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QUANTITATIVE IMMUNOELECTROPHORESIS OF PROTEINS IN HUMAN ERYTHROCYTE MEMBRANES

ANALYSIS OF PROTEIN BANDS OBTAINED BY SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

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SUMMARY

1. We have defined conditions that permit quantitative immunoelectrophoresis in agarose gels of dodecyl sulfate-solubilized erythrocyte membrane proteins.

2. Using human serum albumin, transferrin, MN-glycoprotein (glycophorin) and crude spectrin as test proteins, we found that accurate analyses are possible if samples and gels are 1 % in non-ionic detergent (Berol EMU-043 or Triton X-100) and if no more than 100 nmol free dodecyl sulfate is applied per sample.

3. Dodecyl sulfate-treated membranes analyzed by crossed immunoelectrophoresis using rabbit antibodies against membrane material yielded optimal precipitation patterns in gels containing 1 % of non-ionic detergent.

4. Crossed immunoelectrophoresis in the presence of 1 % of Berol revealed precipitates when 10 protein bands defined and isolated by preparative dodecyl sulfate-polyacrylamide gel electrophoresis were run against anti-membrane antibodies. Seven of these bands showed more than one precipitation arc, indicating the presence of more than one antigenic component.

5. Crossed-line immunoelectrophoresis showed that dodecyl sulfate-polyacrylamide gel electrophoresis bands 1, 2 and 2.1 shared common antigenic components. The MN-glycoprotein was present in bands 3, 4A, 4B and 5, where antigenic components of the major intrinsic erythrocyte membrane protein, band 3, were also found.

6. After absorption of the anti-membrane antibody with intact erythrocytes, immunoelectrophoresis showed the disappearance of the MN-glycoprotein precipitates. An increase in the area below the precipitate corresponding to the major intrinsic protein (band 3) was also observed, indicating exposure of some antigens of this protein on the outer surface of intact cells.

7. After absorption of the antibody preparation with washed erythrocyte membranes, immunoprecipitates were not seen in any experiments, indicating that all antigenic determinants observed are exposed at one or both surfaces of the membrane.

8. Our analyses indicate that the peptide moieties of serum lipoproteins do not constitute a significant component of erythrocyte membranes.

INTRODUCTION

Electrophoretic molecular sieving in polyacrylamide gels laden with sodium dodecyl sulfate (dodecyl sulfate-polyacrylamide gel electrophoresis) is one of the most widely used methods for the analysis of membrane proteins [1]. Another method for membrane protein analysis employing crossed immunoelectrophoresis has recently been described by Bjerrum and Lundahl [2]. The latter type of analysis is performed on agarose gels laden with non-ionic detergents and allows high resolution of proteins according to charge and antigenicity in a quantitative, sensitive manner and good reproducibility [3]. It has been applied to several membrane systems [4–10].

Since immunoelectrophoretic examination of dodecyl sulfate-treated proteins has been shown to be feasible [11–15], we have combined the two analytical procedures in the analysis of erythrocyte membrane proteins. In a previous study we demonstrated the immunoelectrophoretic heterogeneity and cross-reaction of bands 1, 2 and 2.1 from human erythrocyte membranes, defined and isolated by dodecyl sulfate-polyacrylamide gel electrophoresis. We now first define conditions necessary to prevent artifacts due to the presence of dodecyl sulfate in our immunoelectrophoresis system and then describe the immunoelectrophoretic characteristics of dodecyl sulfate-polyacrylamide electrophoresis bands 3–8, including the MN-glycoprotein. We also demonstrate the usefulness of immunoelectrophoresis in the analysis of some vectorial aspects of membrane structure.

MATERIALS AND METHODS

Detergents. Sodium dodecylsulfate (especially pure) was obtained from BDH, London, and the non-ionic detergents Berol EMU-043 and Triton X-100 from MoDoKemi, Stenungsund, Sweden and Rohm and Haas, Philadelphia. U.S.A., respectively.

