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Research Article

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Missing Band 7 Membrane Protein in Two Patients with High Na, Low K Erythrocytes

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ABSTRACT We investigated the erythrocyte membrane proteins of two patients with congenital hemolytic anemia due to increased permeability of the erythrocyte membrane to Na and K (hereditary stomatocytosis and cryohydrocytosis). One-dimensional sodium dodecyl sulfate (SDS) gel electrophoresis resolved the band 7 erythrocyte membrane proteins into three components with approximate molecular weights of 30,000, 28,000, and 26,000. The 28,000-dalton component was decreased in both patients with permeability disorders. Two-dimensional electrophoresis (nonequilibrium pH gradient electrophoresis in the first dimension combined with SDS gel electrophoresis in the second dimension) resolved the 28,000-dalton component from normal erythrocyte membranes into two proteins with different isoelectric points, designated 22×8 and 60×8 . In the patients with hereditary stomatocytosis and cryohydrocytosis, 22×8 was completely absent, whereas 60×8 was detected as usual. In contrast, all the band 7 proteins (including 22×8) were invariably present in a survey of normal subjects and reticulocytosis controls. The unique finding of a missing band 7 protein in the patients with hereditary stomatocytosis and cryohydrocytosis raises the possibility that the absence of this protein is responsible for the increased Na and K permeability in these disorders.

INTRODUCTION

In patients with the various forms of hereditary stomatocytosis and hemolytic anemia, erythrocyte permeability to Na and K is elevated 2- to 50-fold above normal (1). Although these permeability disorders are uncommon, they provide an important opportunity to learn how the erythrocyte membrane controls the passive fluxes of Na and K. Available evidence suggests

that alterations of the erythrocyte membrane proteins may be responsible for the Na and K permeability disorders (2-4). In a previous report, we described a decrease in band 7 on SDS gel electrophoresis¹ of the erythrocyte membrane proteins of one affected patient (3). In the present study, we used two-dimensional electrophoresis to resolve band 7 into four distinct proteins. In two patients with permeability disorders, one of the four band 7 proteins (designated protein 22×8) was completely absent, raising the possibility that the absence of this protein is responsible for the increased Na and K permeability in these disorders.

METHODS

Preparation of erythrocyte membranes. All steps were performed at 4°C, except that erythrocytes from the patient with cryohydrocytosis were washed at 25°C because they hemolyzed at 4°C (6, 7). Heparinized blood was washed three times with 0.154 M NaCl, and the leukocytes were removed by aspiration. Membranes were prepared by hypotonic lysis in 5 mM sodium phosphate, pH 8.0 (5P8),² as described by Dodge and colleagues (8). Unwashed membranes and supernatants were saved after the hypotonic lysis, and membranes were also saved after three or six washes in 5P8. Membranes and supernatants were frozen at -70°C until analyzed. Supernatants were concentrated in an ultrafiltration cell with a YM10 membrane (Amicon Corp., Danvers, MA) before electrophoresis.

Extraction of membranes. Membranes were washed three times with 5P8 and then extracted at 4°C with either nonionic detergent, low ionic strength buffer, or alkali. Nonionic detergent extraction was performed as described by Yu and co-workers (9) with 0.5% Triton X-100 in 56 mM sodium borate, pH 8.0 for 20 min. For the low ionic strength extraction, membranes were dialyzed against 0.1 mM sodium phosphate, 0.1 mM ethylenediamine tetraacetic acid, and 0.2 mM phenylmethylsulfonyl fluoride at pH 8.0 for 20

¹ The protein bands were numbered according to Fairbanks and co-workers (5).

² *Abbreviations used in this paper:* 5P8, 5 mM sodium phosphate, pH 8.0; IEF, isoelectric focusing; NEPHGE, nonequilibrium pH gradient electrophoresis.

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h (procedure modified from Liu and Palek [10]). Membranes were also extracted with 0.1 N NaOH for 15 min as described by Steck and Yu (11).

