of polypeptide fragments on tryptic digestion at low ionic strength.6 This patient was difficult to classify into one of the six clinical variants described by Lux and Wolfe,7 in that both parents had normal red cell morphology, while the propositus showed significant budding and fragmentation of red cells on heating to 45°C, but which was not as pronounced as is found in HPP. Spectrin obtained from three other variants of HE (neonatal elliptocytosis with hemolysis and abnormal thermal sensitivity of red cells, mild HE with no hemolysis, and mild HE with sporadic hemolysis) was found to be normal on tryptic digestion. Whether the above findings represent a primary molecular abnormality of spectrin, or how such an abnormality could lead to an elliptocytic shape, is not known.

In this study we report abnormalities in the proportion of dimers and tetramers in 4°C low ionic strength extracts of red cell membranes in some patients with variants of HE. Such extracts reflect the native oligomeric state of spectrin in the membrane.8 The interconversion of spectrin dimers and tetramers was also studied. This approach was suggested by recent studies of Liu and Palek,9 who found that spectrin tetramers could be converted to dimers in ghosts incubated under hypotonic conditions at 37°C, and that this diminished the stability of membrane skeletons derived from these ghosts to mechanical fragmentation, i.e., dimer formation favored structural instability of the membrane skeleton. In these studies, spectrin tetramers and dimers were separated by column chromatography or more conveniently by nondenaturing polyacrylamide gel electrophoresis, in which very small amounts of spectrin, or how such an abnormality could lead to an elliptocytic shape, is not known.

In this study we report abnormalities in the proportion of dimers and tetramers in 4°C low ionic strength extracts of red cell membranes in some patients with variants of HE. Such extracts reflect the native oligomeric state of spectrin in the membrane.8 The interconversion of spectrin dimers and tetramers was also studied. This approach was suggested by recent studies of Liu and Palek,9 who found that spectrin tetramers could be converted to dimers in ghosts incubated under hypotonic conditions at 37°C, and that this diminished the stability of membrane skeletons derived from these ghosts to mechanical fragmentation, i.e., dimer formation favored structural instability of the membrane skeleton. These studies, spectrin tetramers and dimers were separated by column chromatography or more conveniently by nondenaturing polyacrylamide gel electrophoresis, in which very small amounts of spectrin could be analyzed. A preliminary report by Liu and Palek10 showing abnormalities of dimer–tetramer interconversion in some patients with HE (and HPP) has appeared while the present study was in progress.

MATERIALS AND METHODS

Subjects

Nine patients from 8 different kindreds with variants of HE were studied and classified according to the nomenclature of Lux and Wolfe.7 The clinical and hematologic features of four of these subjects (C.G., T.M., R.L., and J.E.) have been described, and the salient features of all the subjects are summarized. Controls consisted of hematologically normal laboratory staff.

Subject C.G.

C.G. is a 30-yr-old splenectomized white female with a well compensated hemolytic anemia (HB 14.5 g/100 ml, MCV 82 cu μ,
showed elliptocytosis but also significant poikilocytosis, pyknocytosis, and osmotic fragility. The hemoglobin was 10.0 g/100 ml, MCV 70 cu µ, reticulocyte count 5.2%, and the peripheral smear showed elliptocytosis but also significant poikilocytosis, pyknocytosis, and red cell budding. At 11 mo, elliptocytosis was predominant with only occasional fragments and microspherocytes. The hemoglobin was 10.0 g/100 ml, MCV 70 cu µ, and reticulocyte count 6%. At this stage, the red cells showed some fragmentation and membrane budding on heating to 45°C, but not as pronounced as in patient C.G. above. Membrane fragmentation increased progressively on heating at 1°C increments up to 49°C. Osmotic fragility was normal both before and after incubation for 24 hr at 37°C and autohemolysis was increased (10.5% at 48 hr), but corrected by glucose (3.1% at 48 hr). At 22 mo, the hemoglobin is 12.7 g/100 ml, MCV 69 cu µ, and reticulocytes 5%. Elliptocytes are still the predominant cell type, and pyknocytosis and poikilocytosis cannot be seen. There are no siblings and only the mother is available for study. She is hematologically normal and has normal heat stability of her red cells. The clinical and hematologic features of this patient will be described in detail elsewhere (Dr. L. MacDougall, submitted for publication). He probably represents a case of neonatal elliptocytosis with increased thermal sensitivity of red cells. Although this patient shows some features characteristic of HPP, several features suggest that he is rather a variant of HE, viz., the predominant elliptocytosis, relatively high MCV, incomplete fragmentation of red cells on heating to 45°C, increased autohemolysis corrected by glucose, and normal osmotic fragility.

