An Immunochemical Approach for the Analysis of Membrane Protein Alterations in Ca^{2+}-Loaded Human Erythrocytes

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An increase in the intracellular concentration of Ca^{2+} in human erythrocytes results in the formation of γ-glutamyl-ε-lysine cross-linked membrane protein polymers. Following solubilization of the membranes with SDS, these polymers can be isolated on a Lubrol-containing sucrose gradient. Immunoelectrophoresis of the polymeric material with a polyspecific rabbit antibody against human ghosts gave rise to a single, but heterogeneous, precipitate. The polymer was amphiphilic and, on addition to Triton-solubilized erythrocyte membrane proteins, it coprecipitated with spectrin. When the antighost antibody was absorbed with the polymer prior to cross immunoelectrophoresis of normal erythrocyte membrane proteins, the precipitates of glycophorin, acetylcholinesterase, and hemoglobin were normal, whereas the antibody titers against band 3 protein, spectrin, and ankyrin became reduced. Furthermore, a rabbit antibody raised against the isolated human polymer reacted selectively with the same three membrane proteins. No reactions occurred with lysate proteins.

An elevation of intracellular concentration of Ca^{2+} in intact human erythrocytes can be achieved by using ionophore A23187 [1, 2]. Such loading with Ca^{2+} has pronounced effects on the shape of the cell and on the viscoelastic properties of its membrane [3–6]. Simultaneously, as seen on the SDS-PAGE profile, changes occur in the polypeptide composition of the membrane [7–9]. In addition to the appearance of polymeric material on top of the gels, a partial diminution of band 1 and 2 (spectrin) and band 3, as well as the total disappearance of bands 2.1 and 4.1 [7–12] was observed. These Ca^{2+}-induced changes may be subdivided into three categories according to the criterion as to

whether they were caused (a) by oxidation of protein sulfhydryls to disulfides [1, 9, 12], (b) by proteolytic digestion [8, 13], or (c) by transglutaminase-mediated formation of γ-glutamyl-ϵ-lysine cross-links [5, 7, 8, 10, 11]. If the SDS-PAGE electrophoretic analysis was carried out in the presence of a reducing agent, the disulfide-dependent polymerization could be distinguished from the other two processes. With the use of synthetic substrates of transglutaminase, acting as inhibitors of cross-linking, it was possible to focus on the enzyme-mediated cross-linking event without too much interference from proteolysis. Thus, in cells exposed prior to and during the Ca^{2+}-loading process to various amine substrates of transglutaminase, the intactness of spectrin, bands 2.1, 3, and 4.1 could be retained [5, 7, 10, 11]. With labeled amines, such as ^{14}C-histamine, incorporation of the radioactive compound into spectrin and band 3 could be demonstrated, showing that at least these proteins carried transglutaminase-reactive endoglutamine residues [10].

Altogether, these observations indicated that the γ-glutamyl-ϵ-lysine bridge containing polymer contained spectrin, band 3, 2.1, and 4.1 proteins. The participation of band 3 in the enzyme-mediated cross-linking event was also supported by the finding of King and Morrison that, following external labeling with ^{125}I and lysis of the cells in the presence of Ca^{2+}, some radioactivity was present in the high molecular weight polymer [14].

Since no degradative procedure is available as yet for the selective cleavage of γ-glutamyl-ϵ-lysine cross-links in polymeric structures, the direct compositional analysis of the membrane polymer of Ca^{2+}-loaded erythrocytes had to be approached by immunochemical tests. A polyspecific antibody was raised against normal erythrocyte membrane proteins and was used in the present study in various crossed immunoelectrophoretic procedures. Similar studies were carried out with an antibody produced against the purified polymer.

MATERIALS AND METHODS

Erythrocytes from fresh blood (drawn in citrate) of a single donor were washed four times and were suspended in a buffer containing 100 mM KCl, 60 mM NaCl, 10 mM glucose, and 5 mM Tris-HCl at pH 7.4. For the Ca^{2+}-loading experiments, the cells were diluted to a hematocrit value of 20%, followed by the addition of 20 μM ionophore A23187 and 2 mM of $CaCl_2$. The cell suspension was incubated at 37°C for 3 hours, unless otherwise indicated. The reaction was stopped by repeated washings in the incubation buffer. The membranes were isolated by centrifugation and washed four times in 5 mM phosphate buffer of pH 8.0 [15].

Whole lysate was obtained following treatment of washed human erythrocytes in 5 vol of 5 mM phosphate buffer of pH 8.0, and the membranes were removed by centrifugation at $40,000g_{av}$ for 1 hr. All preparations were kept frozen at $-20°C$.

Isolation of Polymer

Membranes from Ca^{2+}-loaded cells, corresponding to about 8 mg of protein, were solubilized in 4.5 ml of 2% SDS and 40 mM DTT. They were applied in equal aliquots onto six 2.5 ml continuous gradients of 5–15% (w/v) sucrose

containing 0.5% of the non-ionic detergent Lubrol PX, 1 mM EDTA, 10 mM phosphate buffer at pH 7.1. A bottom layer of 0.75 ml 60% (w/v) sucrose served as a cushion. After centrifugation at $160,000g_{av}$ for 2 hours at 5°C in a SW 60 rotor in a Beckman L5-65 ultracentrifuge, the tubes were punctured, and 20–25 individual fractions were collected. Those containing polymer (as judged by SDS-PAGE) were pooled, diluted four times with 10 mM phosphate buffer of pH 7.1, and concentrated to one-third of the original volume employing an Amicon XM 100 A Filter.