Electrophoresis. One-dimensional SDS gel electrophoresis was performed as described by Laemmli with a 4.5% acrylamide stacking gel and a 5-15% linear acrylamide gradient separating gel (12). Samples were prepared for electrophoresis by mixing 1 vol of membranes with 2 vol of SDS sample buffer (3 g/dl SDS, 0.38 M dithiothreitol, 8 g/dl glycerol, and 0.19 M Tris-HCl, pH 6.8), and then by boiling for 3 min. 50 μ g protein as measured by the Lowry technique (13) was placed in each sample well. The gels were stained with Coomassie Brilliant Blue, and they were calibrated using the known molecular weights of the major erythrocyte membrane proteins (14).

For two-dimensional electrophoresis of basic and acidic proteins, nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension was combined with SDS gel electrophoresis in the second dimension as described by O'Farrell and colleagues (15). We used pH 3.5-10 NEPHGE gels and electrophoresed for 1600 V h. To achieve the best resolution of acidic proteins, isoelectric focusing (IEF) was combined with SDS gel electrophoresis as described by O'Farrell (16). For both NEPHGE and IEF, \sim 160 μ g protein was loaded on the gel. For the second dimension separation, the SDS stacking and separating gels and the stain were the same as described above.

To identify spot positions on NEPHGE-SDS gels, a coordinate system was established with the origin at the major glucose-3-phosphate dehydrogenase spot. As shown in Fig. 3, spot positions are given as the horizontal (left to right) X vertical (top to bottom) distances in millimeters from the origin. In all the figures, the basic end of the first dimension separation is on the left and the acidic end is on the right.

RESULTS

Hematologic features. We evaluated two patients with congenital hemolytic anemia due to increased permeability of the erythrocyte membrane to Na and K. The hematologic features of the patients are summarized in Table I and are described in detail elsewhere (6, 7, 17). Briefly, both patients had high Na, low K erythrocytes, whereas only the patient with cryohydrocytosis demonstrated *in vitro* lysis of erythrocytes exposed either to the cold (4°C) or to alkaline pH. Both patients exhibited compensatory increases in active cation transport and glycolysis.

One-dimensional electrophoresis of band 7 erythrocyte membrane proteins. In a previous report, we noted a decrease in band 7 on SDS gel electrophoresis of the erythrocyte membrane proteins of the patient with hereditary stomatocytosis (3). In the present study, we used the discontinuous SDS gel system described by Laemmli in order to resolve band 7 into three components with approximate molecular weights of 30,000, 28,000, and 26,000. As shown in Fig. 1, the 28,000-dalton component of band 7 was decreased in the patients with hereditary stomatocytosis and cryohydrocytosis. Further elucidation of this abnormality required two-dimensional electrophoresis, since two proteins of like molecular weight but different isoelectric point are superimposed on SDS gel electrophoresis to form the 28,000-dalton component.

Two-dimensional electrophoresis of band 7 proteins of normal subjects and reticulocytosis controls. We used two techniques of two-dimensional electrophoresis, nonequilibrium pH gradient electrophoresis combined with SDS gel electrophoresis (NEPHGE-SDS), and isoelectric focusing combined with SDS gel electrophoresis (IEF-SDS). As shown in Fig. 2A, NEPHGE-SDS of unwashed membranes resolved six proteins with molecular weights corresponding to the band 7 region. Washing the membranes with 5P8 removed the cytoplasmic proteins 16 \times 11 and 20 \times 10 (compare A and C in Fig. 2). On the other hand, the membrane proteins 8 \times 10, 22 \times 8, 60 \times 8, and 61 \times 7 persisted undiminished after six washes (Fig. 2C). Because NEPHGE-SDS surveys the overall pattern of proteins while IEF-SDS resolves proteins with isoelectric points in the pH range 4-7, only the two most acidic band 7 proteins (60 \times 8 and 61 \times 7) observed on NEPHGE-SDS were also resolved by IEF-SDS (not shown).

The band 7 proteins resolved by one- and two-dimensional electrophoresis were correlated by means of a superimposition gel. In this technique, membrane proteins are solubilized in the agarose, which secures the NEPHGE or IEF gel to the top of the second di-

TABLE I
Hematologic Data

	HGB	MCHC	Reticulocytes	Mean osmotic fragility (fresh)	Cations			GSH	Cold hemolysis
					Na	K	Total		
	g/dl	g/dl	%	% saline	meq/10 ¹³ rbc			mg/dl	
Stomatocytosis*	11	30	11-21	0.53	74	67	141	70	No
Cryohydrocytosis	11	34	3-30	0.47	39	60	99	51	Yes
Normal	12-15	32-36	1-2	0.40-0.45	5-10	80-90	85-100	64-79	No

* Postsplenectomy.