Subject R. Le.

R. Le. is a white female aged 40 yr who has marked elliptocytosis and had an ongoing hemolytic anemia prior to splenectomy in April 1980, but with an apparent “cure” since splenectomy. Red cell indices prior to splenectomy were: hemoglobin 8.2–11.8 g/100 ml, MCV 79–84 cu µ, reticulocyte count 5–11%. Osmotic fragility and autohemolysis were normal. Following splenectomy, the hemoglobin varied between 13.5 and 14.3 g/100 ml and reticulocyte count 1.5–1.8%. Incubation of her red cells shows only mild fragmentation beginning at 48°C, with almost complete splitting at 49°C. The parents of this patient are deceased, the examination of her three children reveals no hematologic abnormality. Although we lack evidence that other family members have mild HE, she probably represents a case of mild HE with sporadic hemolysis.

Subjects F. La. and J. La.

These are a father and son, respectively, with striking elliptocytosis on peripheral smear. J. La. has a compensated hemolytic anemia (Hb 16.0 g/100 ml, MCV 85 cu µ, reticulocyte count 4.5%) and has a normal osmotic fragility and autohemolysis. The father (F. La.) presented with similar features 5 yr ago and has since had a splenectomy with apparent “cure.” At present his hemoglobin is 16.5 g/100 ml and reticulocyte count 1.5%. They represent a family of mild HE with sporadic hemolysis.

Subjects M. K., S. H., J. E., and M. E.

These are four unrelated adult white females with prominent elliptocytosis and normal hemoglobin values and reticulocyte counts, and show classical autosomal dominant transmission of the disorder. These patients represent mild HE with no hemolysis.

Subjects A. T. and M. R.

These are adult female patients with classical hereditary spherocytosis. A. T. has not been splenectomized and has a compensated hemolytic anemia (Hb 12.5 g/100 ml, reticulocyte count 5%). M. R. is splenectomized with a hemoglobin of 14.5 g/100 ml and reticulocyte count 1%.

Subject J. P.

J. P. is a hematologically normal adult male who was splenectomized following abdominal trauma.

Preparation of Spectrin Extracts

Heparinized venous blood was collected and cooled immediately to 0°C. Membranes were prepared by hypotonic lysis of packed red cells in 10 mM Tris-HCl buffer, pH 7.6, containing 1.0 mM EDTA, as previously described. Crude spectrin extracts were obtained by incubating membranes (2–3 mg membrane protein/ml) in 0.1 mM EDTA, pH 7.6, with 0.2 mM phenylmethyl-sulphonylfluoride (PMSF) for 10 min at 37°C or dialyzed against the same solution for 24 hr at 4°C. The suspension was centrifuged at 150,000 g for 30 min and the supernate kept on ice and used without further purification.

Spectrin Dimer–Tetramer Conversion

Transformation of spectrin tetramers to dimers was achieved by incubating the crude 4°C spectrin supernate under isocionic conditions (5 mM sodium phosphate, 150 mM NaCl, 5 mM mercaptoethanol, 5 mM EDTA, 0.2 mM PMSF, pH 7.5) for periods up to 1 hr at 37°C as described by Liu and Palek. Transformation of spectrin dimers to tetramers was done by incubation of the crude 37°C spectrin extract in the same isocionic medium but for 2.5 and 3 hr at 30°C.
the gel and the possibility that some of the complex might not have entered the gel.