Antibodies

Rabbit antihuman erythrocyte membrane antibody, prepared according to [16], was obtained as an immunoglobulin fraction in 0.1 M NaCl and 15 mM NaN_3 from Dako Corporation, Santa Barbara, California. Antialbumin activity was removed by incubating 1 ml of antibody overnight with 50 μg of human albumin (Pentex Inc, Kankakee, Illinois) at 4°C, followed by centrifugation at $40,000g_{av}$ for 1 hr. Absorption with isolated human erythrocyte membrane polymer was performed in the same way, by adding 0.6 ml polymer (80 μg) to 0.1 ml of antibody. Rabbit immunoglobulin against the human erythrocyte lysate, after removal of membranes, was obtained as previously described [16, 17].

Two rabbits were immunized against the membrane polymer and isolated according to the description of Siefring et al [10] from human erythrocytes after 18 hr of treatment with Ca^{2+}. The SDS was removed prior to immunization. Doses of 0.1 ml containing 0.1 mg protein and 0.05 ml of Freund's incomplete adjuvant (Statens Serum Institute, Copenhagen) were injected according to the schedule of Harboe and Ingild [18]. Three consecutive bleedings between 3 and 6 months were pooled (60 ml), and immunoglobulins were isolated in a final volume of 14 ml containing 62 mg protein per ml of 0.01 M NaCl, containing 15 mM NaN_3.

Chemicals

Triton X-100 or polyoxyethylene(9-10)p-t-octylphenol, scintillation grade, was purchased from Packard; Lubrol PX or polyoxyethylene(10)palmitoyl-stearoyl esters, DTT and phenylmethylsulfonylfluoride were from Sigma; DOC and CTAB from Merck; SDS and Sudan Black B from Aldrich; Ionophore A23187 from Calbiochem. Agarose was obtained either from Marine Colloids (type LE) or from Litex (type HSA).

Immunoelectrophoretic Methods

Definitions and procedures for rocket, fused rocket, crossed immunoelectrophoresis and crossed immunoelectrophoresis with an intermediate gel are given in the reference by Bjerrum and Bøg-Hansen [16]. The agarose gels contained 0.5% of Triton. For immunoelectrophoretic analyses, the membranes were solubilized in 1% Triton to a protein concentration of 2 mg/ml, after sonication by three bursts, each lasting 5 sec (Branson sonifier 200) [16]. Two different buffers were employed—one containing 100 mM glycine and 38 mM Tris at pH 8.7, and another containing 200 mM glycine adjusted to pH 10 with NaOH.

Charge-shift crossed immunoelectrophoresis was performed as described [19]. To detect Sudan black binding, crossed immunoelectrophoresis was carried out in the presence of 0.05% Triton and 0.002% Sudan black B [20]. The method of Steck and Yu [21] was used for SDS-PAGE employing either tube or slab gels (1.5 mm thick); combined SDS-PAGE and crossed immunoelectrophoresis was carried out in a manner previously described [22].

Assays

Staining of the immunoplates for esterase activity was performed as described in [16]. Protein was determined according to Lowry et al [23] using the modification of Bonsall and Hunt [24], with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Only some of the membrane proteins of human erythrocytes act as antigens when tested in crossed immunoelectrophoresis with a rabbit antibody raised against the entire ghost [22, 25–27]. This can also be shown for membrane proteins obtained from red blood cells following incubation with ionophore A23187 and Mg^{2+} (Fig. 1A). In addition to positive staining of a precipitation line for cholinesterase (not shown), seven precipitates were visible, labeled both according to the immunochemical identification pattern, given in
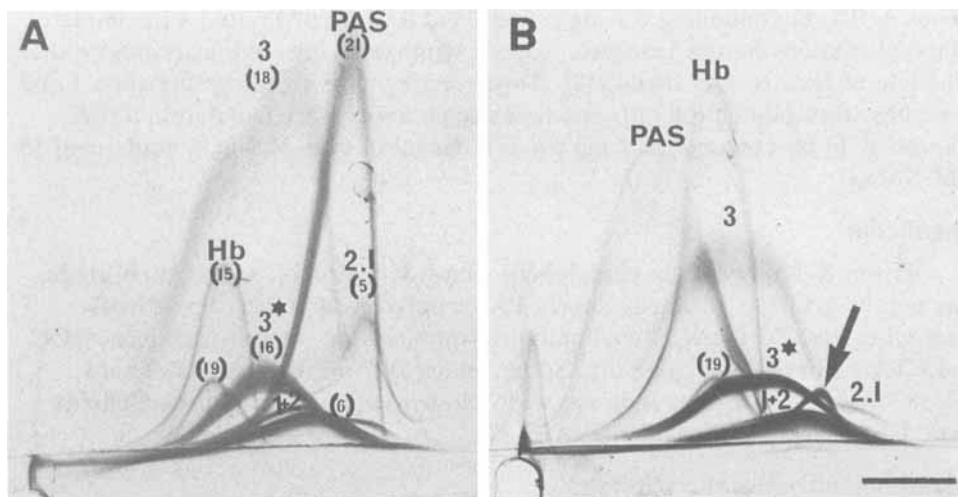

