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RED CELL MEMBRANE IN HEMOLYTIC DISEASE

STUDIES ON VARIABLES AFFECTING ELECTROPHORETIC ANALYSIS

NANCY SAUBERMAN [a,*], NORMAND L. FORTIER [a], GRANT FAIRBANKS [b], RONALD J. O'CONNOR [a] and L. MICHAEL SNYDER [a]

[a] *Hematology Division, St. Vincent Hospital, Worcester, MA and* [b] *Worcester Foundation for Experimental Biology, Shrewsbury, MA (U.S.A.)*

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Summary

Significant alterations in the spectrin: band 3 and band 4.1a : band 4.1b ratios and an occasional decrease in the peak height of band 4.2 with respect to band 4.1 were found in electrophoretic patterns of red cell membranes from patients with hereditary xerocytosis. Electrophoretic comparison of whole cell, cytoplasm and membrane polypeptides implied that atypical partitioning at hemolysis could account for some, but not all, of the alterations seen in membrane patterns of xerocytes. A decrease in band 4.2 peak height as well as a variation in the profile of band 3 were produced in controls by specific manipulations of the electrophoresis protocol. Metabolic depletion of normal cells produced the type of alterations in bands 3 and 4.1 found in xerocyte membranes, whereas Heinz body production, addition of calcium to the hemolysis buffer and incubation of membranes in detergent under conditions designed to promote proteolysis did not. The presence of a higher peak height of band 4.1b with respect to that of band 4.1a in membranes of patients with various other red cell disorders correlated with an increase in the percentage of reticulocytes in peripheral circulation. The appearance of both band 3 and 4.1 abnormalities in the patterns of control cells which had been enriched in young cells by density gradient centrifugation suggested that these alterations in hemolytic disease are related to the predominance of young cells in the population.

* To whom correspndence should be addressed.
Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Introduction

Hereditary xerocytosis is a congential hemolytic anemia associated with the appearance of large, flat, dehydrated red cells in peripheral circulation [1,2]. At least one lesion of this disease exists in the membrane of these cells since they possess an enhanced permeability to potassium cations. Even though the sodium-potassium pump functions at a rate several times that of the normal, it is still not able to compensate for the cation leakage of these cells, hence their dehydration.

High resolution electrophoretic techniques have been very successful in detecting protein changes in membranes of bacteria [3,4] and virus-transformed mammalian cells [5]. In the case of known congenital functional disorders of the red cell, however, no electrophoretic anomalies in membrane proteins have been found consistently, even where chemical analysis has demonstrated variations in lipid, protein or carbohydrate content. The potential use of this technique for resolving minor variations in proteins has been demonstrated by de Jong et al. [6] who report that single amino acid substitutions can significantly alter electrophoretic mobility by affecting SDS-binding properties of the protein.

In the present study we used two different gel systems to verify the presence of several alterations in the electrophoretic pattern of xerocyte membranes. We investigated some possible sources of artifacts which have been used to account for discrepancies between membrane patterns obtained in different laboratories. The age and metabolic stability of erythrocytes in addition to retention of cytoplasmic polypeptides were evaluated as possible contributors to the electrophoretic alterations consistently seen in xerocyte membranes.

Methods

Preparation of membranes. Blood was collected in heparinized tubes and kept on ice no more than 1 h before being processed further. Cells were washed in Tris/saline (10 mM Tris/base, 150 mM NaCl adjusted to pH 7.35 with HCl at 25°C or pH 8 at 4°C) and hemolyzed in 10 mM Tris buffer (pH 8) according to the procedure of Fairbanks et al. [7]. Other buffers used in place of Tris in specified portions of this study include borate (1.83 mM $Na_2B_4O_7$, 28.66 mM H_3BO_3, pH 8, sodium phosphate (5 mM, pH 8), sodium Hepes (20 mM, NaOH to pH 7.5) and ammonium bicarbonate (125 mM NH_4Cl, 25 mM $NaHCO_3$, pH 7.7). The ratio of washed, packed cells to the volume of buffer at hemolysis was 1 : 30 unless otherwise indicated. In one experiment to determine the effect of hemolysis ratio on the xerocyte electrophoresis pattern, 1 : 10, 1 : 70 and 1 : 100 ratios were also used.

Xerocyte and control membranes were prepared using several alternative washing and hemolyzing solutions to determine whether the xerocyte alterations suggested by their electrophoresis pattern were artifacts of the preparation procedure. Washing solutions include: autologous plasma and Krebs-Ringer bicarbonate [8] ±10 mM glucose. In a separate experiment, 10 mM glucose ±0.25 mM dithiothreitol was added to the regular Tris hemolysis buffer.

Production of Heinz bodies in control cells was accomplished by a modifica-

tion of Jacob and Jandl's procedure [9], in which cells were washed, resuspended to a hematocrit of 10% using glucose (15 mM) in phosphate-buffered saline with 1000 units/ml of glucose oxidase, and incubated at 37°C for 2 h. Heinz bodies were stained and counted according to a modification of Beutler's procedure [10]. After incubation the cells were washed twice with Tris/saline and hemolyzed as described above.

Figure legends describe other manipulations referred to in the text.

Electrophoresis. Unless otherwise specified, electrophoresis of membrane samples was carried out on 6-mm cylindrical gels as described by Laemmli [11]. Some specific details of the electrophoresis protocol used in this laboratory are described here. Fresh samples * were combined (four parts sample to one part solution) with a detergent solution of the following composition: 1.25 M sucrose (Ultra Pure, Schwarz Mann), 0.1 M dithiothreitol (Sigma), 0.25 M Tris-HCl (Sigma), 5% SDS (Ultra Pure, Bio-Rad) and 0.01% bromophenol blue (Bio-Rad). Dissolved samples were then divided and frozen in duplicate (use of a single sample for repeated electrophoretic analyses results in severe background staining and the appearance of high molecular weight aggregates at the top of the gel). Hemolysate and supernatant samples were placed in a boiling water bath for 3 min immediately after addition of the detergent solution to inactivate any proteases present. To these hemoglobin-rich samples, additional dithiothreitol (to 40 mM) was added after cooling but before freezing them.

The lower gel was poured to a height of 8 cm and overlaid with 50 μl of isobutanol at least 12 h prior to use. The isobutanol was thoroughly rinsed off the gel surface with distilled water immediately after polymerization was complete (45 min) leaving only enough water to keep the gel surface moist overnight. (Failure to remove isobutanol resulted in the stained gel having a faint, elliptical band around the outside below spectrin.) Before pouring the 1.5 cm upper gel, the residual water layer was carefully removed with cotton. This gel was overlaid with distilled water and used within 4 h of polymerization. When the effect of gel SDS concentration on the electrophoresis pattern was studied, the lower gel SDS concentration was varied between 0.05 and 1.0% SDS.

Samples to be electrophoresed were removed from the freezer and placed directly in a boiling water bath for 2 min. They were then cooled on ice and made 40 mM in dithiothreitol, assuming all residual dithiothreitol had evaporated or become inactive during boiling. Approximately 25 μg of protein was then applied to each gel. High protein loading (50 μg) was necessary to detect periodic acid-Schiff-positive bands. To avoid the precipitation of SDS and dithiothreitol which occurred when samples were allowed to sit on ice after boiling, samples were warmed for a few seconds and mixed on a Vortex mixer immediately after loading them onto gels.

The tray buffer was a modification of Maizel's Tris/glycine, pH 8.3, system [12]. In addition to 0.05 M Tris and 0.38 M glycine, SDS and ethylenediaminetetraacetic acid (EDTA) were added to a concentration of 0.1% and 2 mM, respectively. The total dissolution of EDTA in this buffer was found to be essential in achieving a pH of 8.3 directly without titration. Both migration

* Ghosts were first diluted 1 : 10 with Tris hemolysis buffer.

speed and resolution of components were found to be sensitive to this factor. The gels were run at 2.24 W/gel (2.8 mA/gel starting current) until the tracking dye had migrated exactly 7.5 cm into the lower gel.