Abbreviations used in this table: HGB, hemoglobin; MCHC, mean corpuscular hemoglobin concentration; rbc, erythrocyte.

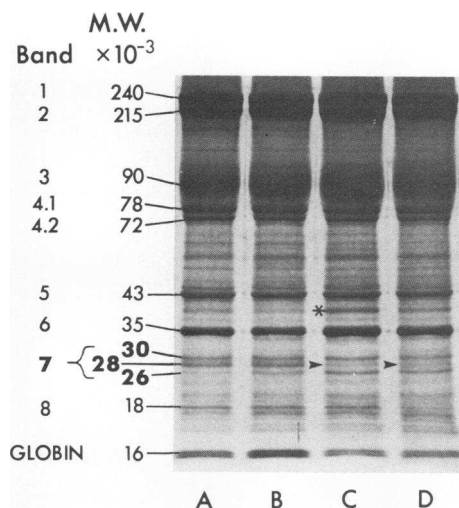


FIGURE 1 One-dimensional SDS gel electrophoresis of the erythrocyte membrane proteins. Band 7 is resolved into three components with approximate molecular weights of 30,000, 28,000, and 26,000. The 28,000-dalton component is present in the electrophoretic patterns of the normal subject (A, 1% reticulocytes) and the reticulocytosis control (B, 14% reticulocytes, a postsplenectomy patient with glucose-6-phosphate dehydrogenase deficiency and chronic hemolysis). The 28,000-dalton component is present but diminished (arrows) in the electrophoretic patterns of the patients with cryohydrocytosis (C, 19% reticulocytes) and hereditary stomatocytosis (D, 21% reticulocytes, postsplenectomy). The increase in the 26,000-dalton component of band 7 seen in the reticulocytosis control (B) and in both patients (C and D) is a feature of young erythrocyte age. The patient with cryohydrocytosis (C) also showed an increase in a 38,000-dalton band (asterisk) that was not observed in the reticulocytosis controls. M. W., molecular weight.

mension SDS gel. The resultant gel displays lines (depicting the one-dimensional pattern) that intersect spots (depicting the two-dimensional pattern) of the same molecular weight (Fig. 2B). The correlation of one-dimensional SDS gel electrophoresis and two-dimensional NEPHGE-SDS gel electrophoresis is presented schematically in Fig. 3. Of particular note, the SDS migration of proteins 22×8 and 60×8 is identical to the 28,000-dalton component of band 7 (Fig. 2B and 3).

The extraction of the band 7 proteins was studied to characterize them as integral or peripheral membrane proteins. Normal membranes were extracted with either nonionic detergent, low ionic strength buffer, or alkali, and then the extracts and the residues were analyzed by one- and two-dimensional electrophoresis (gels not shown). All the band 7 proteins were present in the Triton residue. Trace amounts of proteins 60×8 and 61×7 were also detected in the Triton extract. After low ionic strength extraction, proteins 8×10 and 22×8 were found exclusively in the

residue, whereas proteins 60×8 and 61×7 were seen only in the extract. Only proteins 22×8 and 61×7 were clearly resolved on the NEPHGE-SDS gels of the alkali extracts and residues. Protein 22×8 remained exclusively in the alkali residue, whereas protein 61×7 was present only in the alkali extract. In general, proteins 8×10 and 22×8 were not solubilized by the extraction media, whereas proteins 60×8 and 61×7 extracted with the peripheral membrane proteins.