Membrane material. Human erythrocyte membranes were prepared from outdated citrated blood as in ref. 17. The final membrane suspension had a protein concentration of approx. 4 mg/ml. Membranes which were not used immediately were stored at −20 °C. For immunochemical analysis the membranes were solubilized to a final concentration of 2.5 mg/ml in 0.01 M glycine, 0.0038 M Tris (pH 9.2, 5 °C) containing 0.5 % (w/v) sodium dodecyl sulfate or 1 % (w/v) of Berol EMU-043. Crude spectrin was prepared from isolated membranes by EDTA extraction (5 mM EDTA, 0.5 mM NaN_3, pH 7.4, 72 h) followed by gel filtration on Sephadex G-200 [18, 19]. MN-glycoprotein was prepared according to Marchesi [20] and its purity was determided as described in ref. 21. Protein concentration was 0.5 mg/ml, determined according to ref. 22.

Serum proteins. Human albumin (reinst., batch 4464) and human transferrin (reinst., batch 1168) were purchased from Behringswerke AG, Marburg/Lahn, Germany. Denaturation with sodium dodecyl sulfate was performed at 37 °C for 3 h.

Antibodies. Rabbit antibodies against human serum protein (Code 100 SF, lot

033), against human albumin (lot 071, 4 mg protein ml) and against human transferrin (lot 030, 10 mg protein/ml were purchased from Dakopatts A/S, Copenhagen F, and rabbit antibodies against human α-1-lipoprotein (Batch no. 2348P) and against human β-lipoprotein (Batch no. 2076X) were purchased from Behringwerke AG, Marburg/Lahn, Germany. Non-absorbed antibodies against human erythrocyte membrane material were prepared as described earlier [4]. Immunoglobulin concentration was 27 mg/ml. This preparation contained precipitating antibodies against albumin and haptoglobin as the only serum proteins [23]. Trasylol (a polyvalent protease inhibitor) was obtained from Bayer AG, Leverkusen, Germany and was added to the antibodies to give a concentration of 500 units/ml [24]. Antibodies similar to those used in the work are now available from Dakopatts A/S, Copenhagen F, Denmark.

Preparative dodecyl sulfate-polyacrylamide gel electrophoresis. We employed the gel-slab technique described by Knüfermann et al. [25]. Slab dimensions were $0.5 \times 20 \times 20$ cm. For each preparation, 10 mg of ghost protein solubilized in 50 mM phosphate buffer (pH 8.0) containing 3 % (w/v) sodium dodecyl sulfate, and prestained with *o*-phthalaldehyde, were applied to a gel slab. The electrophoresis buffer contained 0.1 % sodium dodecyl sulfate. After electrophoresis the separated bands were visualized under ultraviolet light, the fluorescent gel strips cut out and squeezed through a syringe and extracted with 8 vol of 5 mM detergent-free phosphate buffer overnight at 4 °C. The samples were then concentrated over Amicon PM 10 membranes to 0.1–0.4 mg protein/ml and stored at −20 °C. Analytical dodecyl sulfate-polyacrylamide gel electrophoresis was performed as in ref. 1.

Immunoelectrophoretic methods. Before immunoelectrophoresis, the non-ionic detergents Berol EMU-043 or Triton X-100 were added to the protein samples to final concentrations of 1 % (w/v), and the samples stirred at 4 °C for 15 min. After centrifugation at $2.3 \cdot 10^6$ $g \cdot$ min the supernatant fluids were used for immunoelectrophoresis at 10 °C in 1 % (w/v) agarose (Batch no. 060, Litex, Glostrup, Denmark) with relatively high electroendosmosis ($0.08\ cm^2 \cdot V^{-1} \cdot h^{-1}$; ref. 26) containing 0.038 M Tris and 0.10 M glycine, pH 8.9 (16 °C). In most experiments a non-ionic detergent was added to sample and gel to a final concentration of 1 % (w/v) for Berol EMU-043 and 1 % (v/v) for Triton X-100, respectively. Rocket, crossed immunoelectrophoresis and crossed immunoelectrophoresis with an intermediate gel were performed according to refs 27, 4 and 28, respectively. Line and crossed-line immunoelectrophoresis [29] was performed as described in ref. 28.