The Coomassie blue and periodic acid-Schiff staining procedures of Fairbanks et al. [7] were used in this study except that Coomassie blue was not included in destaining solutions. When a series of comparative gels were scanned at 525 nm on a Helena Quickscan, Jr., the gain was held constant while the zero knob was adjusted for each gel at the position of lowest background staining. The location of the bromophenol blue tracking dye, indicated by an arrow on each scan, corresponds to a relative migration of 1.0. Relative mobility of polypeptides with respect to the tracking dye is designated on the horizontal axis below each scan. For the quantitative studies shown in Table I, densitometric tracings were divided into the major bands as indicated by arrows at the baseline in Fig. 2 and integrated stain intensities between the divisions were recorded.

Results

Electrophoretic analysis of xerocyte membranes

Membrane samples from xerocyte and control red cells were analyzed by two independent sodium dodecyl sulfate-polyacrylamidel gel electrophoresis techniques: the continuous Tris/acetate/EDTA system of Fairbanks et al. [7] (Fig. 1) and the discontinuous Tris/glycine/EDTA system of Maizel [12] and Laemmli [11] (Fig. 2). The alterations in the region extending from band 3 to 4.2 are most clearly viewed on the latter gel system. It is these that were the

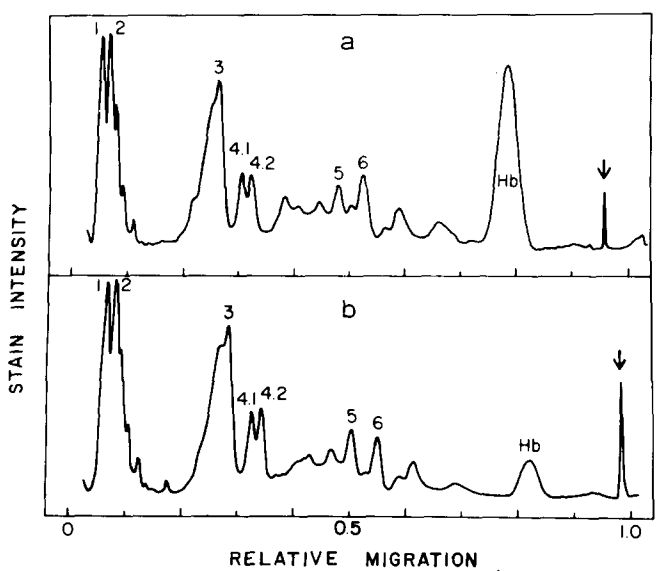

Fig. 1. Densitometric tracings from electrophoretic separation of membrane polypeptides in a continuous SDS gel system. Equal volumes of membrane samples were run on Tris/acetate/EDTA gels according to the procedure of Fairbanks et al. [7] with 0.2% instead of 1.0% SDS in the gel [30]. (a) Xerocyte, and (b) control.

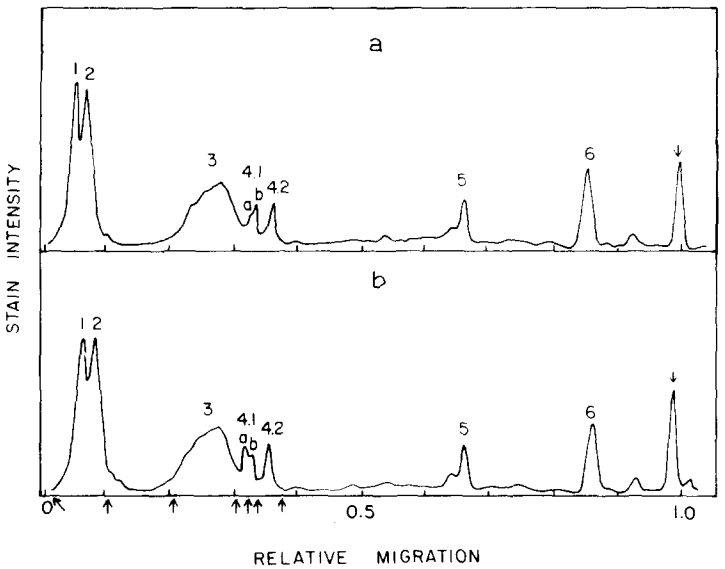

Fig. 2. Densitometric tracings from electrophoretic separation of membrane polypeptides in a discontinuous SDS gel system. Equal volumes of membrane samples were electrophoresed according to a modification of the Maizel-Laemmli method (see Methods). Arrows at baseline depict divisions used in integrating stain intensities of individual bands for Table I. (a) Xerocyte, and (b) control.

main subject of investigation in this study. The elevated amount of glyceraldehyde-3-phosphate dehydrogenase, band 6, in xerocyte membranes has been the subject of a past study [13]. The apparent increase in retention of hemoglobin by xerocyte membranes in Fig. 1 is not typical of ghosts which have been washed three times with hemolyzing buffer.

In the Maizel-Laemmli gel system, band 3 extends over the 90 000—130 000 region [14]. It exhibits significantly different contours in the two different gel systems, having a distinct front and trailing portion in Fig. 1 in contrast to its more symmetrical, diffuse migration in Fig. 2. This profile variation is probably related to differences in sample preparations as is discussed below under Variations in electrophoresis protocol. In both figures, however, the xerocyte band 3 contour may be described as having more distinct shoulders at the higher molecular weight edge than that of the control.

In Fig. 1a, band 4.1 is a single band with a slightly lower peak height than band 4.2, as opposed to the relative heights of the corresponding control bands (Fig. 1b). On the other gel system, the 4.1 band is resolved into two bands previously designated 4.1a and 4.1b by King and Morrison [15]. Xerocyte patterns contrast with controls here in having an elevated 4.1b with respect to 4.1a. However, the 4.2 alteration is not seen on these gels.

In order to quantify the alterations in the 3—4.1 region of xerocyte gels, the relative stain intensities of bands 1—3, 4.1a and 4.1b in discontinuous gels were determined. Table I shows that both spectrin : band 3 and 4.1a : 4.1b ratios in the xerocyte pattern were lower than normal (minor variation between gel runs made a statistical analysis of actual band values impossible).

Periodic acid-Schiff staining of xerocyte hemolysate, supernatant and ghosts

TABLE I

XEROCYTE STAIN INTENSITY RATIOS FROM GELS OF ISOLATED MEMBRANES

Discontinuous electrophoresis (see Methods) was used. The ratios of stain units/band and their standard deviations are followed by the number of separate ghost isolation and the total number of gels used in the calculation. *P*, the probability of having a statistical difference between xerocyte and control values by chance.

Band ratio	Ratio of stain units/band		*P*
	Control	Xeroxyte	
(1 + 2)/3	0.725 ± 0.0999 (7,66)	0.693 ± 0.108 (5,62)	0.09
4.1a/4.1b	2.53 ± 0.657 (7,75)	1.06 ± 0.388 (5,69)	<0.01

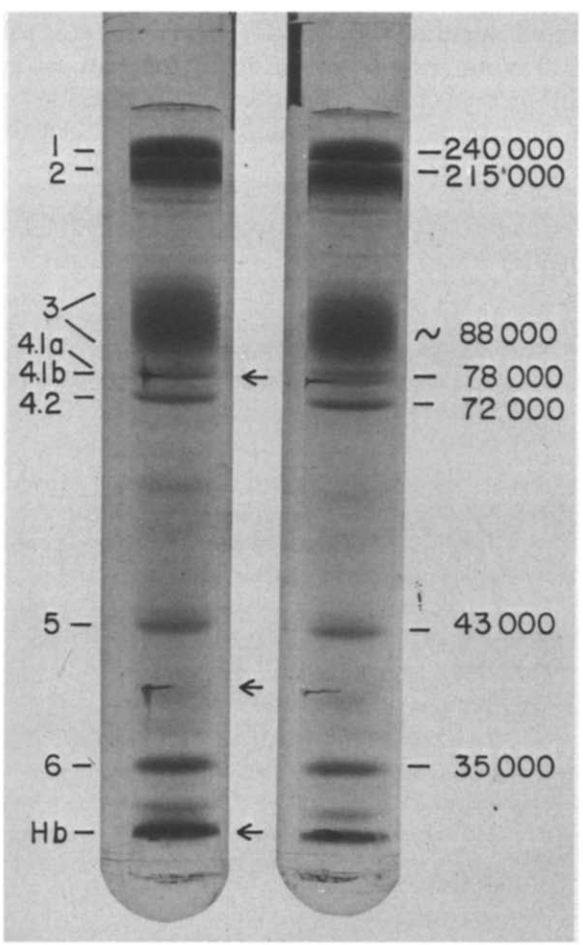

Fig. 3. Discontinuous SDS gel electrophoresis. Arrows indicate the location of periodic acid-Schiff-positive bands which were marked with India ink prior to superstaining with Coomassie blue. (a) Xerocyte, and (b) control.

did not reveal any gross quantitative abnormalities in the three major bands. By marking these bands with ink and superstaining with Coomassie blue, PAS-1 was found to migrate at or slightly below the position of band 4.1b (Fig. 3). However, two independent lines of evidence rule out any PAS contribution to the 4.1b alteration in xerocyte gels: (i) boiling a membrane sample prior to electrophoresis shifts PAS-1 to PAS-2 with no effect on the 4.1b profile and (ii) electrophoretic patterns of low ionic strength membrane extracts and Triton X-100 extraction residues show a 4.1b profile which differs from that of the control even though no PAS-staining components are present (see Solubility of altered polypeptides below).