Fig. 1. Crossed immunoelectrophoresis of human erythrocyte membrane proteins (15 μg) in Triton at pH 8.7. Control cells, incubated with ionophore and 2 mM Mg^{2+} for 3 hr are shown in panel (A), Ca^{2+}-loaded cells in (B). Precipitates are labeled both according to the designation of Bjerrum and Bøg-Hansen [26] in round brackets and by the numbering system derived from SDS-PAGE: 1 and 2 (spectrin); 2.1 (ankyrin, including possible degradation products); 3 (band 3 protein) in addition to its complex 3*; PAS (glycophorin) and Hb (hemoglobin). In panel (B) the arrow points to the position of undegraded glycophorin. Electrophoresis in the first dimension was performed at 10 V/cm until the bromophenol blue-stained albumin marker migrated to a distance of 4.5 cm toward the anode on the right. Electrophoresis in the second dimension was carried out at 2 V/cm overnight, with the anode on top. The gels contained 0.5% Triton. The rabbit antihuman erythrocyte membrane antibody content in the second dimension was 5 μl/cm². The horizontal bar represents 1 cm.

brackets, of Bjerrum and Bøg-Hansen [26] and also according to the nomenclature derived from SDS-PAGE analysis. Precipitate (5) corresponds to band 2.1 and 2.3 (ie, ankyrin and ankyrin related products); (6) to band 1 and 2 (spectrin); (15) to Hb (hemoglobin); (18) to band 3; (21) to PAS 1 and 2 (glycophorin). Precipitate (16) corresponds to some complex of band 3, which is not dissociated in presence of Triton (P.J. Bjerrum and O.J. Bjerrum, unpublished results); hence it is labeled 3*. The identity of precipitate (19) is not known.

Treatment of the cells with Ca^{2+} and ionophore caused appreciable changes in the crossed immunoelectrophoretic pattern (Fig. 1B). Significantly, the 2.1 precipitation was much diminished, and the amount of hemoglobin was considerably elevated in comparison to the control in Figure 1A; glycophorin became more than 90% degraded. Concomitantly, a slower migrating form of PAS appeared. There was also a change in the shape of band 3 precipitate. Activity staining for cholinesterase (not shown) was unaltered. However, in spite of the fact that polymeric material due to transglutaminase action could be shown by SDS-PAGE to be present in the Triton extract of the ghosts from Ca^{2+}-loaded cells, the experiment in Figure 1B did not allow immunochemical identification of the polymer. The precipitation peak above the site of application, in the bottom left corner on Figure 1B, was not a reproducible feature.

Typical SDS-PAGE crossed immunoelectrophoretic patterns of membrane proteins from control and Ca^{2+}-loaded erythrocytes are presented in Figures 2A and 2B, respectively. Following treatment with SDS, in comparison to the findings in Figure 1, fewer precipitation lines were seen and staining for cholinesterase was negative. It could be seen that Ca^{2+} loading caused an added degradation of band 3, as evidenced particularly by the increase in height of the band-3-related component near position 4.5. Glycophorin was shown to be almost totally degraded to lower molecular weight products. These experiments did not facilitate the immunochemical detection of the polymer (marked as X in Fig. 2B), however, because during immunoelectrophoresis in the second dimension it remained in the SDS-polyacrylamide gel. We thus turned to an examination of the isolated polymer itself, and made a special effort to remove any SDS from it, prior to immunochemical analysis.

The SDS-solubilized ghosts from Ca^{2+}-loaded erythrocytes were centrifuged in a sucrose gradient that contained Lubrol. This simple and relatively rapid procedure offered the combined advantage of allowing essentially a complete exchange of SDS for the non-ionic detergent [29] and the isolation of the highest molecular weight polymeric species.* As shown in Figure 3C (Nos. 3–9, marked X), these do not penetrate the gel in the SDS-PAGE employed. Fused rocket immunoelectrophoresis (Fig. 3A) also revealed the presence of polymer in the most dense fractions (Nos. 3–9). The line of precipitation seemingly fused with that of the spectrin in the lighter fractions, which was identified by its position and staining intensity. As seen in Figure 3B, the control run with ghosts from normal cells did not yield any antigenic material in the corresponding dense fractions (ie, Nos. 3–9).