Absorption of antibodies. For absorption with ghosts, antibodies against human erythrocyte membrane material, dialyzed against 0.154 M NaCl, were stirred with isolated membranes for 30 min at 20 °C (2.5 mg antibodies/mg membrane protein). The supernatant remaining after centrifugation (Sorvall RC-2B, rotor SE 12, 20 000 rev./min for 1 h) was used for the immunoelectrophoretic studies.

For absorption with intact erythrocytes, freshly drawn heparinized blood was centrifuged and the erythrocytes washed five times in 0.154 M NaCl. 25 ml of packed erythrocytes, obtained by centrifugation at $250 \times g$ for 15 min (erythrocyte cell volumes 17.5 ml) were mixed with 54 mg of antibodies in 12.5 ml of saline and incubated for 15 min at 20 °C with gentle shaking. The final supernatant obtained after centrifugation at $200 \times g$ for 15 min was used. Antibodies diluted to the same extent served as controls. Twice absorbed antibodies were used for some experiments.

RESULTS

Effect of dodecyl sulfate on immunoprecipitation patterns in quantitative immunoelectrophoresis was determined by experiments in which (1) the detergent was applied as a sample behind the antigen-containing gel in line immunoelectrophoresis and (2) proteins extensively treated with dodecyl sulfate were analyzed. Albumin, transferrin, spectrin and MN-glycoprotein were used as model substances. Fig. 1A shows the results of line immunoelectrophoresis when 25–1600 nmol of dodecyl sulfate was applied in wells behind an intermediate gel containing albumin in detergent-free

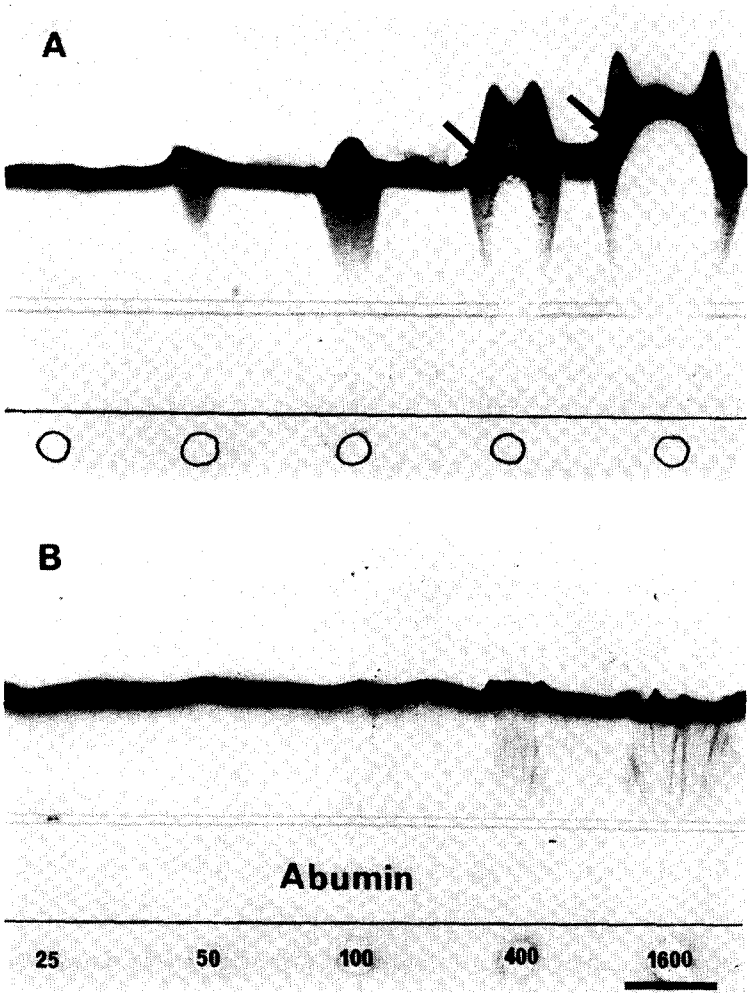

Fig. 1. Effect of dodecyl sulfate on line immunoelectrophoresis of human albumin (A) in gels without detergent and (B) in gels containing 1 % of Berol. 35 μg of albumin was distributed in the intermediate gel. The amount of anti-albumin was 1.25 $\mu l \cdot cm^{-2}$. 25–1600 nmol of sodium dodecyl sulfate were applied to the wells below the intermediate gel, as indicated. Arrows indicate additional precipitates in (A). The electrophoresis was performed at 2 $V \cdot cm^{-1}$ for 18 h at 16 °C. The bar represents 1 cm.