Distribution of alterations at hemolysis

Electrophoresis of hemolysates and supernatants from xerocyte and control membrane isolations were carried out to determine whether the majority of the polypeptides responsible for the abnormalities seen in bands 3 and 4.1 of the ghosts originated in the cytosol or the membrane. Even prior to fractionation, xerocytes have both quantitative and qualitative differences from the control pattern throughout the band 3—4.2 zone. From inspection of the slab gel in Fig. 4, it appears that the cytosolic proteins make a significant contribution to the xerocyte hemolysate pattern alterations in the band 3 region. In fact, it

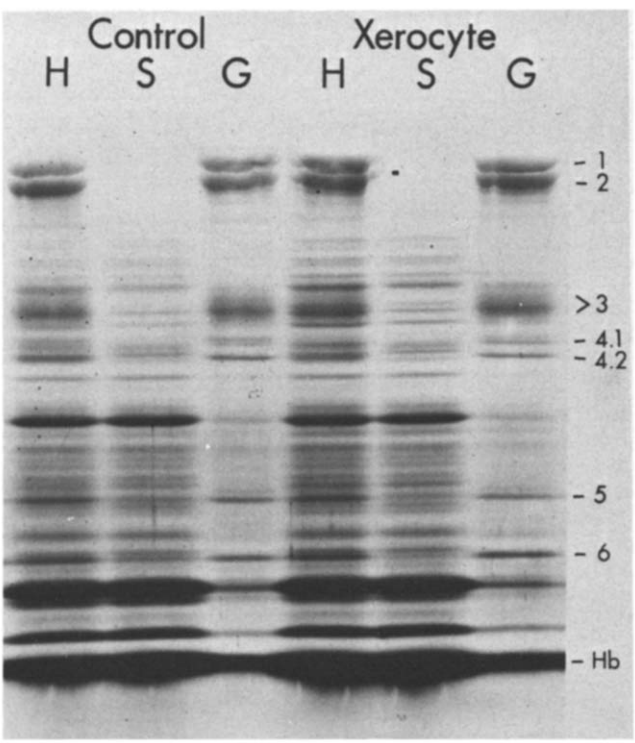

Fig. 4. Electrophoretic comparison of hemolysate (H), supernatant (S) and ghosts (g). Slab gel electrophoresis was carried out according to the procedure described by Laemmli [11] on a 6—8—10% step gradient slab. Sample preparation was that of Fairbanks (Fairbank, G., unpublished results).

seems that the partitioning of certain of these band 3 polypeptides to the supernatant at hemolysis results in the isolated membrane looking much more like the control than the hemolysate does.

In the 4.1 region this is not the case. The degree of 4.1b elevation is preserved during ghost preparation with very little if any of this polypeptide being lost to the supernatant as was also the case for the control.

Solubility of altered polypeptides

In an attempt to detect any gross abnormalities in the mode of binding of altered xerocyte polypeptides to the membrane, the protein contents of supernatants and residues from Triton X-100 and EDTA extractions of xerocyte and control membranes were analyzed by electrophoresis (Figs. 5 and 6). It was anticipated that in control membranes there would be a clear division between two types of proteins: (i) the tightly bound, lipid-soluble proteins preferring the Triton supernatant or the EDTA pellet and (ii) the less tightly bound, 'peripheral' proteins released by low ionic strength treatment but not by Triton. This was indeed the case for most of the polypeptides in the band 3—4.2 region. The majority of band 3 was Triton soluble and EDTA insoluble, as was band 4.2. The small portion of band 3 released from the membrane by EDTA treatment appeared to be that same portion which was detergent insoluble. The resolution of these 4—5 band 3 'satellites' in the high gain scan of the EDTA extract (Fig. 6 inserts) revealed quantitative differences in xerocyte and control patterns.

The solubility of band 4.1 was the least well-defined of any described so far. It was not completely released by low ionic strength extraction and yet it was clearly insoluble in Triton. The differences in 4.1a : 4.1b profile characteristic of xerocyte and control ghost patterns were seen in the EDTA supernatant and pellet as well as the Triton pellet (Figs. 5c, d and 6).

Variations in hemolysis protocol

The protein content of isolated membranes has been shown to depend on the type and pH of hemolysis buffer, and on the ratio of buffer to the volume of packed cells at hemolysis [16—18]. In our hands these variables (see Methods) had no effect on the band 3—4 region of either xerocyte or control membrane gel patterns (data not shown). Washing cells in plasma from normal donors or supplementing the wash buffer with various combinations of glucose, phosphate and dithiothreitol likewise had not effect.

The presence of calcium (3 mM) at hemolysis has been reported to cause a number of changes in the electrophoretic pattern of ghost proteins which have been attributed to proteolysis, retention of cytoplasmic components and the aggregation of certain membrane proteins, including 4.1 [15,19,20]. This treatment affected xerocyte and control membranes equally according to electrophoretic analysis (Fig. 5), producing aggregates at the top of the gel (not visible in scan) and at 180 000 daltons, more pronounced shoulders in band 3, a decrease in both bands 4.1a and 4.1b, an increase in band 4.5, and a nonspecific elevation in background staining over the entire gel. Band 4.2 was not affected by calcium. All of these pattern changes were observed at a lower