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*In order to achieve a clean separation of the polymer from spectrin, treatment of the membrane with SDS was found to be essential prior to sucrose-density centrifugation. Solubilization with non-ionic detergent was not possible.
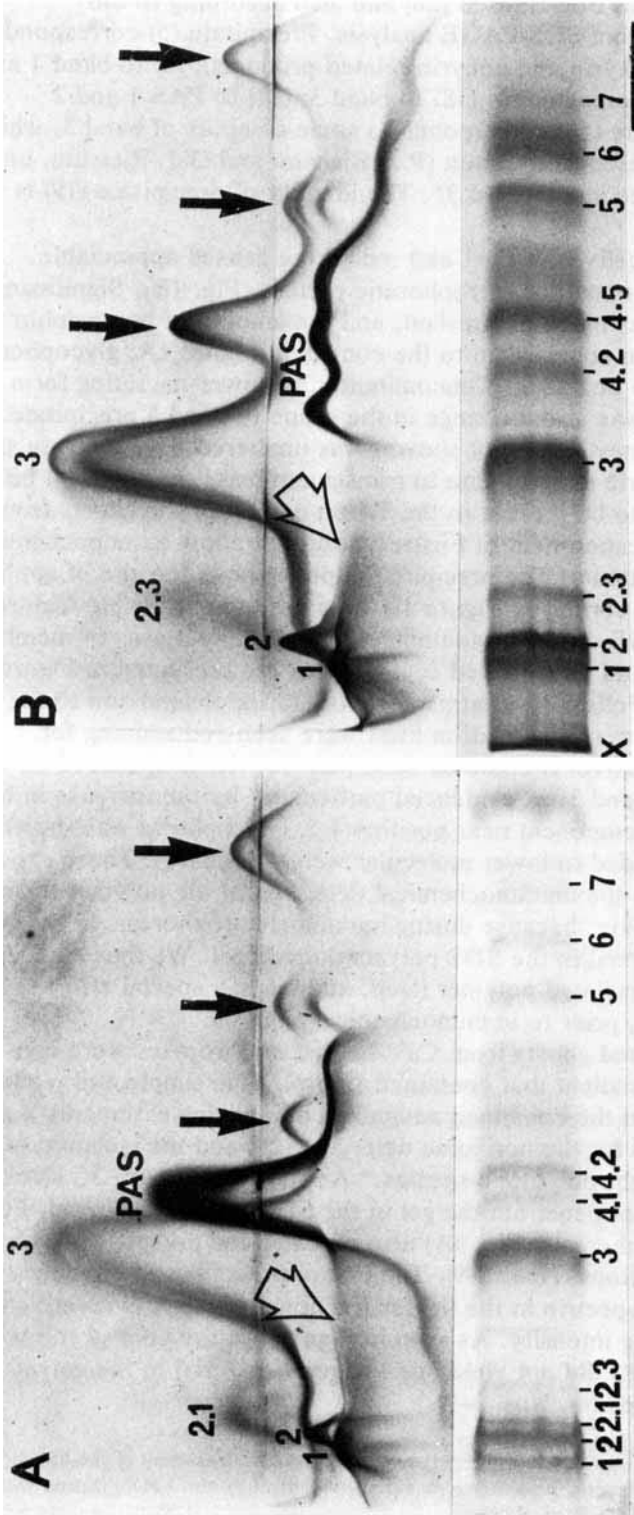
Fig 2   SDS PAGE-crossed immunoelectrophoresis of 50 μg of erythrocyte membrane proteins from (A) control cells and (B) from Ca²⁺ loaded cells after 2 hr of incubation Immunoprecipitates are designated as 1 and 2 (spectrin, including its degradation products, marked by an open arrow), 2 1 and 2 3 (ankyrin related proteins), 3 (including its degradation products, marked by solid arrows), and PAS (glycophorin) For easier recognition, photographs of Coomassie blue-stained SDS PAGE gels were inserted to demonstrate the resolution in the first dimension The polymeric material in panel (B) is denoted as X and other bands are labeled according to [28] SDS PAGE in the first dimension was performed until the pyronin marker dye migrated 7 5 cm to the anode on the right The gel was then placed on the top of a 2 mm thick agarose gel containing 3 5% Lubrol The upper portion of the plate contained 2% Lubrol and the antihuman erythrocyte membrane antibody (10 μl/cm²) Second dimension immunoelectrophoresis was performed as described in the legend to Figure 1 The horizontal bar indicates 1 cm
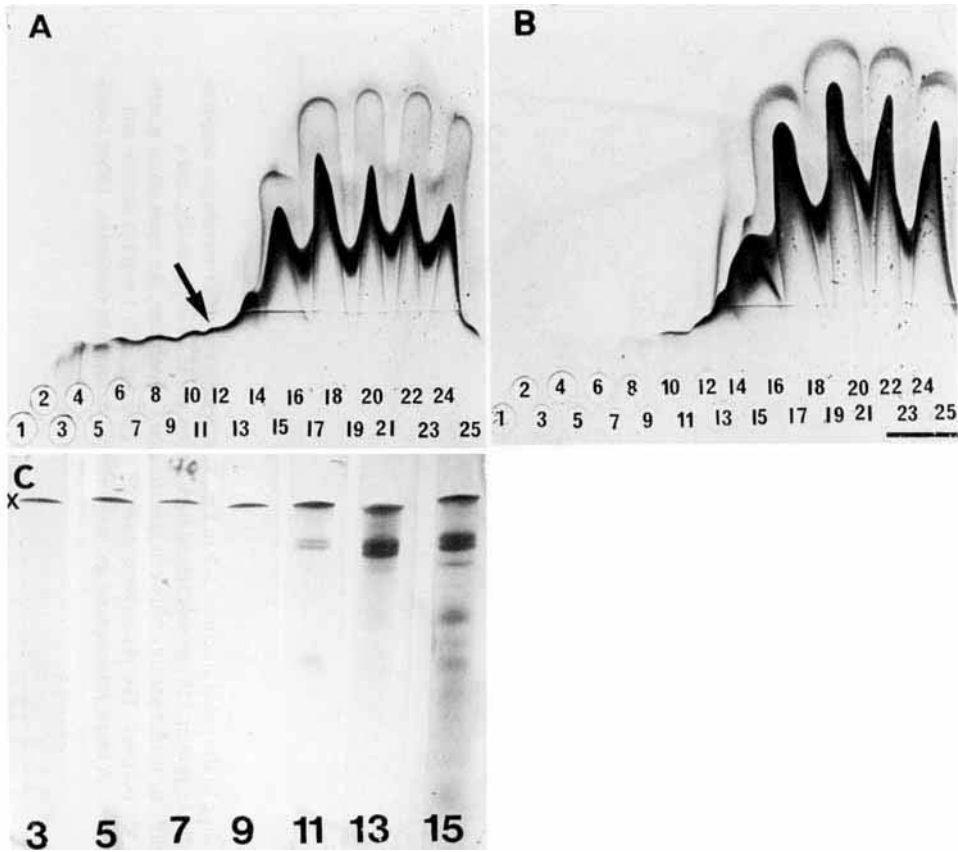
Fig. 3. Separation of SDS-solubilized human erythrocyte membrane proteins (1.3 mg) on Lubrol-containing sucrose gradient. The fractions, numbered from the bottom of the gradient, were monitored by fused rocket immunoelectrophoresis (panels A and B; using 10-μl aliquots) and with SDS-PAGE (panel C; using 80-μl aliquots). The analysis of membrane material from the Ca^{2+}-incubated cells (3 hr) are shown in (A) and (C). Immunoelectrophoresis with control membranes is presented in (B). The arrow indicates fusion between the precipitates of the dense material and spectrin. Only polymer (X) is seen in fractions 3–9. Conditions for immunoelectrophoresis were as described for Figure 1.