gel. With dodecyl sulfate > 50 nmol, the precipitation line trails, distorts and gives rise to rockets, together with additional precipitates (arrow). This detergent artifact is similar to the non-artifactual patterns found when proteins showing identity or partial identity with albumin were placed in the wells [30, 31]. Comparable results were obtained with similar concentrations of transferrin and spectrin, where as little as 10 nmol and 1 nmol of dodecyl sulfate, respectively, disturbed the precipitation lines. However, the incorporation of 1 % of a non-ionic detergent (Berol or Triton) into the agarose gel (Fig. 1B) returned the precipitation line to its normal form except at high levels of dodecyl sulfate. In the presence of 1 % non-ionic detergent, the precipitation line becomes perturbed at 400, 100 and 10 nmol with albumin, spectrin and transferrin, respectively. When dodecyl sulfate was electrophoresed in the first dimension gel of crossed-line immunoelectrophoresis behind a line of albumin or transferrin, 500–1000 nmol of detergent could be applied without any visible disturbance if 1 % of Berol was included in the gel. With spectrin the limit was 200–500 nmol.

Crossed immunoelectrophoresis of the above model proteins after exposure to 0.1 M sodium dodecyl sulfate (25 mol per mol protein) for 3 h at 37 °C revealed abnormally high migration velocities and produced blurred, irregular precipitates with camel arcs or shoulders; these are generally characteristic of heterogeneous proteins [32, 33]. Removal of the excess detergent by gel filtration or dialysis resulted in sharper and more regular precipitates. However, incorporation of 1 % of non-ionic detergent in the agarose gels and in the samples caused the precipitation patterns to return to normal. Except for a slight increase in migration velocity, normal precipitation patterns (antibody concentration as in Fig. 1) were observed with transferrin plus 100 nmol dodecyl sulfate and for albumin and MN-glycoprotein plus 300 nmol dodecyl sulfate. Spectrin yielded two congruent precipitation arcs in the presence of 300 nmol dodecyl sulfate (see Figs. 2B and 2C). These two precipitates are also observed with Berol-solubilized membranes by certain antibody preparations [23]. Normally they coprecipitate (cf. precipitate S in Fig. 2C).

These model experiments lead us to conclude that immunoelectrophoretic analysis of albumin, transferrin, membrane MN-glycoprotein and spectrin is possible if the applied amount of dodecyl sulfate does not exceed 100 nmol, and if a non-ionic detergent is incorporated in samples and gels.

Immunoelectrophoretic analyses of dodecyl sulfate-solubilized membrane material

Fig. 2 shows the result of crossed immunoelectrophoresis of dodecyl sulfate-solubilized human erythrocyte membrane proteins. The membranes employed were in buffer (pH 8.9) containing 0.5 % (w/v) of sodium dodecyl sulfate. The weight ratio of sodium dodecyl sulfate to membrane protein was 2.5 : 1. When the solubilized membrane proteins were analysed in detergent-free gels with rabbit anti-membrane antibody, five blurred precipitation arcs were observed (Fig. 2A, arrows). Furthermore, the migration velocities of the different components were very similar, resulting in decreased resolution. With lower concentrations of dodecyl sulfate, more precipitates could be recognized but the degree of solubilization was decreased. Incorporation of 1 % of non-ionic detergent in the agarose gels resulted in slower migration of the components and gave rise to the formation of a greater number of precipitates of increased sharpness. In the experiment of Fig. 2B, where the same dodecyl sulfate-

solubilized membrane material as in Fig. 2A was analysed in the presence of Berol, 7 immunoprecipitates were observed (arrows). The pattern and the areas delimited by the precipitation arcs are now similar to those obtained with the human erythrocyte