To detect possible variations in the pattern of the band 7 proteins in the population, we surveyed 5 normal subjects, 2 patients with hereditary xerocytosis, and 27 patients with erythrocyte disorders other than primary permeability defects. All the band 7 proteins (8×10 , 22×8 , 60×8 , and 61×7) were invariably present on the NEPHGE-SDS gels of these control subjects. The amount of protein at each spot position on the two-dimensional gels was not quantitated. We studied the following patients (the number of patients and the percentage of reticulocytes are indicated in parenthesis): hereditary xerocytosis (two patients, 11%, 16%), hereditary spherocytosis presplenectomy (one patient, 11%), hereditary spherocytosis postsplenectomy (two patients, 1%, 1%), hereditary pyropoikilocytosis presplenectomy (one patient, 6%), hereditary pyropoikilocytosis postsplenectomy (two patients, 1%, 3%), Rhnnull phenotype (one patient, 21%), hemoglobin SS disease (two patients, 21%, 1%), hemoglobin SC disease (one patient, 2%), hemoglobin CC disease (one patient, 1%), hemoglobin H disease (one patient, 2%), β -thalassemia major pretransfusion therapy (one patient, 3%), iron deficiency anemia on therapy (one patient, 3%), glucose-6-phosphate dehydrogenase deficiency with chronic hemolysis postsplenectomy (two patients, 28%, 24%), glucose-6-phosphate dehydrogenase deficiency with hemoglobins Constant Spring and E trait (one patient, 8%), pyruvate kinase deficiency (one patient, 11%), traumatic cardiac hemolytic anemia (one patient, 9%), hemolytic-uremic syndrome of childhood (one patient, 2%), congenital microangiopathic hemolytic anemia and thrombocytopenia postsplenectomy (one patient, 1%), Fanconi's aplastic anemia off therapy (one patient, 1%), Bartter's syndrome (five patients, 1-2%).

Two-dimensional electrophoresis of band 7 proteins in hereditary stomatocytosis and cryohydrocytosis. Unlike the above control subjects who displayed a constant pattern of band 7 proteins, the patients with hereditary stomatocytosis and cryohydrocytosis presented a unique finding. As shown by the NEPHGE-SDS gels in Fig. 2E and F, both patients with permeability disorders completely lacked protein 22×8 . We considered that this finding might be due to proteolysis. Therefore, membranes were prepared after leukocytes were exhaustively removed by both cotton