Crossed immunoelectrophoresis with the isolated polymer is shown in Figure 4A. The asymmetric shape of the precipitate on reacting with the antibody to normal erythrocyte ghost suggests electrophoretic heterogeneity. There is no corresponding precipitate with the control Triton-solubilized normal ghost membrane in Figure 4C. Addition of the isolated polymer to the control material changed the shape of the spectrin and band 3 precipitation lines (compare Fig. 4B with 4C). Partial immunochemical identity of the polymer with spectrin is evident, as the fusion of the lines is not complete (note the spur at the arrow in Fig. 4B), suggesting the presence of additional nonspectrin epitopes on the polymer. A shoulder on the band 3 precipitate (indicated by an open arrow in Fig. 4B) would be expected if the polymer also contained band 3, thereby reducing the concentration of antiband 3 molecules available for reaction with free band 3.
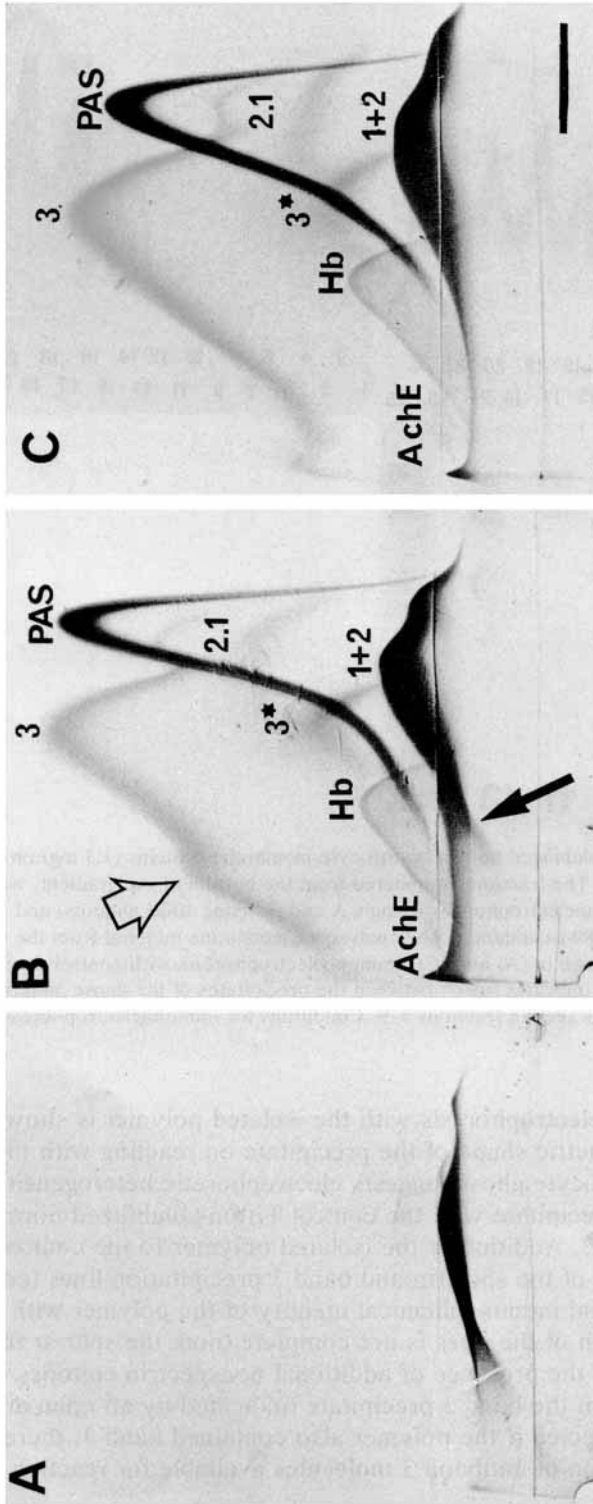
Fig. 4. Crossed immunoelectrophoresis of pure polymer (corresponding to the poled fractions 3–9 in Fig. 3) in relation to other membrane constituents. Polymer (4 μg) is shown in panel (A), control erythrocyte membrane protein (18 μg) solubilized with Triton at pH 10 in panel (C), and a mixture of the two in (B). The solid arrow in (B) points to a spur signifying partial identity between spectrin and the polymer; the open arrow points to a shoulder on the band 3 precipitate introduced by the addition of the polymer. The plates were stained for esterase with 1-napthyl acetate and Fast Red TR; the precipitate is designated as AchE (acetylcholinesterase). A blank intermediate gel was inserted to increase resolution. Other conditions and designations correspond to those in Figure 1.