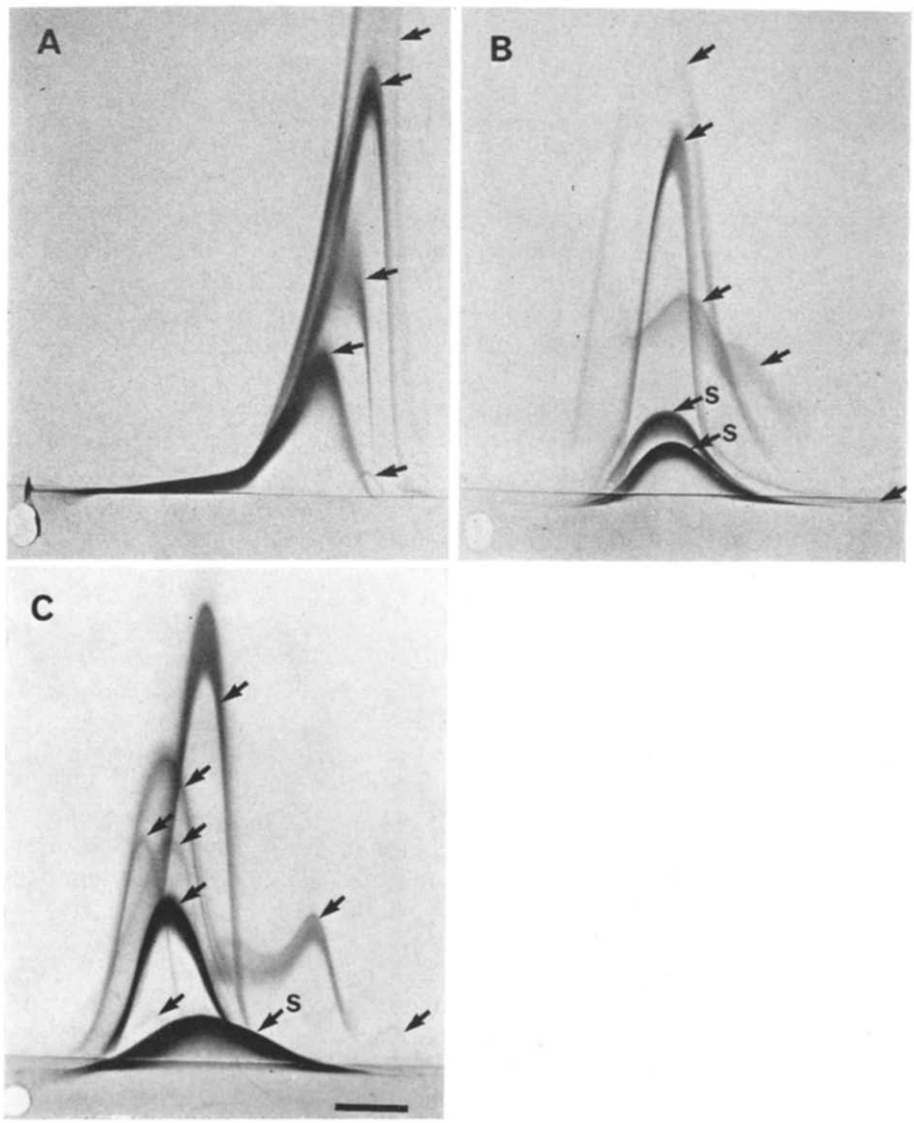

Fig. 2. Crossed immunoelectrophoresis (pH 8.9, 16 °C) of detergent-solubilized human erythrocyte membrane proteins. (A) 18 μg of dodecyl sulfate-solubilized membrane proteins analysed in detergent-free gel. (B) 18 μg of the same sample as in (A) made 1 % with respect to Berol and analysed in the presence of 1 % Berol. (C) 25 μg of Berol EMU-043-solubilized membrane protein analysed in the presence of 1 % of Berol. The amount of the anti-membrane antibodies was 5.3 $\mu l \cdot cm^{-2}$.Electrophoresis in the first dimension was performed at 10 $V \cdot cm^{-1}$ for 45 min in (A) and (B), and for 60 min in (C). Electrophoresis in the second dimension was at 2 $V \cdot cm^{-1}$ for 16 h. Note the better resolution in (B) and (C) compared to that in (A). Precipitation arcs are marked with filled arrows. S corresponds to spectrin. The bar represents 1 cm.

membrane material solubilized in 1 % of Berol with 9 precipitation arcs (Fig. 2C) [23]. The latter precipitation pattern differed to some degree from earlier published patterns [3, 4] because of inhibition with Trasylol of the proteolytic enzyme plasmin (present in the antibody preparation), which otherwise tended to degrade the proteins during electrophoresis in the antibody-containing gel [24].