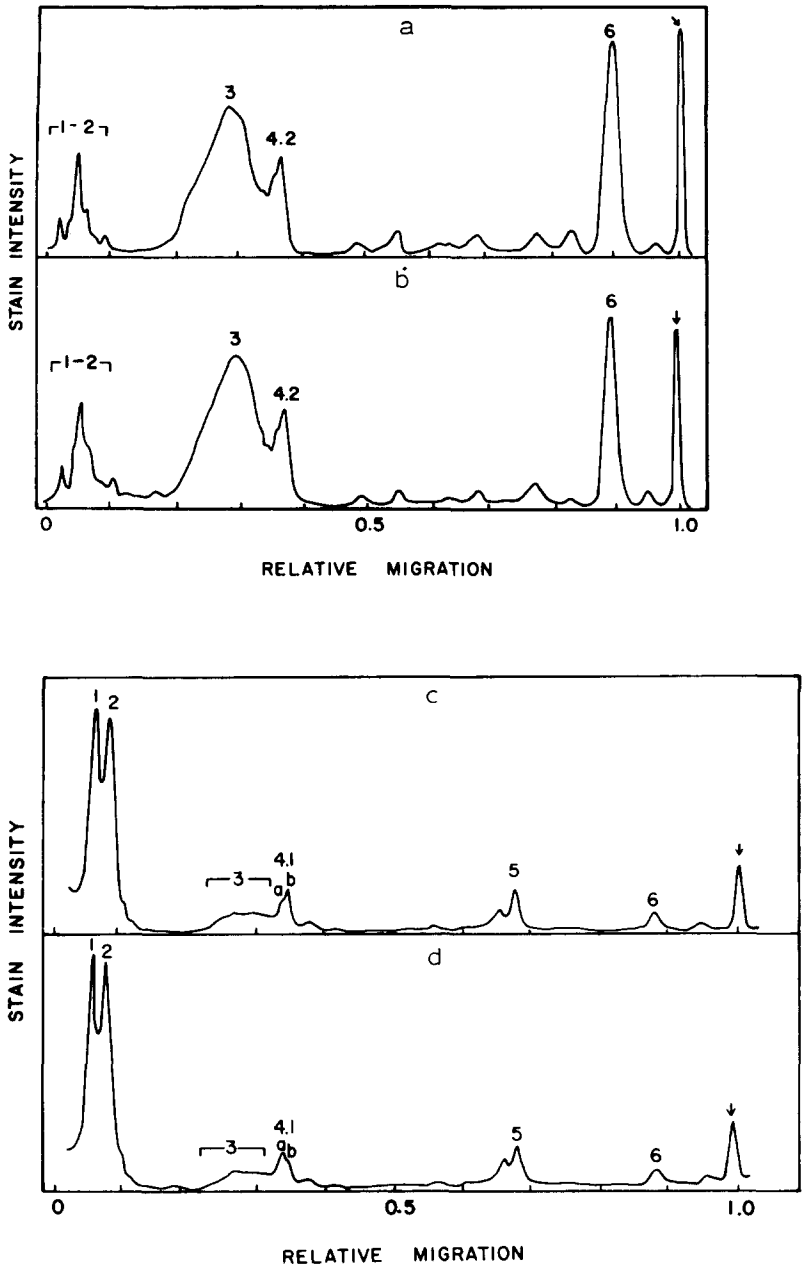

Fig. 5. Triton extraction of xerocyte ghosts. Xerocyte and control ghosts were counted in a Model S Coulter Counter after the addition of 10 ml of Isoton (Scientific Products) to 44.7 μl of packed ghosts. The packed ghosts were then diluted to a concentration of $2.58 \cdot 10^9$ ghosts/ml with hemolyzing buffer and made 2% in Triton X-100 by adding 1 ml of hemolyzing buffer and 0.5 ml of a 20% Triton solution. Following centrifugation at $1.2 \cdot 10^5 \times g \cdot h$, supernatants and pellets were dissolved in the usual SDS solution (see Methods). The pellet was rinsed twice with distilled water, adjusted to 2 ml with hemolyzing buffer and dissolved with 0.5 ml of the SDS solution. The extract (80 μl) and pellet (10 μl) solutions were then applied to the gel. It was necessary to add twice the normal amount of dithiothreitol to the extract solutions after boiling to prevent aggregation. (a) Xerocytes extract; (b) control extract; (c) xerocyte pellet, and (d) control pellet.

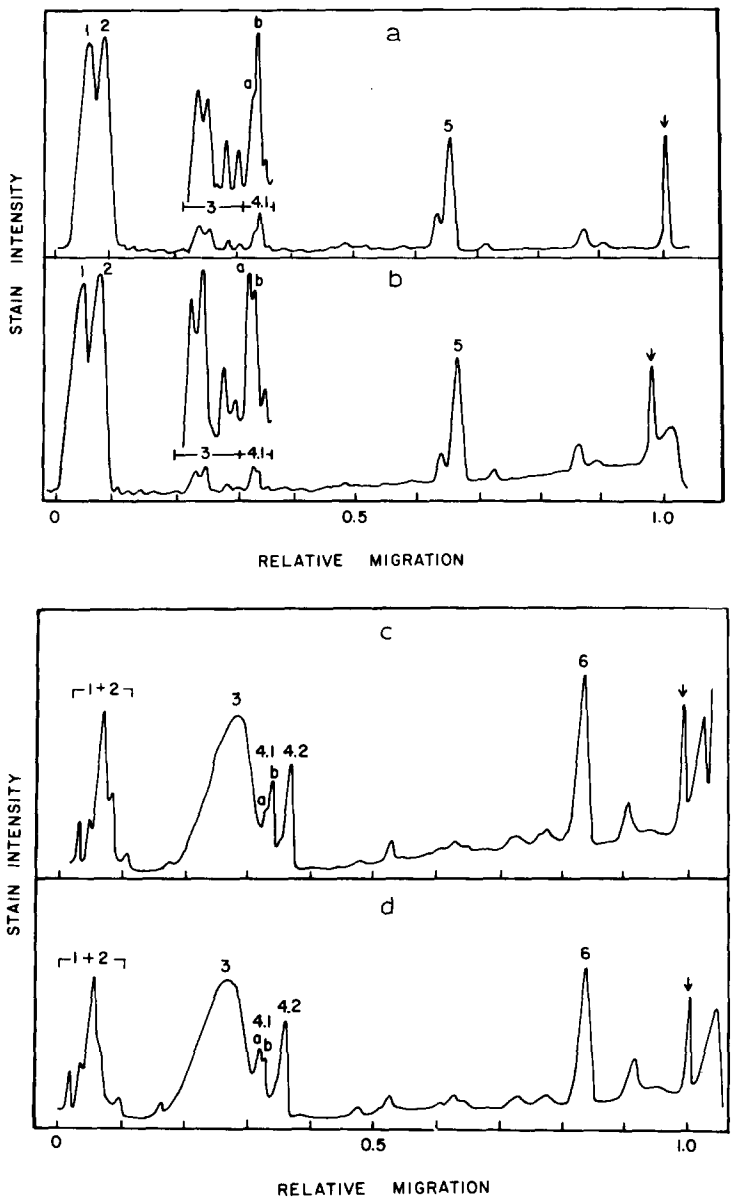

Fig. 6. Low ionic strength extraction of xerocyte ghosts. The low ionic strength extraction procedure of Fairbanks et al. [7] was performed (Tris hemolyzing buffer was substituted for phosphate) and the suspension was passed twice through a 25 gauge needle prior to centrifugation at $1.2 \cdot 10^5 \times g \cdot h$. Gels were loaded with 100 μl of xerocyte (a) or control (b) extracts and 10 μl of xerocyte (c) or control (d) pellets. High-gain scans of extract patterns are shown in inserts.

calcium concentration (1 mM), although to a proportionally smaller degree (data not shown).

Variations in electrophoresis protocol

Sample preparation. Variations in the procedure for preparing electrophoresis

samples had significant effects on the profiles of bands 3, 4.1 and 4.2 in the membrane pattern. When control samples which had been frozen in the usual detergent solution (see Methods) were thawed and applied directly to a gel, the front edge of band 3 had a sharp, vertical contour, 4.1a was equal to 4.1b in peak height and 4.2 was only slightly higher than 4.1 (Fig. 8a). Addition of dithiothreitol to the thawed sample prior to electrophoresis exaggerated the band 3 'front', lowered 4.1b with respect to 4.1a and elevated 4.2 (Fig. 8b).

The unboiled xerocyte sample (Fig. 8a insert) exhibited an even more extreme variation from the normal in bands 3 and 4.1. Addition of dithiothreitol to the unboiled xerocyte sample (Fig. 8b insert) increased 4.2 relative to 4.1 so that these bands more closely resembled the bands in Fig. 2a. As in the control, dithiothreitol addition sharpened the leading edge of band 3.

In Fig. 8c and d, the effect of boiling control samples without and with the subsequent addition of dithiothreitol is shown. After boiling band 3 is approximately symmetrical with a diffuse leading edge extending into 4.1. The hypersharp leading edge is absent from both xerocyte and control patterns (Fig. 8c and d) (xerocyte pattern not shown). Addition of dithiothreitol to samples after boiling, as is the usual protocol in this laboratory, increased or sharpened band 4.2 and 5 and removed the 'fuzzy' background staining in the entire region of the gel above band 3 (Fig. 8d).