In view of the possibility that an amphiphilic integral membrane protein, such as band 3, might be a component of the polymer, charge-shift electrophoretic experiments were carried out [19, 30] with deoxycholate (DOC) or cetyltrimethylammonium bromide (CTAB) added to Triton during electrophoresis in the first dimension as seen in (Fig. 5A to C). The charged detergent molecules imposed a change in the migration of the polymer. Under conditions where hemoglobin, a hydrophilic protein, migrated 20 mm, the polymer experienced an apparent charge-shift of 11 mm toward the anode with DOC and 10 mm toward the cathode with CTAB. As a general rule, amphiphilic membrane proteins undergo charge-shifts larger than 5 mm under similar circumstances [19]. The detergent-binding property of the polymer was also indicated by the fact that immunoelectrophoresis in the presence of Triton and Sudan black B gave rise to a black colored precipitation line [20]. Yet another indication that the polymer contained an integral membrane component was derived from the observation that it could not be extracted from the ghosts of Ca^{2+}-loaded erythrocytes either by treatment with EDTA (1 mM, pH 7.4) or by raising the pH to 13.
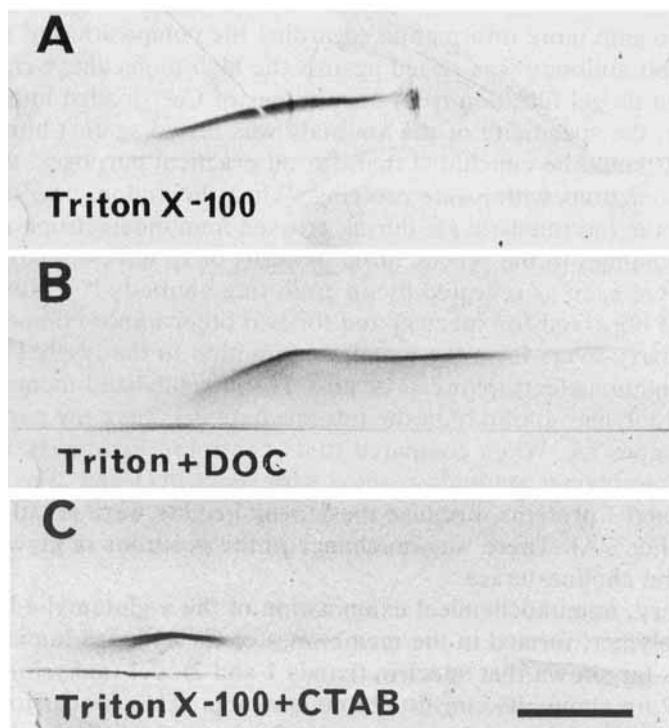

Fig. 5. Examination of the amphiphilic behavior of the purified membrane polymer by charge-shift crossed immunoelectrophoresis. In panels A, B, and C, 2 μg of polymer (corresponding to the material used in Fig. 4) was analyzed. Electrophoresis in the first dimension was performed at 100 V/cm until the hemoglobin marker migrated 2 cm to the anode to the right in 0.5% Triton X-100 alone or with 0.2% deoxycholate (DOC) and 0.0125% cetyltrimethylammonium bromide (CTAB) added. Electrophoresis in the second dimension against antihuman ghost antibody was carried out as described for Figure 1.

In the next phase of our work, the polymer obtained by sucrose density centrifugation was used for absorbing the antighost antibody. Its residual titer was then assessed by crossed immunoelectrophoresis (Fig. 6). When the absorbed antibody was included in the intermediate gel (as in Fig. 6B) instead of the original one (as in Fig. 6A), there was an upward displacement in the heights of precipitation lines for spectrin (1 and 2), band 2.1 and band 3 proteins. This was consistent with the idea that these protein components were present in the polymeric material. A rough approximation of the decrease in titers could be obtained by relating the peak heights in Figure 6B to those in Figures 6A and 6C. The latter shows the positions of precipitation lines without any antibody in the intermediate gel (ie, zero titer). Based on differences in peak heights between Figures 6A and 6C for defining 100% titers, following absorption of the antibody to the polymer (as shown in Fig. 6B), the residual titers were approximately 14% for spectrin band 2 and for band 2.1, 48% for spectrin band 1, and 74% for the band 3 protein. The specificity of removing antibodies against these proteins is underlined by the fact that no changes in titers were observed in the heights of precipitates for glycophorin, hemoglobin, and cholinesterase following absorption onto the polymer.