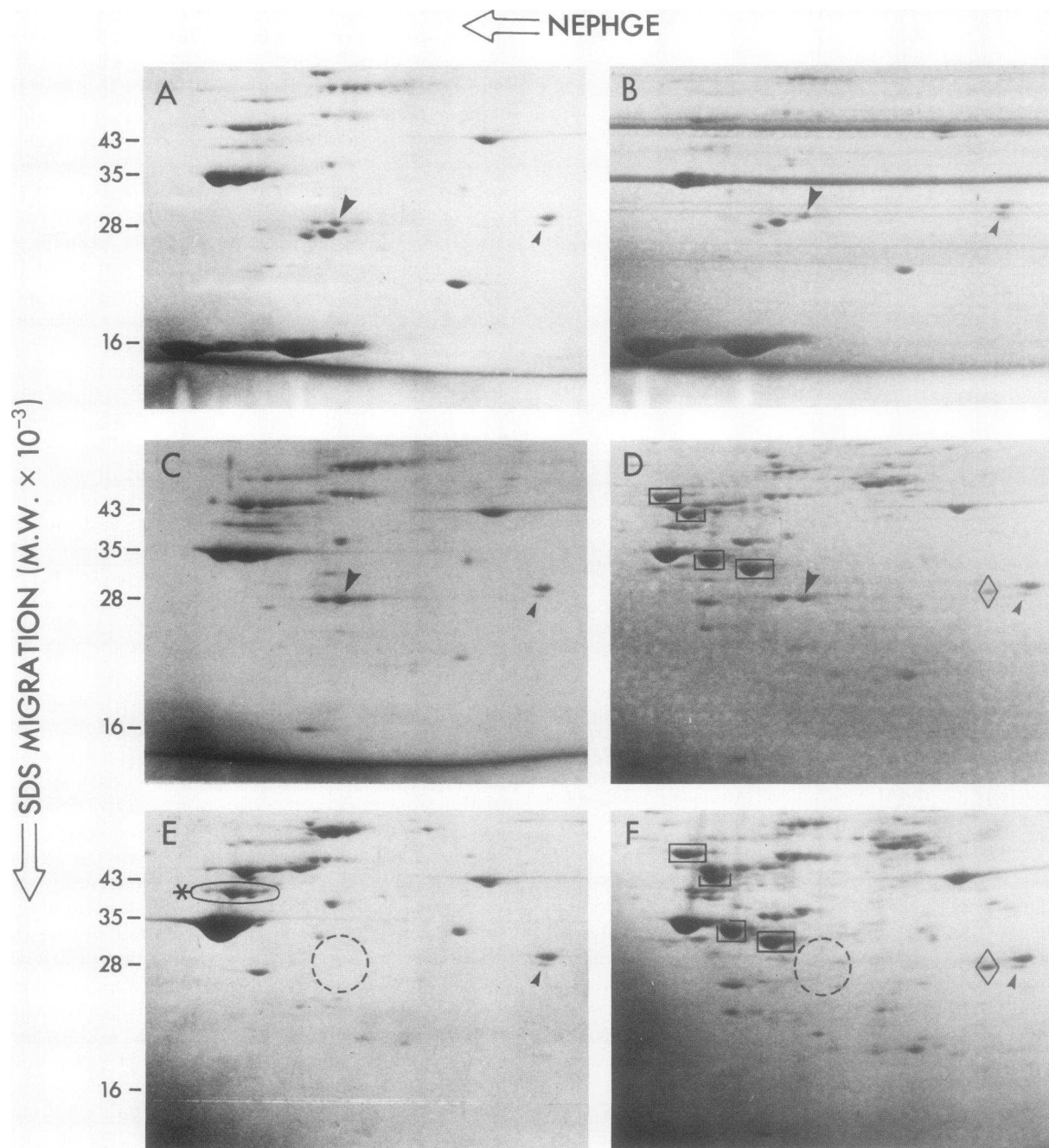


FIGURE 2 Two-dimensional NEPHGE-SDS gel electrophoresis of the erythrocyte membrane proteins. Only the low molecular weight region of the gels is shown. A is the electrophoretic pattern of unwashed membranes from a normal subject. The proteins 22×8 (large arrow) and 60×8 (small arrow) have the same SDS migration but different isoelectric points. B is a superimposition gel formed by solubilizing erythrocyte membranes in the agarose, which secures the NEPHGE gel to the top of the second dimension SDS gel. The lines depict the one-dimensional pattern of normal unwashed membranes, and the spots depict the two-dimensional pattern of normal unwashed membranes. Proteins 22×8 (large arrow) and 60×8 (small arrow) intersect the line formed by the 28,000-dalton component of band 7, and therefore these two proteins constitute the 28,000-dalton component on one-dimensional electrophoresis. Proteins 22×8 (large arrows) and 60×8 (small arrows) are present in the electrophoretic patterns of washed membranes from a normal subject (C) and a reticulocytosis control (D, same patient

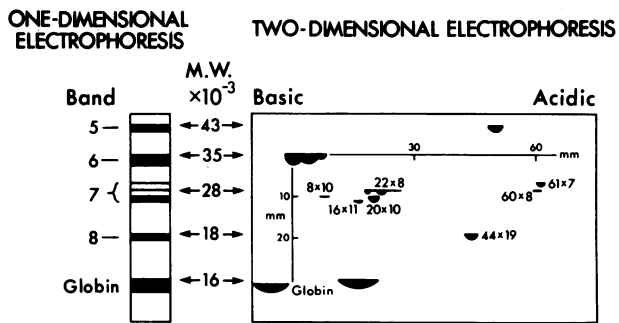


FIGURE 3 Schematic correlation of one-dimensional SDS gel electrophoresis and two-dimensional NEPHGE-SDS gel electrophoresis of normal unwashed erythrocyte membranes. Spot positions are identified on NEPHGE-SDS by a coordinate system with the origin at the major glucose-3-phosphate dehydrogenase spot. Protein 61×7 corresponds to the 30,000-dalton component of band 7. Proteins 22×8 and 60×8 are superimposed on one-dimensional electrophoresis to form the 28,000-dalton component of band 7. Proteins 8×10 , 16×11 , and 20×10 are superimposed on one-dimensional electrophoresis to form the 26,000-dalton component of band 7, but only protein 8×10 persists in washed membranes. Additional proteins are detected if more protein is loaded on the gels.

wool filtration and aspiration, and 0.2 mM phenylmethylsulfonyl fluoride was added to the 5P8. The NEPHGE-SDS gel pattern was unchanged by these precautions against proteolysis. Protein 22×8 was not lost during membrane preparation, since it was not detected in either the unwashed membranes or the supernatants collected after hypotonic lysis of the erythrocytes. Lastly, protein 22×8 was invariably absent from membranes prepared over a 3-yr period (eight separate membrane preparations for the patient with hereditary stomatocytosis and three separate preparations for the patient with cryohydrocytosis).