These observations indicate that immunoelectrophoretic analyses can be reliably performed on isolated protein bands obtained by preparative dodecyl sulfate-polyacrylamide gel electrophoresis.

Immunoelectrophoretic analysis of the major bands obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of erythrocyte membrane proteins

The method used for the preparative isolation of membrane proteins by dodecyl sulfate-polyacrylamide gel electrophoresis has been described recently [25]. Re-electrophoresis of the individual bands used in immunoelectrophoresis experiments showed that each band had been obtained in an electrophoretically homogeneous form [16, 25].

Rocket immunoelectrophoresis in the presence of Berol, using rabbit anti-membrane antibody (Fig. 3A), showed several precipitation lines in most bands (arrows). This heterogeneity is more obvious in crossed immunoelectrophoresis, which reveals multiple precipitates for bands 3, 4A, 4B and 5, but not 6, 7 and 8 (Figs 4A and 5).

The various dodecyl sulfate-polyacrylamide electrophoresis bands can be

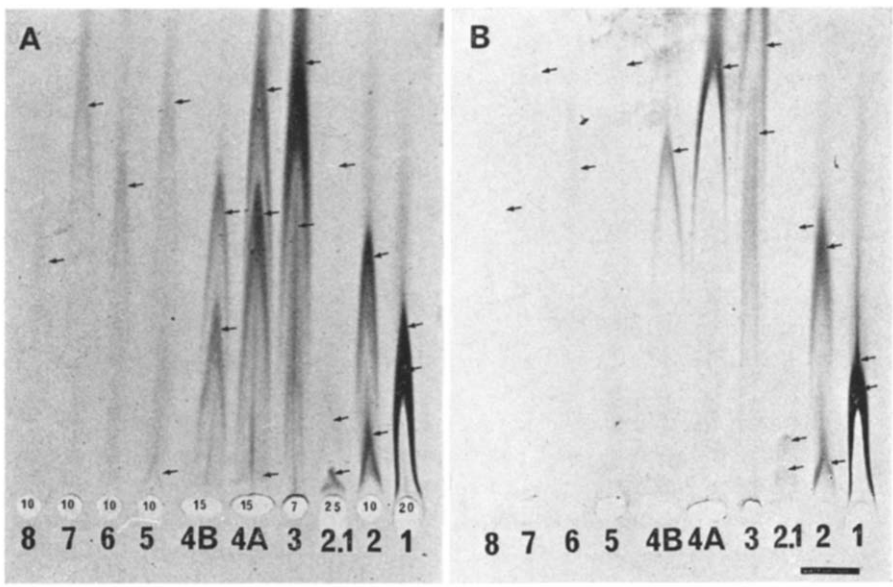

Fig. 3. Rocket immunoelectrophoresis of isolated bands from preparative dodecyl sulfate gel electrophoresis of human erythrocyte membranes (A) analysed with normal anti-membrane antibodies. (B) analysed using antibodies absorbed with intact erythrocytes. Arrows indicate precipitates. The samples contained approx. 0.05 % dodecyl sulfate and 1 % of Berol. Applied volumes in μl are indicated on the figure. Experimental conditions were as in Fig. 1B. The antibody content of the gel was 10 $\mu l \cdot cm^{-2}$. The bar represents 1 cm.