In order to gain more information regarding the composition of the polymer, a rabbit antibody was raised against the high molecular weight components isolated by gel filtration from membranes of Ca^{2+}-loaded human red cells [10]. First, the specificity of the antibody was tested against human erythrocyte lysate. It could be concluded that, for all practical purposes, there were no cross-reactions with lysate proteins. When the antipolymer antibody was present in the intermediate gel during crossed immunoelectrophoresis with the lysate, no changes in the precipitation patterns of 15 different components of the lysate were seen as revealed by an antilysate antibody [7]. However, retardation was observed for spectrin and for two other minor components, thought to be carry-overs form the membrane fraction to the lysate [16].

Crossed immunoelectrophoresis against Triton-solubilized membrane proteins, with antipolymer antibody in the intermediate gel, gave the pattern presented in Figure 7A. When compared to its control in Figure 7B, it is evident that the antipolymer antibody reacted with spectrin (1 and 2) as well as with band 2.1 and 3 proteins, because these peak heights were greatly diminished (see Fig. 7A). There was no change in the positions of glycophorin, hemoglobin, and cholinesterase.

In summary, immunochemical examination of the γ-glutamyl-ϵ-lysine cross-linked polymer, formed in the membranes of Ca^{2+}-loaded human erythrocytes, has thus far shown that spectrin (bands 1 and 2), 2.1 (ankyrin), and the band 3 protein are among its constituent components. This conclusion has been rather firmly established by a dual approach of using the purified polymer for the absorption of a polyspecific antibody against normal ghosts (Fig. 6) and by direct determination of specificity of an antibody against the polymer, with normal membrane antigens (Fig. 7).