Although the absence of protein 22×8 was the major and unifying finding in the two patients with permeability disorders, additional features were noted on the one- and two-dimensional gels. The postsplenectomy patient with hereditary stomatocytosis displayed extra protein spots that were also observed in the postsplenectomy control with reticulocytosis (Fig. 2D and F, boxes and diamonds). One of the extra protein spots was present in the band 7 region and was designated protein 52×8 (Fig. 2D and F, diamonds). These extra protein spots were not seen in the non-

splenectomized patient with cryohydrocytosis. In the latter patient, the increase in the 38,000-dalton band seen on one-dimensional electrophoresis (Fig. 1C, asterisk) was confirmed by an increase in the corresponding spot on two-dimensional electrophoresis (Fig. 2E, asterisk). In regard to possible abnormalities of high molecular weight proteins, the NEPHGE-SDS gels were not helpful since proteins $> \sim 60,000$ daltons failed to enter the first-dimension NEPHGE gels. However, the high molecular weight proteins from the patients with permeability disorders appeared normal on one-dimensional SDS gel electrophoresis (Fig. 1) and two-dimensional IEF-SDS gel electrophoresis (not shown).

The band 7 proteins of the family members were analyzed by one-dimensional SDS gel electrophoresis and two-dimensional NEPHGE-SDS gel electrophoresis. All the band 7 proteins (including 22×8) were present in the individuals available for study: the mother of the patient with hereditary stomatocytosis, and both parents and two siblings of the patient with cryohydrocytosis. Protein 52×8 , which we observed only in postsplenectomy samples with reticulocytosis, was not detected in the hematologically normal mother of the patient with hereditary stomatocytosis. The 38,000-dalton band was not increased in the family members of the patient with cryohydrocytosis.

DISCUSSION

Alterations of the erythrocyte membrane proteins have been well documented in congenital hemolytic anemias with abnormal cell shape. Deficiencies or altered interactions of the proteins of the membrane skeleton have been demonstrated in hereditary spherocytosis, hereditary pyropoikilocytosis, and hereditary elliptocytosis. Spectrin is either deficient (18) or fails to bind protein 4.1 (19, 20) in some patients with spherocytosis, whereas spectrin self-association is defective in pyropoikilocytosis (21). In some individuals with elliptocytosis, there is either a deficiency of protein 4.1 (22) or a reduction of the membrane binding sites for ankyrin (23). In three patients with march hemoglobinuria, Banga and colleagues (24) identified a missing band 7 protein on one-dimensional SDS gel electrophoresis. Banga speculated that the absence of this 29,000-dalton protein predisposed the erythrocytes to traumatic disruption during severe exercise.

as in Fig. 1B). In the patients with cryohydrocytosis (E) and hereditary stomatocytosis (F), protein 60×8 (small arrows) is noted as usual, but protein 22×8 is completely absent (circles). In the patient with cryohydrocytosis (E), there is an increase in the spot (asterisk) corresponding to the 38,000-dalton band seen on one-dimensional electrophoresis (Fig. 1C, asterisk). In the postsplenectomy reticulocytosis control (D) and the postsplenectomy patient with hereditary stomatocytosis (F), additional protein spots (boxes and diamonds) are noted which most likely relate to splenectomy and young erythrocyte age. One of these additional proteins (D and F, diamonds) is present in the band 7 region and is designated 52×8 . M. W., molecular weight.