Concentration of SDS in gel. Alterations in proteins migrating in the 4.1–4.2 zone have been noted in the electrophoretic analysis of abnormal patients with various hemolytic disorders [21,22]. Bouvin and Galand have pointed out

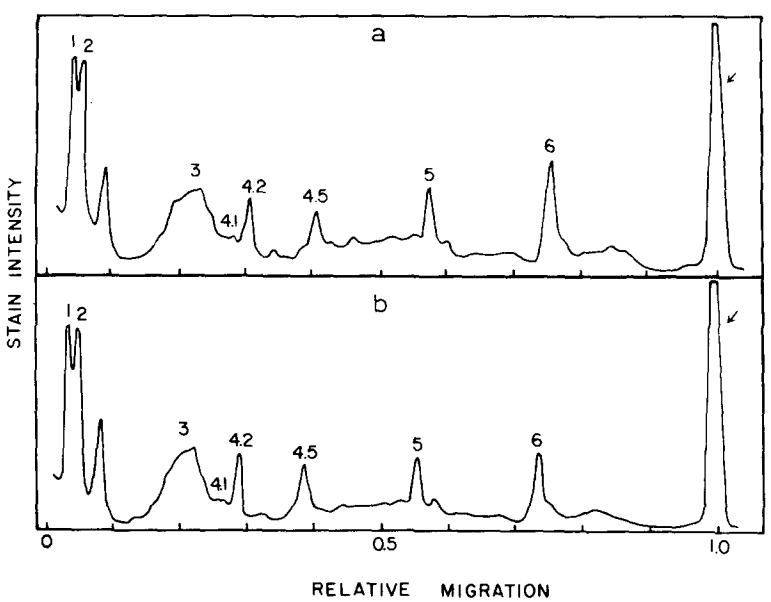

Fig. 7. Effect of hemolysis in the presence of 3 mM calcium. Washed xerocyte and control cells were hemolyzed in Tris hemolysis buffer which had been made 3 mM in calcium. The ghosts were then collected and washed twice in calcium-free buffer. Densitometric tracings of gels of xerocyte (a) and control (b) membranes are shown.

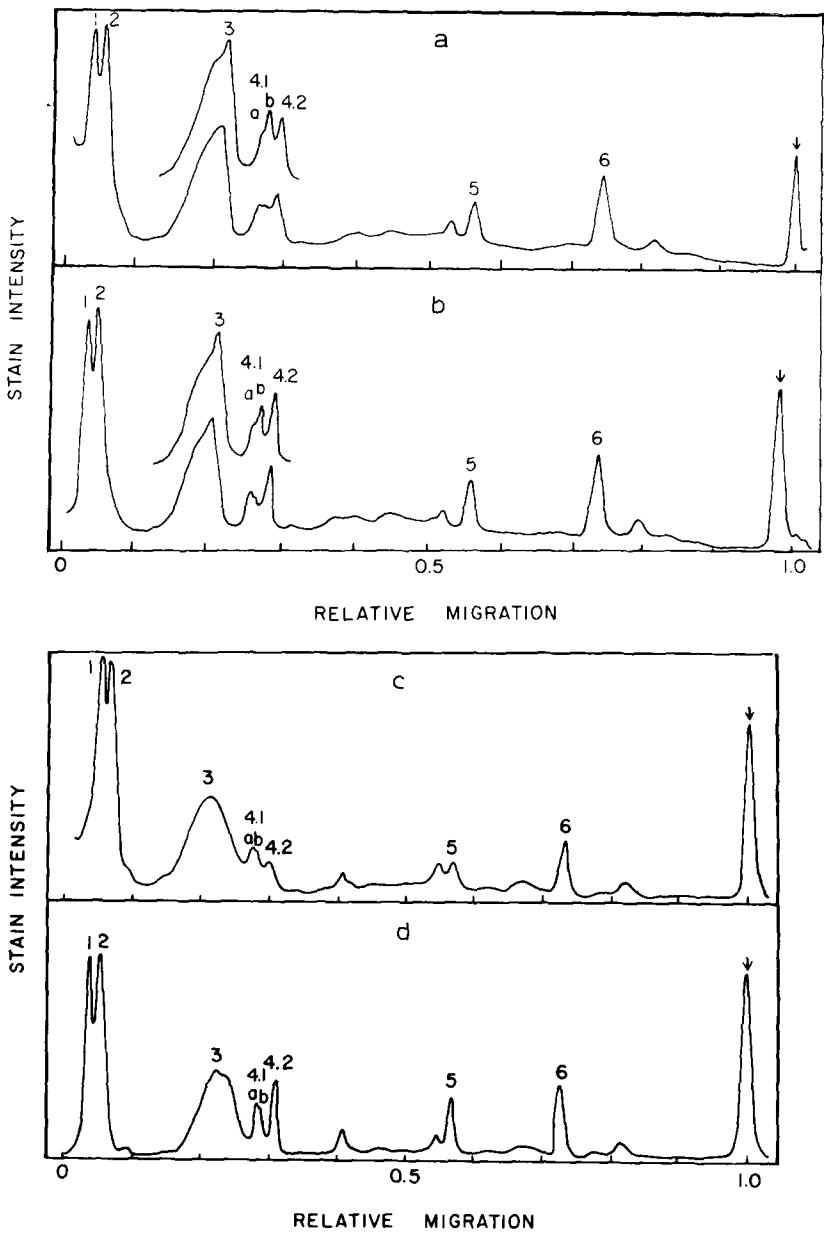

Fig. 8. Effect of altering membrane solubilization protocol. Membrane samples were frozen in the usual detergent solution and then either applied to the gel directly after thawing (a and b) or after boiling (c and d). Additional dithiothreitol (to approximately 40 mM) was added after boiling to samples b and d. Xerocyte tracings are presented as inserts above control patterns in a and b.

that the precise contour of this region in some gel systems is a function of SDS concentration [23]. We found that band 4.2 gradually broadens as the SDS concentration in the gel is increased from 0.1 to 1% SDS, until the 4.1 profile is completely obscured at 1%, the highest SDS concentration (not shown).

Metabolic depletion

Xerocytes were found to lose ATP at least 20% faster than control cells while being washed three times in Tris/saline in preparation for ghost isolation [2,13]. In order to assess the contribution of this phenomenon to the electrophoretic anomalies in the band 3—4 electrophoretic zone, normal and xerocyte cells were stored under sterile conditions for 24 h at 37°C, prior to membrane isolation and electrophoresis. Metabolic depletion appeared to alter the electrophoretic pattern of band 3 in both normal and xerocyte membranes (Fig. 9).

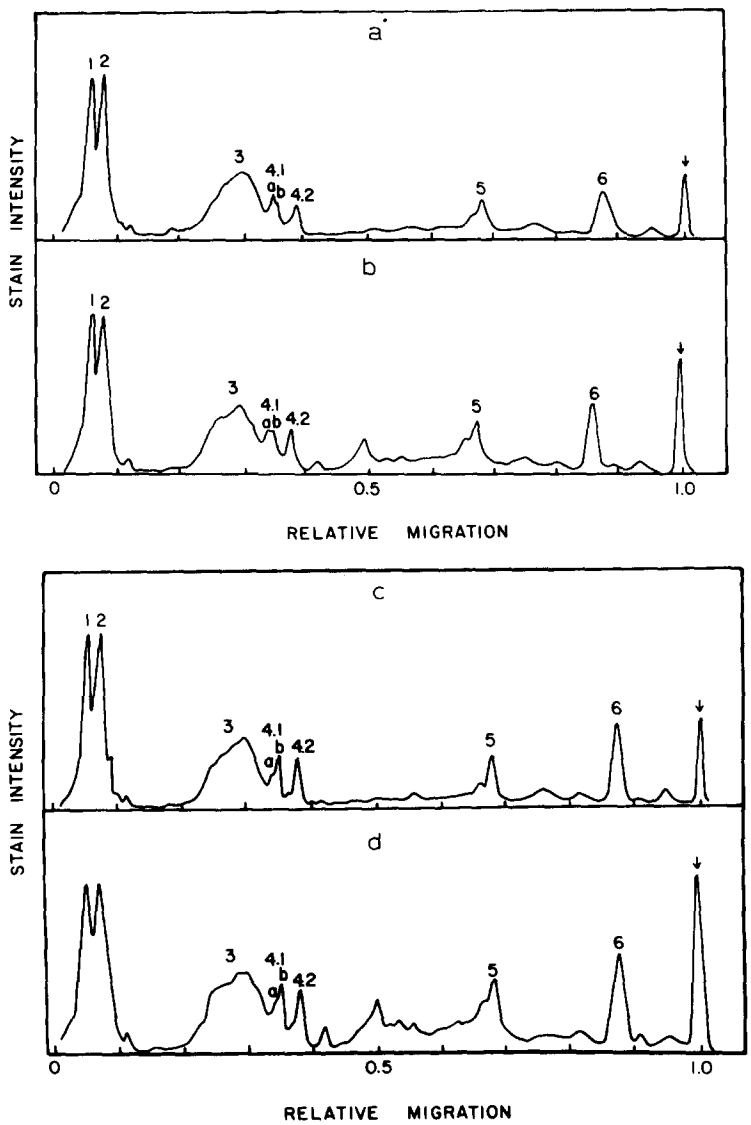

Fig. 9. Effect of metabolic depletion. Cells were washed with modified Krebs-Ringer bicarbonate buffer [8] containing 0.5 mM $CaCl_2$, 20 mM sodium Hepes (pH 7.4) and 2 mg% gentamicin suspended at 10% hematocrit in the same buffer, and incubated at 37°C for 24 h with gentle shaking. Membranes from normal (a) and depleted (b) control cells and from normal (c) and depleted (d) xerocytes.