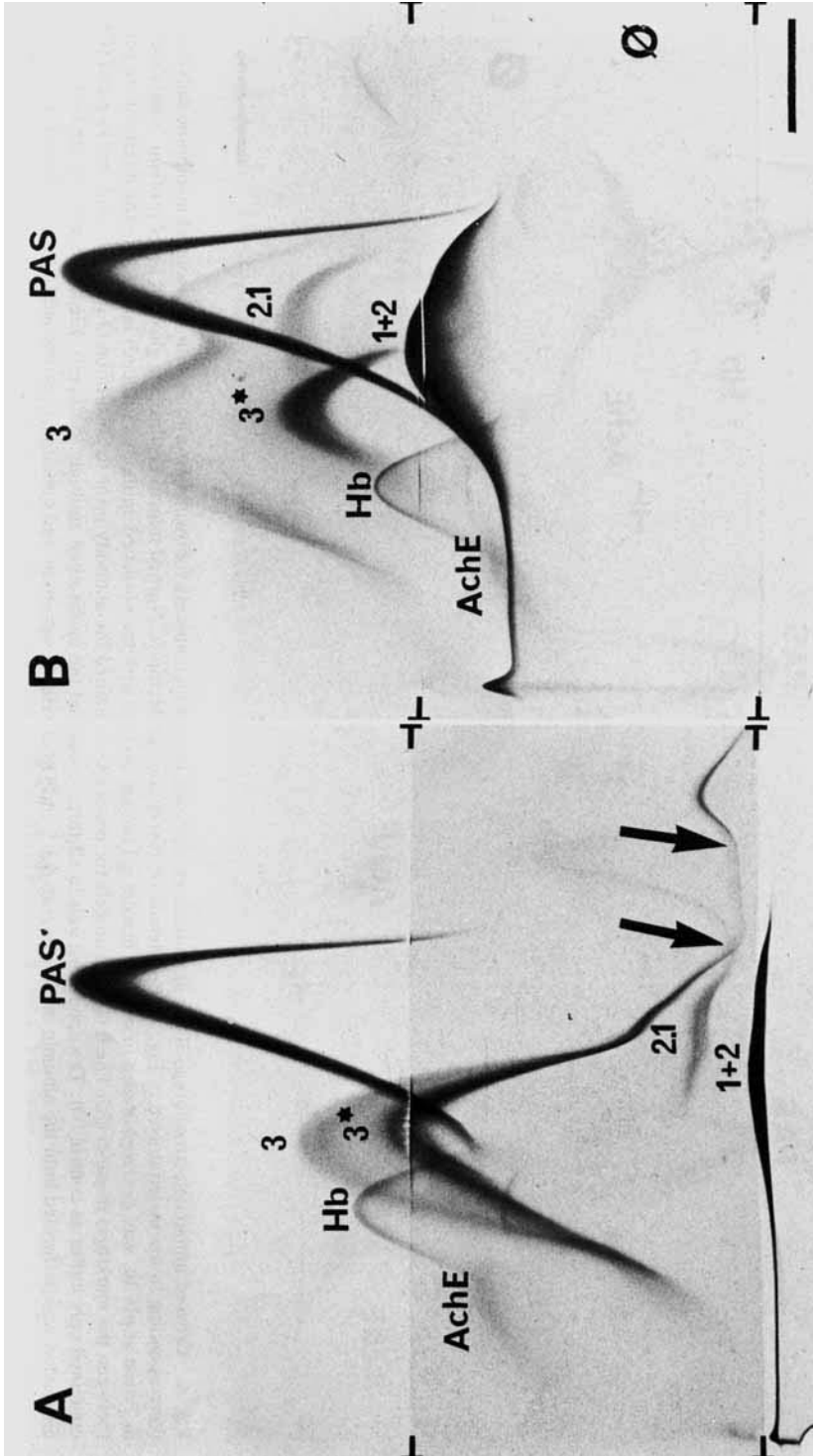
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Fig. 6.   Crossed immunoelectrophoresis using antihuman erythrocyte membrane antibody following absorption with the isolated membrane polymer (corresponding to the material used in Fig. 4). For details of absorption see Methods. Normal human erythrocyte ghost (8 μg of protein), solubilized in Triton at pH 10, was electrophoresed first in the bottom gel (anode on top) with the absorbed antibody (3 μl/cm²) included in the intermediate gel (between the markings) in panel (B). The intermediate gels for panel (A) contained the antibody prior to absorption (3 μl/cm²)m and that in panel (C) contained only buffer as control (∅). The uppermost gels in all three contained the unabsorbed antibody (5 μl/cm²). Electrophoresis in the first dimension was performed until the albumin marker migrated 3.5 cm. Otherwise, designations and conditions correspond to those in Figure 4.
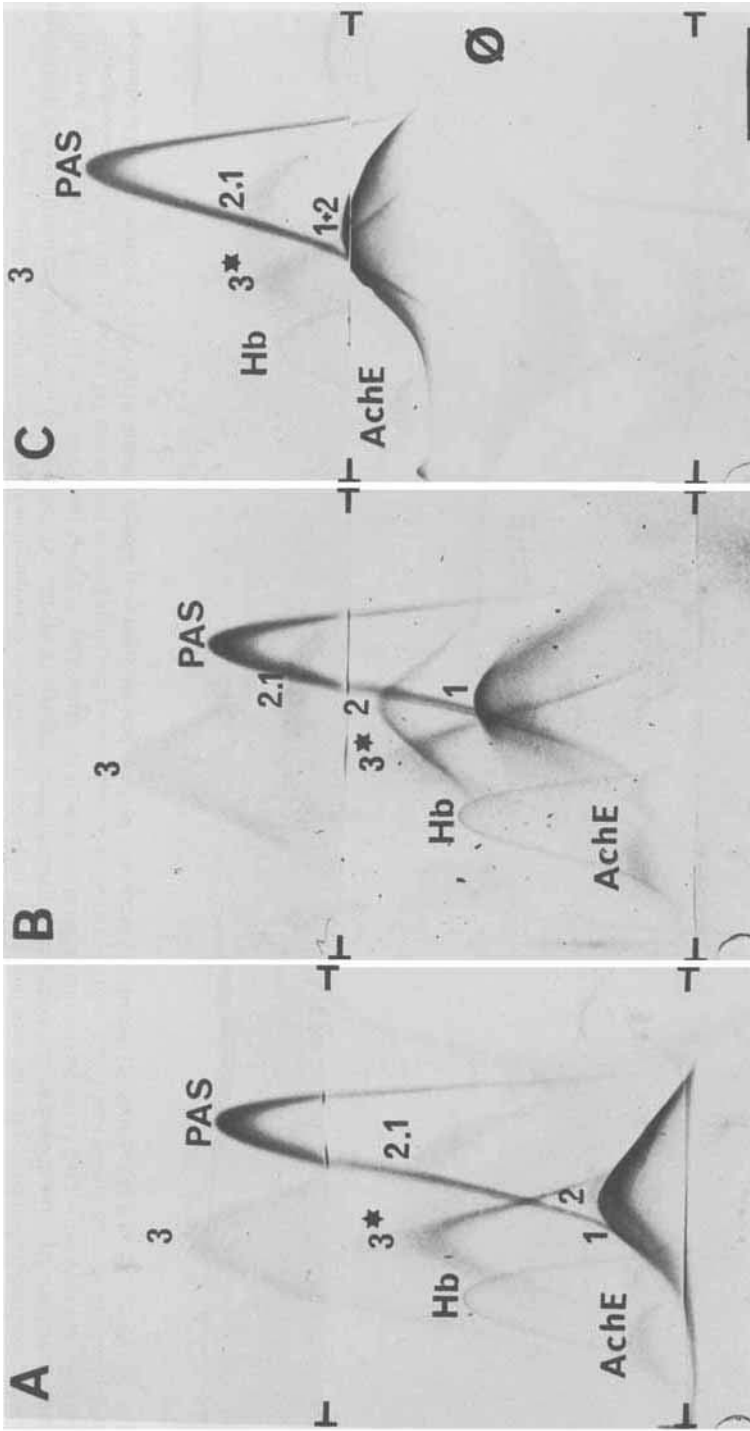
Fig. 7.   Specificity of a rabbit antibody against the membrane polymer purified from Ca^{2+}-loaded human erythrocytes. Norman human erythrocyte membrane proteins solubilized into Triton at pH 10 (20 μg of protein) were electrophoresed in the bottom gel (anode to the right). Immunoelectrophoresis was then carried out in the second dimension either against the antipolymer antibody (40 μl/cm^2) as in (A), or against buffer (∅) as in (B), in the intermediate gels. The uppermost gels contained antinormal ghost antibody (6 μl/cm^2). Arrows point to fusion between precipitates of degradation products of ankyrin and band 3 protein with their parent molecules. Designations and conditions correspond to those in Figures 1 and 5.

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