**Verification of Experimental System**

The results reported are critically dependent on the assumption that the proportions of spectrin dimers and tetramers are not perturbed by the analysis system, e.g., during preparation and storage of spectrin dimers and tetramers or during gel electrophoresis. In particular, it is necessary to maintain the extracts at 0°-4°C at all times after extraction, since even brief exposure to warmer temperatures can result in conversion. This was examined by isolating pure tetramers or dimers by gel filtration chromatography (Sepharose Cl-4B) of 0°C and 37°C low ionic strength extracts as described by Liu and Palek. The purified tetramers and dimers were then subjected to the same handling procedures as the extracts, electrophoresed on 3% acrylamide gels, and the proportion of dimers and tetramers determined as described above. Purified tetramers and dimers obtained from three control and one HE subject (C.G.) handled in this way showed no interconversion whatsoever. The importance of such measures is borne out by our findings prior to the storage of spectrin dimers and tetramers or during gel electrophoresis. This was examined by isolating pure tetramers or dimers by gel filtration chromatography (Sepharose Cl-4B) of 0°C and 37°C low ionic strength extracts as described above. Purified tetramers and dimers determined as described above. The purified tetramers and dimers were then subjected to the same handling procedures as the extracts, electrophoresed on 3% acrylamide gels, and the proportion of dimers and tetramers determined as described above. Purified tetramers and dimers obtained from three control and one HE subject (C.G.) handled in this way showed no interconversion whatsoever. The importance of such measures is borne out by our findings prior to the application of these precautions, when the range obtained from the proportion of tetramers (or dimers) in 4°C low ionic strength extracts in control subjects was unacceptably wide and could have obscured minor changes in the test population.

Spectrin extractability of red cell membranes was determined as previously described. 

**RESULTS**

**Proportion of Spectrin Dimers and Tetramers in Red Cell Membranes**

Low ionic strength extraction at 4°C reflects the native oligomeric state of spectrin in the membrane. The proportion of spectrin tetramers in 4°C extracts in 10 controls, 9 HE subjects, 2 HS subjects, and a splenectomized control is shown in Table 1. Values are also shown for the asymptomatic mother and asymptomatic father of two of the HE variants.

Three subjects, C.G. (unclassified HE with increased thermal sensitivity of red cells), T.M. (neonatal echinocytosis with increased thermal sensitivity of red cells), and M.M. (asymptomatic mother of T.M.) showed an increase in the proportion of dimers in such extracts. Densitometric tracings of the separation of the oligomeric species of spectrin in these extracts as well as a control extract by non-denaturing polyacrylamide gel electrophoresis is shown in Fig. 1. In addition to the spectrin dimers and tetramers, a high molecular weight complex (HMW), which only partly enters the gel, and small but variable amounts of complexes with mobility intermediate between spectrin tetramers and HMW are seen. The latter represent higher spectrin oligomers, as recently described by Morrow and Marchesi. Equal amounts of protein were loaded on the gels when comparing patients and controls in individual experiments. Since the densitometric tracings in these experiments show an increased area under the dimer peak in subjects C.G., T.M., and M.M., the greater relative proportion of dimers in these subjects cannot be due to an increased tendency of spectrin tetramers to form HMW, which might not have entered the gel, but rather to a greater tendency of spectrin tetramers to dissociate into dimers.

**Spectrin "Extractability" of Red Cell Membranes**

The finding of an increased proportion of dimers in 4°C extracts of red cell membranes of C.G., T.M., and M.M. is meaningful only if the overall efficiency of extraction of spectrin (as determined by SDS-PAGE of whole membranes and extracts) is similar to control preparations. Spectrin "extractability" of red cell membranes was determined as previously described.