Patterns from depleted samples had more pronounced shoulders than those from unincubated samples, with the xerocyte leading control in degree of alteration. An elevation in band 4.1b of the depleted control (Fig. 9a and b) was also apparent. However, metabolic depletion, just as cellular ageing, had little if any effect on the peak height of xerocyte 4.1b (Fig. 9c and d).

Oxidation

The peroxide-stimulated production of Heinz bodies, which in intact cells is associated with an increase in permeability to K^+ [24] was also examined for possible effects on the electrophoretic pattern of control membranes. Normal cells were incubated in a glucose oxidase-glucose medium until 30% of the cells had five or more Heinz bodies. Since the isolated membranes prepared from treated cells were identical electrophoretically to those of control cells, except for a two-fold increase in hemoglobin (not shown), we conclude that oxidative stress on normal red cells, sufficient to precipitate hemoglobin onto the membrane surface, had no electrophoretically detectable effect on the migration of bands 3, 4.1, or 4.2.

Proteolysis

When normal and xerocyte ghosts were incubated in 0.1% SDS and 150 mM NaCl for 4 h in an attempt to stimulate endogenous protease activity [7], the band 3 region was noticeably altered and some faint bands appeared below spectrin on the gels (not shown). Incubation of control membranes in 1% SDS for 24 h at 37°C affected the same proteins. More drastic changes in the pattern of control ghosts were made by inclusion of the buffy coat in the 1% SDS incubation medium (Fig. 10a and b). Band 4.2 was significantly reduced by this treatment but 4.1 remained intact.

Other red cell disorders

When membranes of red cells from several known hematological disorders were isolated and electrophoresed, many were found to have an altered band 3 and a higher 4.1b peak height relative to 4.1a. The results shown in Table II suggest that an elevated 4.1b peak height corresponds to an increase in reticulocyte count rather than being specific to any one type of functional disorders *. The level of reticulocytes in circulation may be elevated in hematological disorders involving abnormalities of red cell production and/or destruction. Electrophoresis of ghosts from a hereditary spherocytosis patient before and after splenectomy (Fig. 11a and b) and from a patient with pernicious anemia before and after vitamin B-12 therapy (Fig. 11c and d) enabled us to observe the independent response(s) of the band 4.1 profile to production and destruction.

Prior to splenectomy the hereditary spherocytosis patient was overproducing red cells (18% reticulocytes) as a result of excessive destruction by the spleen

* Band 3 alterations are not given since it has been difficult to determine the normal contour for electrophoretic tracings of ghost samples in this region. Mueller and Morrison also mentioned this difficulty in a paper specifically on the subject of band 3 variants [14].

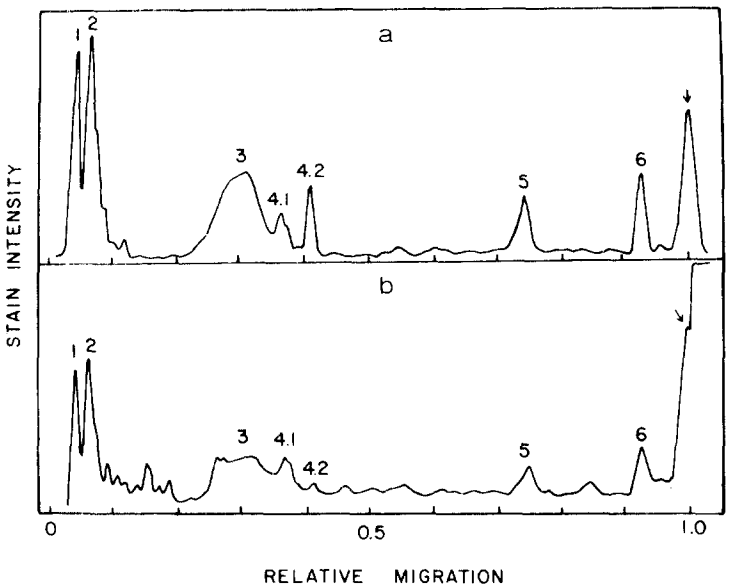

Fig. 10. Effect of incubating ghosts with buffy coat. Red cells were washed without aspirating the buffy coat and ghosts were prepared without aspiration of the button lying beneath the ghosts after centrifugation of the hemolysate. These and ghosts prepared in the usual manner were then incubated at 37°C for 1 h in the electrophoresis detergent solution (see Methods). Control ghosts without (a) and with (b) buffy coat.

TABLE II

COMPARISON OF 4.1b TO 4.1a LEVELS IN MEMBRANES FROM A VARIETY OF RED CELL DISORDERS

The disorders are presented in the order of increasing reticulocyte counts. Total bilirubin is given as an approximation of total red cell destruction occurring in the patient at the time of membrane preparation. All patients had direct bilirubin levels within normal limits indicative of no biliary obstruction. The comparative peak heights of 4.1a and 4.1b are indicated as follows: +, when 4.1b > 3.1a; ±, when 4.1b = 4.1a, and —, when 4.1b < 4.1a. G6PD, glucose-6-phosphate dehydrogenase.

Disorders	Hematocrit (%)	Reticulocyte count (%)	Total bilirubin (mg/100 ml)	4.1b > 4.1a
Control	37—52	0.5—1.5	<1.3	--
Hereditary spherocytosis * (1 month post-splenectomy)	36	0.8	1.0	
Congenital microcytic hypochromic anemia	32	1.8	1.1	—
Pernicious anemia **	15	3.8	4.1	±
Xerocytosis	33	3.8	3.0	+
Paroxysmal nocturnal hemoglobinuria	22	4.0	4.1	+
Hereditary spherocytosis * (1 week post-splenectomy)	33	6.0	1.6	+
Cold agglutinin hemolytic anemia	28	6.5	3.2	+
G6PD deficiency	38	9.0	5.6	+
Autoimmune hemolytic anemia	25	12.5	3.3	+
Idiopathic hemolytic anemia	32	12.5	2.1	+
Pernicious anemia ** (4 days post vitamin B-12)	16	15.0	—	+
Hereditary spherocytosis *	26	18.0	3.4	+

* Hereditary spherocytosis before and after (1 week and 1 month) splenectomy (same patient).