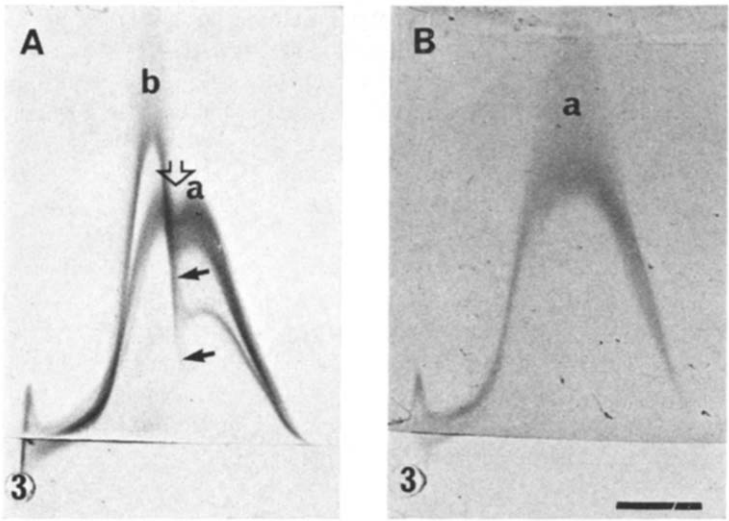

Fig. 4. Crossed immunoelectrophoresis of 10 μl of band 3 isolated by preparative dodecyl sulfate polyacrylamide gel electrophoresis of human erythrocyte membrane proteins using (A) normal antibodies against erythrocyte membrane proteins and (B) antibodies absorbed with intact erythrocytes. The antibody content of the gels was 10 $\mu l \cdot cm^{-2}$. Filled arrows indicate splitting and flying (unending) precipitates. The open arrow indicates a reaction of partial identity. Electrophoresis in the first dimension was performed at 10 $V \cdot cm^{-1}$ for 35 min. Experimental conditions were otherwise as for the experiment of Fig. 2B. a and b indicate precipitates. The bar represents 1 cm.

divided into three groups on the basis of their precipitation patterns in crossed immunoelectrophoresis. The first group comprises bands 1, 2 and 2.1, in which the antigenic components have identical migration velocities in the first-dimension electrophoresis. The precipitation arcs are symmetrical and congruent [16]. Our present results differ from those in ref. 16, in that bands 1, 2 and 2.1 were stabilized against degradation by plasmin contaminating the antibody [24] by use of the protease inhibitor Trasylol. Band 1 yields two adjacent congruent precipitation arcs, band 2 produces two separated, congruent precipitation arcs each composed of two precipitation lines, and band 2.1 gave rise to 3 congruent precipitation arcs.

The second group comprises bands 3–5, whose individual main components have different first-dimension migration velocities (Figs 4A and 5). All bands in the group revealed two main precipitation arcs, a major broad one a and a smaller, more distinct precipitate b. Additional minor precipitation arcs are also seen c and d (Fig. 5). The relative height of precipitate b compared to precipitate a decreases from band 3 to band 5. Both major precipitation arcs appear to be heterogeneous, each one being composed of 2–4 distinct precipitation lines. For bands 3, 4A and 4B some of the precipitation lines show partial identity [31]. The anodic leg of peak b does not reach the base line and splits into several lines, some of which fuse with precipitate a (Figs 4 and 5, arrows). Furthermore, in the case of band 3, where precipitate a crosses precipitate b, a characteristic depression of precipitate a is seen (Fig. 5A, open arrow). This indicates a partial identity between precipitates a and b or complexing between the corresponding antigens.