### Table 1. Proportion of Spectrin Tetramers and Dimers in 4°C and 37°C Membrane Extracts Before and After Conversion

<table>
<thead>
<tr>
<th>Subjects</th>
<th>4°C Membrane Extracts (% Spectrin Dimers)</th>
<th>Tetramer Conversion</th>
<th>37°C Membrane Extracts (% Spectrin Dimers)</th>
<th>Dimer Conversion</th>
</tr>
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<tbody>
<tr>
<td>Controls (n = 10)</td>
<td>(mean ± SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T.M. (neonatal HE)</td>
<td>47, 48</td>
<td>89, 90</td>
<td>93, 90</td>
<td>78, 76</td>
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<tr>
<td>M.M. (asymptomatic mother of T.M.)</td>
<td>26, 30</td>
<td>79, 78</td>
<td>93, 91</td>
<td>63, 65</td>
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<tr>
<td>C.G. (unclassified HE variant)</td>
<td>22, 20</td>
<td>91, 92</td>
<td>90, 92</td>
<td>79, 78</td>
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<tr>
<td>C.D. (asymptomatic mother of C.G.)</td>
<td>10</td>
<td>71</td>
<td>90</td>
<td>63</td>
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<tr>
<td>D.D. (asymptomatic father of C.G.)</td>
<td>8</td>
<td>70</td>
<td>90</td>
<td>60</td>
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<tr>
<td>R.L.e. (HE with sporadic hemolysis)</td>
<td>9</td>
<td>91</td>
<td>91</td>
<td>70</td>
</tr>
<tr>
<td>J.L.e. (HE with sporadic hemolysis)</td>
<td>9</td>
<td>70</td>
<td>90</td>
<td>56</td>
</tr>
<tr>
<td>F.L.e. (HE with sporadic hemolysis)</td>
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<td>75</td>
<td>91</td>
<td>56</td>
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<tr>
<td>J.E. (mild HE — no hemolysis)</td>
<td>7</td>
<td>77</td>
<td>94</td>
<td>59</td>
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<tr>
<td>S.H. (mild HE — no hemolysis)</td>
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<td>74</td>
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<td>62</td>
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<tr>
<td>M.K. (mild HE — no hemolysis)</td>
<td>6</td>
<td>—</td>
<td>92</td>
<td>59</td>
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<tr>
<td>M.P. (mild HE — no hemolysis)</td>
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<td>—</td>
<td>93</td>
<td>59</td>
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<tr>
<td>A.T. (nonsplenectomized HS)</td>
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<td>95</td>
<td>63</td>
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<tr>
<td>M.R. (splenectomized HS)</td>
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<td>81</td>
<td>95</td>
<td>61</td>
</tr>
<tr>
<td>J.P. (splenectomized control)</td>
<td>4</td>
<td>81</td>
<td>93</td>
<td>54</td>
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</table>
membranes of C.G., T.M., and M.M. and 4 controls varied between 76% and 85%.

Conversion of Spectrin Tetramers to Dimers

Spectrin tetramers in 4°C low ionic strength extracts can be converted to dimers by incubation in buffer of relatively high ionic strength at 37°C. The progressive formation of dimers over 60 min in 4°C extracts treated in this way is shown in Fig. 2. The range of observed values in 10 controls (2 SD above and below the mean) is shown by the dotted lines. In these subjects, the proportion of dimers formed decreased markedly between 30 and 60 min, presumably as equilibrium was reached. The initial change (0–15 min) in the proportion of dimers formed in T.M. (neonatal elliptocytosis) was slower than in the controls (presumably due to the presence of a large proportion of dimers at zero time), but at equilibrium the proportion of dimers was considerably greater in T.M. than in the controls. M.M. (asymptomatic mother of T.M.), although starting with an increased proportion of dimers, had an equilibrium position at 60 min within the control range. Subject C.G. (hemolytic HE with increased red cell thermal sensitivity) showed an increased initial change in the proportion of dimers and a high proportion of dimers at equilibrium. Subject R.L.e. (mild HE with sporadic hemolysis), who had a normal proportion of dimers at zero time, had not reached equilibrium at 30 min. The proportion of dimers at 60 min was increased and similar to that found in T.M. and C.G. Two patients with mild HE and no hemolysis (J.E. and S.H.) and two patients with mild HE and sporadic hemolysis (F.La. and J.La.) had values within the control range, as did the asymptomatic mother and father of C.G. The curves for J.E. and J.La. are shown in Fig. 2.

Individual values for the proportion of dimers found at 60 min after conversion of spectrin tetramers are shown in Table 1, which also includes values for a splenectomized control (J.P.), two patients with hereditary spherocytosis, A.T. (not splenectomized) and M.R. (splenectomized), and the asymptomatic parents of C.G.

Conversion of Spectrin Dimers to Tetramers

Low ionic strength extraction of red cell membranes at 37°C favors the formation of spectrin dimers. The proportion of dimers in such extracts in the test subjects was similar to that found in 10 control subjects (Table 1). Conversion of spectrin dimers to tetramers in such extracts was achieved by incubation at 30°C in a normal saline buffer for periods up to 3 hr. Equilibrium was achieved at 2.5 hr, and only these values are shown in Table 1. Three subjects (C.G., T.M., and R.L.e.) showed an increased proportion of dimers at 2.5 hr, indicating an impairment of association of dimers to tetramers. Of some interest is the finding in the extracts of M.M. (mother of T.M.) in which the proportion of dimers at equilibrium was at the upper limits of the control range.
DISCUSSION