** Pernicious anemia before and after 4 days vitamin B-12 therapy (same patient).

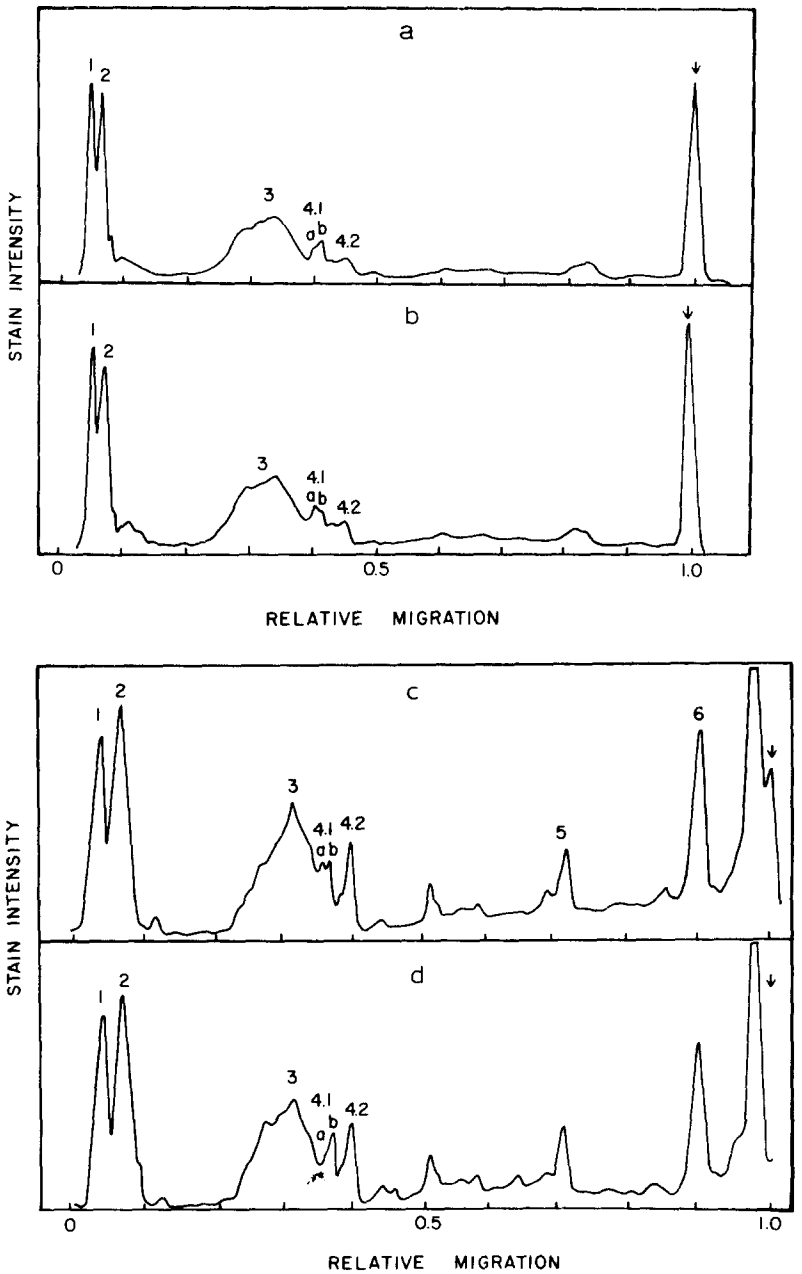

Fig. 11. Effects of splenectomy and B-12 therapy. Membranes isolated from blood drawn from a patient with hereditary spherocytosis before (a) and after (b) splenectomy; and from a patient with pernicious anemia before (c) and after (d) vitamin B-12 therapy.

(total bilirubin = 3.4 mg/100 ml). Although surgical removal of spleen brought the level of destruction to within normal limits within only one week (1.6 mg/100 ml bilirubin), both production (6% reticulocytes) and 4.1b remained elevated. Analysis one month post-splenectomy revealed a normal 4.1 profile

and reticulocyte count (0.8%) (Fig. 11a and b).

In the particular case of pernicious anemia (vitamin B-12 deficiency) studied, excessive production (3.8%) and destruction (4.1 mg bilirubin/100 ml) of red cells was occurring. The elevated 4.1b in the patient's pattern prior to treatment (Fig. 11c) may have stemmed from either abnormality. However, the simultaneous increase in reticulocyte count (to 15%) and 4.1b (Fig. 11d) after four days of vitamin B-12 therapy suggests a positive correlation between 4.1b elevation and red cell production, as was also implied by the data from the hereditary spherocytosis patient described previously.

Age-related alterations

The elevated reticulocyte count of patients with xerocytosis (Table II) is an indication that the mean age of their circulating erythrocytes is younger than that of controls. Since others had noted differences in both the quantity and electrophoretic patterns of reticulocyte and erythrocyte membrane proteins [26–28], it was conceivable that the minor electrophoretic pattern alterations seen in xerocyte membranes resulted from reticulocyte protein contamination. To explore this possibility, intact red cells of xerocyte and control patients were separated by density gradient centrifugation into more buoyant, younger cells and less buoyant, older cells. Reticulocyte enrichment did not appear to affect the membrane pattern of xerocytes (Fig. 12a and b). However, membranes isolated from the top fraction of control erythrocytes (3.9% reticulocytes) had more band 3 and a slightly reduced 4.1a : 4.1b ratio when compared to either the unfractionated (1%) or the bottom fraction (0.2%) (Fig. 12c and d). These alterations in the pattern of membranes from the control top fractions were qualitatively identical to, although not extreme as, those seen in the pattern of either xerocyte fraction.

Table III shows that the correlation between 4.1 peak height and reticulocyte count is imperfect. Membranes prepared from the oldest xerocytes with a reticulocyte count of 3% have the abnormal 4.1 profile, whereas those from umbilical cord blood and from the youngest control cells, which have counts of 3.8% and 3.9%, respectively, still have 4.1b lower than 4.1a. Even though the height of band 4.1b does decrease with age, the reticulocyte-enriched sample from the control population still does not show 4.1b in excess over 4.1a.

TABLE III

XEROCYTE MEMBRANES AS CONTRADICTIONS TO RETICULOCYTE BAND 4.1 PROFILE CORRELATION

Membrane sample	Reticulocyte count	4.1b > 4.1a
Control, oldest (bottom fraction)	0.2	—
Control, unfractionated	0.8	—
Xerocyte, oldest (bottom fraction)	3.0	+
Xerocyte, unfractionated	3.8	+
Umbilical cord blood	3.8	—
Control, youngest (top fraction)	3.9	—
Xerocyte, youngest (top fraction)	12.4	+

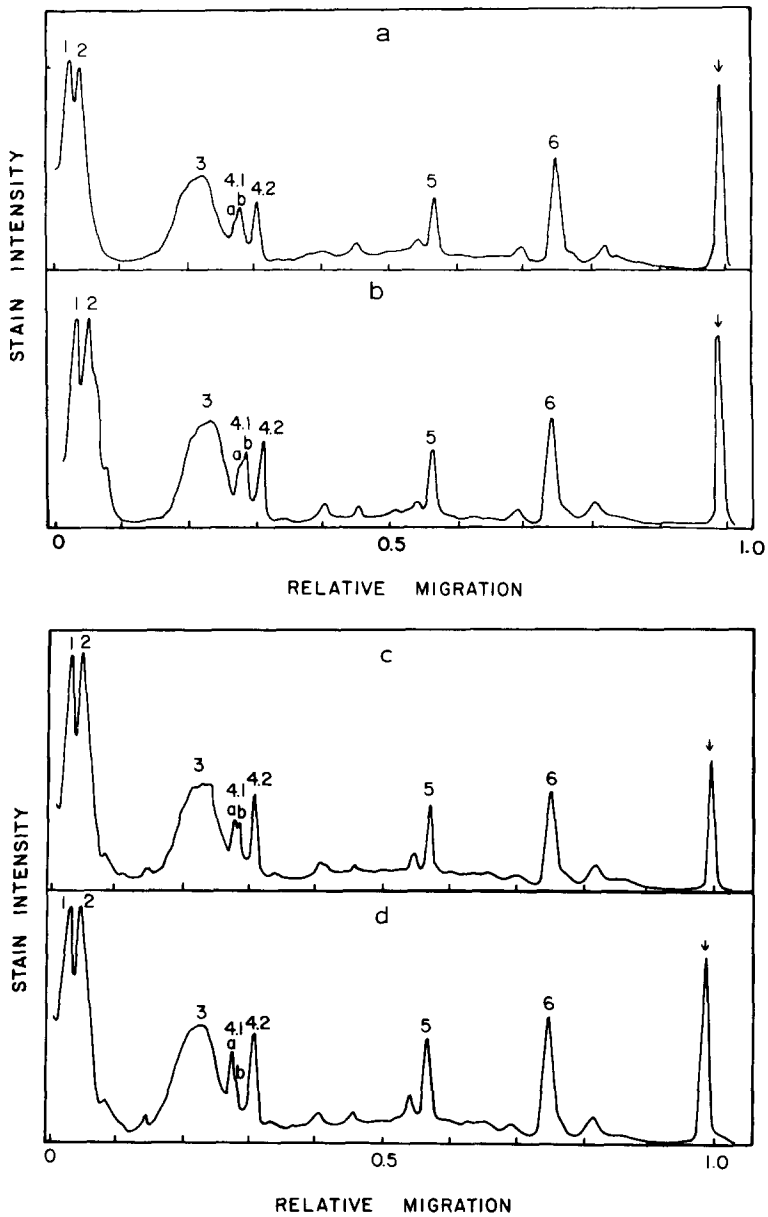

Fig. 12. Analysis of membranes from young and old cell fractions. From 5 to 15 ml of red cells packed to a 60% hematocrit were centrifuged at 15 000 rev./min for 1 h in the fixed-angle rotor of a Sorvall RC2B centrifuge in a modification of Murphy's method [31]. An aliquot of red cells (usually 1 ml) was then carefully removed from the top and bottom of the tube with a plastic pipet. Reticulocyte counts were made on the sample according to Wintrobe [32]. Ghosts were isolated from these cells by the usual method. Xerocyte top (a) and bottom (b) fractions and control top (c) and bottom (d) fractions.