Investigations by ourselves (3) and others (2, 4) have demonstrated several erythrocyte membrane protein abnormalities in hereditary stomatocytosis. The absence of protein 22 × 8 was the major and unifying finding in our two patients with permeability disorders, but other features were also noted. For example, our postsplenectomy patient with hereditary stomatocytosis demonstrated an extra protein spot in the band 7 region which was designated 52 × 8 (Fig. 2F, diamond). Protein 52 × 8 has approximately the same isoelectric point and molecular weight as aberrant protein described by Bienzle and co-workers (2) in one of two patients with hereditary stomatocytosis. Bienzle examined Triton extracts of erythrocyte membranes by means of two-dimensional IEF-SDS gel electrophoresis. Although Bienzle concluded that the aberrant protein was unique to stomatocytosis, we found a protein similar to that described by Bienzle in both our stomatocytosis patient and the postsplenectomy control with reticulocytosis (52 × 8, Fig. 2D and F, diamonds). Thus, our evidence suggests that this extra band 7 protein is related to splenectomy and young cell age and is not specifically associated with stomatocytosis. The existence of protein 22 × 8 in Bienzle's two cases cannot be ascertained, since this protein is not solubilized by Triton and is not resolved by IEF-SDS gel electrophoresis. Other investigators have studied patients with hereditary stomatocytosis using one-dimensional SDS gel electrophoresis alone. Miller and colleagues (25) found a normal electrophoretic pattern in three affected siblings. More recently, Schroter and co-workers (4) reported an absent protein in the band 2 region in a single patient.

The mode of inheritance of the primary permeability disorders is unknown, but the available evidence is consistent with autosomal dominant transmission (1). In the family with cryohydrocytosis described by Miller and colleagues (26, 27), the proband and his son had hemolytic anemia, but the parents and the sister of the proband were hematologically normal. Our family studies of hereditary stomatocytosis and cryohydrocytosis showed that all of the band 7 proteins were present in the parents and the siblings available for study. However, because our two-dimensional analysis of the erythrocyte membrane proteins was not quantitative, the possibility of a reduced amount of protein 22 × 8 in the parents cannot be excluded. Because only a single individual is affected in the pedigrees we studied, no inferences regarding inheritance can be made.

The absence of protein 22 × 8 in our patients with permeability disorders raises the possibility that this protein performs a transport function in normal erythrocytes. Studies of erythrocyte Na and K permeability

indicate a prominent role for proteins, but no specific membrane protein has been linked to permeability. Sutherland and co-workers (28) and Knauf and Rothstein (29, 30) demonstrated that chemical modification of sulfhydryl and amino groups of membrane proteins alters cation permeability. We corrected the permeability defect in hereditary stomatocytosis by treating the erythrocytes *in vitro* with dimethyl adipimidate, a bifunctional imidoester that reacts with the free amino groups of proteins and aminophospholipids (3, 31). Although we have not demonstrated a causal relationship between protein 22 × 8 and erythrocyte Na and K permeability, such a relationship would be analogous to the role established for band 3 as the anion exchange protein (32).

The membrane organization of the band 7 proteins is unique. As pointed out by Yu and colleagues (9) and Steck and Yu (11), band 7 cannot be classified as an integral or a peripheral membrane protein. These two classes of membrane proteins are solubilized by non-ionic detergent (Triton) and protein perturbants (low ionic strength buffer and alkali), respectively. Steck found that band 7 (resolved as a single band on one-dimensional SDS gel electrophoresis) was not readily extracted from the erythrocyte membrane by either nonionic detergent or the protein perturbants. In agreement with Steck's observations, we found that two of the band 7 proteins (8 × 10 and 22 × 8) resolved by NEPHGE-SDS gel electrophoresis were not solubilized by any of the extraction media. On the other hand, the remaining two band 7 proteins (60 × 8 and 61 × 7) were extracted by the protein perturbants along with the other peripheral membrane proteins.

Protein-protein interactions of band 7 have been recognized. For example, dimers and trimers of band 7 are found when membranes are cross-linked with dimethyl-3,3'-dithiobispropioninimidate (33). Also, band 7 is accessible on the cytoplasmic surface of the membrane and can be phosphorylated (34). Another erythrocyte protein, cylindrin, may serve as a model for the band 7 interactions (35-38). Cylindrin is a macromolecule isolated from both the erythrocyte membrane and cytosol. It has a molecular weight of 747,000 and has a hollow cylinder appearance on transmission electron microscopy. As shown by Malech and Marchesi, cylindrin is composed of five subunits with molecular weights ranging from 21,500 to 29,000 daltons (38). Although the cylindrin subunits and the band 7 proteins have similar molecular weights, there is no known relationship between them. Nevertheless, it is conceivable that the band 7 proteins, like the cylindrin subunits, are assembled in the erythrocyte in a macromolecular complex. If such a band 7 complex spanned the membrane to form a Na and K perme-

ability channel, the absence of one of the band 7 proteins might then lead to a permeability defect.

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