The introduction of nondenaturing gel electrophoresis by Liu and Palek9 to determine the distribution of spectrin dimers and tetramers in low ionic strength extracts of red cell membranes is an important and useful technique. Small quantities of protein derived from only a few milliliters of blood can be rapidly analyzed, in contrast to the much larger amounts required for accurate quantitation on agarose columns. One of the disadvantages of this technique, however, is that some of the spectrin in crude extracts is present as higher oligomeric complexes that cannot be directly determined, as some of these aggregates may be excluded from the gel.14 The spectrin conversion experiments performed in the present study were done on crude extracts. At equilibrium, the proportion of spectrin dimers or tetramers would not be influenced by the amount of high molecular weight complex present, although the absolute amount of spectrin dimers or tetramers formed would be affected. Since in our studies we have measured only the proportion of spectrin dimers or tetramers at various time intervals during the conversions, we cannot comment on the quantitative amounts of dimers or tetramers formed but only on the relationship between these two molecular species. Rates of formation of dimers or tetramers in the conversion studies are thus relative to the initial proportion of these species in the extracts.

At 4°C, low ionic strength extracts are thought to represent the native state of spectrin in red cell membranes.8 Two of our patients (T.M. and C.G.) showed a significant increase in the proportion of dimers in 4°C extracts of red cell membranes. M.M., the asymptomatic mother of T.M., also had an increased proportion of dimers in extracts of her red cell membranes, which was a little more than half that found in T.M. She is presumably a heterozygous carrier of a gene determining some of the hematologic features of T.M. Unfortunately, the father is not available for study. The absence of any hematologic abnormality in the mother raises the question as to whether T.M. might have HPP in which parents of affected subjects are characteristically asymptomatic. Although we cannot completely exclude this possibility, for reasons outlined earlier, the hematologic features of T.M. suggest that he is a variant of HE, rather than HPP.

Of particular interest in the current studies are the data on the dissociation and association reactions of tetrameric and dimeric spectrin, respectively. In the four subjects with mild HE and no hemolysis and one kindred with mild HE and sporadic hemolysis, the interconversion of spectrin dimers and tetramers was indistinguishable from the controls. Three other hemolytic variants of HE, however, had abnormal association and dissociation reactions, as determined by an increased proportion of dimers at equilibrium. Patient R.L.e. deserves special comment, as she had a normal proportion of dimers in the 4°C extracts of her red cell membranes, and one might not expect an abnormality of spectrin on interconversion studies. One possibility is that R.L.e.’s spectrin has a greater susceptibility to damage during extraction, affecting subsequent interconversion. Another alternative, which we have not tested, is that R.L.e.’s spectrin tetramer–dimer equilibrium has a different dependence on protein or salt concentration or temperature than normal. The normal proportion of dimers found at equilibrium on interconversion of spectrin tetramers and dimers in M.M. (asymptomatic mother of T.M.) is also unexpected, as she has an increased proportion of dimers in her native membranes. At present we cannot offer a satisfactory explanation for this finding. The instability of spectrin tetramers in M.M. (as manifest by the increased percentage of dimers in her red cell membranes) is not associated with hemolysis and suggests that other, as yet ill-understood, factors may contribute to hemolysis in the other patients with defective spectrin tetramer formation. These findings are similar to those of Liu et al.,15 who have recently documented an increased proportion of dimers and defective
tetramer formation in the asymptomatic mothers of two patients with HPP, as well as in the propositi who had overt hemolytic anemia. Membrane skeletons prepared from red cell ghosts of these asymptomatic mothers were, however, unstable as they showed increased fragility on mechanical shaking. The asymptomatic parents of C.G. on the other hand, have normal proportions of dimers in 4°C extracts of their red cell membranes and show no abnormality of spectrin association or dissociation.

The altered association and dissociation reactions of spectrin in some of the HE patients suggests a primary or secondary (e.g., posttranscriptional) abnormality in the structure of spectrin in these patients, but does not exclude the possibility that such a defect exists in the remaining HE subjects. The decreased ability of spectrin dimers to associate to form tetramers presumably alters the propensity of spectrin to form higher oligomeric states. Evidence has recently been presented\(^\text{14}\) that such higher oligomers of spectrin are an integral component of the membrane skeleton and that they form by self-association at high protein concentration. Such high concentrations of spectrin are thought to be determined by a high affinity interaction of spectrin with ankyrin at the cytoplasmic surface of the membrane. We postulate that aberrations in the formation of higher oligomeric states of spectrin may play an important role in determining the abnormal properties of red cell membranes in some patients with HE. Studies are currently in progress to test this hypothesis.

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REFERENCES

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