Erythrocyte creatine has recently been shown by Fehr and Knob [25] to be a better estimate of red cell age than reticulocyte count, particularly in milder hemolytic diseases. In the present study, creatine levels were found to correlate better with the 4.1 band profile that the reticulocyte count did (Table IV).

TABLE IV

CREATINE LEVELS OF TOP AND BOTTOM CELL FRACTIONS

	Reticulocyte count (%)	Creatine (μmol/g Hb)	4.1b > 4.1a
Control			
Top fraction	2.8	2.0	—
Bottom fraction	0.15	1.1	—
Xerocyte			
Top fraction	14.0	3.9	+
Bottom fraction	2.5	3.2	+

Discussion

Table V is a summary of alterations in the electrophoresis pattern of bands 3, 4.1 and 4.2 which occurred as a result of the 15 separate manipulations of intact cells or their isolated membranes described herein. Generalizations about the effects of each manipulation are listed under 'comments' in the table. The presence of several consistencies within each column make it possible to draw some tentative conclusions about the nature of the polypeptide(s) comprising each band. These are described below.

Band 3

The elevated polypeptides in the band 3 zone of the xerocyte pattern seem to be cytoplasmic components which are released from the membrane at low ionic strength. Retention of these band 3 'satellites' at hemolysis may be induced in control cells by hemolysis in the presence of calcium, reticulocyte enrichment and metabolic depletion.

The portion of this zone, the lower two-thirds, which is affected by boiling and addition of reducing agent to the dissolved membrane immediately prior to electrophoresis may be the major integral membrane protein described by Yu et al. [29] as being extractable with non-ionic detergents. The band 3 found in our Triton extracts and that left behind after low ionic strength extraction has a smoother, more symmetrical electrophoretic contour compared to that of the membranes before extraction. Since xerocyte band 3 contours are normal in both of these extract and residue patterns, we suspect that this major integral protein is not responsible for abnormal band 3 contours in patterns from younger and depleted controls and from patients with other hemolytic anemias described here.

Band 4.1

Bands 4.1a and 4.1b are polypeptides which are only partially extractable after incubation at low ionic strength. They both appear to participate in the calcium-induced formation of high molecular weight aggregates such that the band intensity ratio of the residual 4.1 polypeptides resembles that of corresponding ghosts hemolyzed under normal conditions. Band 4.1b is slightly elevated in ghosts from both younger and metabolically depleted control cells. Patients with any disorder resulting in an increased percentage of reticulocytes

TABLE V

SUMMARY OF ALTERATIONS IN ELECTROPHORETIC PROFILES OF BANDS 3, 4.1 AND 4.2

Increase, ↑; decrease, ↓; change, +; no change, —. DTT, dithiothreitol.

	3	4.1b/4.1a	4.2 *	Comments
1. Xerocytosis (Figs. 1 and 2)	↑	↑	(↓)	3 shoulders more pronounced; 4.2 lower than 4.1 on continuous gels only
2. Presence of cytoplasmic proteins (Fig. 4)	↑	—	—	3 alterations concentrated in supernatant; 4.1 in supernatant is minor
3. Triton extraction (Fig. 5)	↑	—	—	3 alterations concentrated in residue; extract normal
4. Low ionic strength extraction (Fig. 6)	↓	—	—	3 alterations concentrated in extract; residue normal
5. Variations in hemolysis conditions	—	—	—	No change
6. Ca^{2+} present at hemolysis (Fig. 7)	↑	—	—	3 shoulders more pronounced; 4.1 lower, no profile change
7. Not boiling sample before electrophoresis (Fig. 8b and d)	+	—	—	Leading edge of 3 sharp, like in Fig. 1
8. No DTT added prior to electrophoresis (Fig. 8c and d)	+	↑	↓	Decreased resolution of 3; 4.1 increased; 4.2 decreased
9. Elevation of SDS in gel	+	—	↓	Decreased resolution of 3; 4.2 decreased
10. Metabolic depletion (Fig. 9)	↑	↑	—	3 shoulders more pronouned; 4.1b higher
11. H_2O_2 treatment of intact cells	—	—	—	No change
12. Ghost incubation with buffy coat + SDS (Fig. 10)	↓	—	↓	3 and 4.2 decreased; 4.1 unaffected
13. Splenectomy for patient with hereditary spherocytosis (Fig. 11a and b)	?	↓	—	Effect on 3 unclear; 4.1b lower
14. Vitamin B-12 for patient with pernicious anemia (Fig. 11c and d)	↑	↑	—	3 shoulders increased 4.1b increased
15. Enrichment of younger cells (Fig. 12)	↑	↑	—	3 shoulders more pronounced; 4.1b higher

* No change should not be considered definitive since excess DTT was added to all samples prior to electrophoresis.

in peripheral circulation will probably have 4.1b elevated with respect to 4.1a in their membrane pattern. An apparent elevation in 4.1b occurs when dithiothreitol is not added to a sample after boiling and immediately prior to electrophoresis. However, this may be an artifact of increased background staining in this region (see description of band 4.2 below) and completely independent of the ageing-metabolic effect just described.

Band 4.2

The absence of this polypeptide in the hemolysate supernatant indicates that it is a protein which is firmly bound to the membrane. Consistent with this is its Triton extractability also noted by Yu et al. [29]. The electrophoretic migration of band 4.2 appears to be sensitive to the presence of dithiothreitol

in the sample and the concentration of SDS in the gel. Its diffuse migration in the absence of dithiothreitol contrasts with the sharp peak resulting from dithiothreitol. There may be a minimum amount of reducing agent necessary for sharp migration, a migration which does not contribute to background staining in the region of 4.1b described above. This minimum concentration may be higher for xerocyte membranes since they occasionally appear to have less than the normal amount of 4.2. Even though the quantity of reducing agent also affects the control pattern, the more extreme response in the case of the xerocytes may indicate an increased amount of dithiothreitol-reversible aggregation occurring during membrane preparation. Correlation of this increased lability to altered age or metabolic state of xerocyte cells is not possible with the present data since excess dithiothreitol was added to both metabolically depleted and younger cell membrane preparations. However, reports in the literature concerning a decreased band 4.2 peak height in electrophoretic profiles of membranes from patients having either hereditary spherocytosis or biliary obstruction [21,22], both in association with reticulocytosis, imply that this 4.2 alteration is also an age-dependent phenomenon.

Conclusion

The studies described herein demonstrate that high resolution electrophoretic analysis performed under well-controlled conditions can detect subtle differences in red cell membrane polypeptide composition associated with hemolytic disease. However, our experience indicates most strongly that the valid interpretation of such results in relation to the primary functional abnormality of the membrane requires a very extensive and refined study of many physiological variations that also result in pattern alterations. We suggest, in particular, that electrophoretic comparisons in future studies of hemolytic disease be accompanied by appropriate consideration of the contribution of chronologically young cells in the population, the frequently observed metabolic lability of the abnormal cells and the altered pattern of partitioning between cytoplasm and membrane that may be encountered.

Acknowledgments

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