The third group comprises bands 6–8, all of which gave rise to a faint single

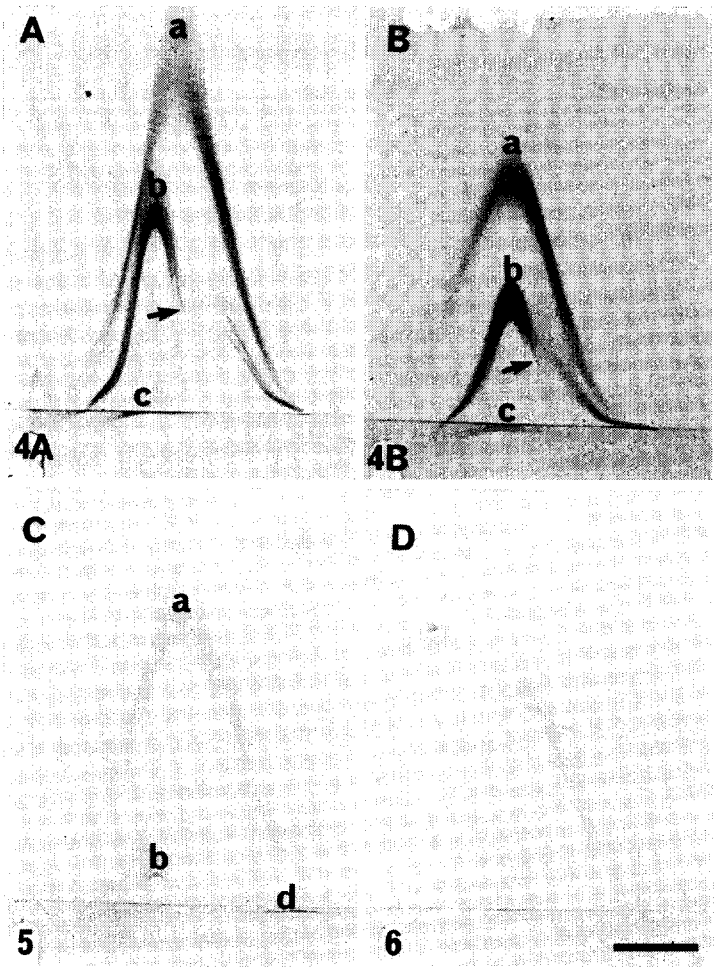

Fig. 5. Crossed immunoelectrophoresis of bands 4A, 4B, 5 and 6. (A) 25 μl of band 4A, (b) 35 μl of band 4B, (C) 25 μl of band 5, and (D) 25 μl of band 6. The amount of anti-membrane antibodies was 10 $\mu l \cdot cm^{-2}$. Experimental conditions and the other figure designations were as for the experiment of Fig. 4.

symmetrical precipitation arc in crossed immunoelectrophoresis (Fig. 5D).

The precipitation patterns of the individual bands were reproducible with four membrane preparations. The height of each precipitate was proportional to the amount of protein applied. Control experiments performed without antibody, or with γ-globulins from a non-immunized rabbit, demonstrated that none of the observed precipitates were due to non-specific aggregation or precipitation of proteins during the electrophoresis. Also, without addition of Berol to the gel, sample bands 1–5 revealed immunochemical heterogeneity, but fewer and fainter precipitates were seen.

Addition of antibodies against human serum protein did not change the height of any of the precipitates shown in Fig. 3 or reveal new precipitates. However, in some membrane preparations, albumin was found as contaminant in bands 4–8, possibly deriving from contaminated instruments.

Immunochemical examination for serum lipoproteins in the membrane preparation

Rabbit antibodies to human serum α-1- and β-lipoproteins were employed and, because non-ionic detergents hamper immunochemical analyses of serum lipoproteins [33], the experiments were performed in detergent-free gels. (1) A faint precipitate was observed when dodecyl sulfate-solubilized membrane material was tested in double-diffusion. (2) No immunoprecipitates were seen when dodecyl sulfate-solubilized membrane proteins were subjected to crossed immunoelectrophoresis using the same antibodies. 20 μg of membrane protein were analysed in gels containing from 0.5 μl to 2.5 μl antibody solution per cm^2 of gel [3]. (3) Moreover, no immunoprecipitates were observed when the EDTA extract (without dodecyl sulfate) was examined. (4) Furthermore, the sensitive intermediate gel technique [28] failed in the demonstration of antigenic determinants in membrane material common with serum lipoproteins. Thus no changes of the precipitation pattern of crossed immunoelectrophoresis of dodecyl sulfate-solubilized membrane proteins (Fig. 2A) were observed when antibodies against the serum lipoproteins (25 $\mu l \cdot cm^{-2}$) were incorporated in the intermediate gel. (5) Our isolated dodecyl sulfate bands of the erythrocyte membrane proteins did not yield precipitates with these antibodies in either double diffusion or rocket immunoelectrophoresis. (6) Finally, examinations of our anti-erythrocyte membrane antibodies for activity against the two serum lipoproteins also gave negative results in direct examination and with the intermediate gel technique. In crossed immunoelectrophoresis of 1 μl of fresh human serum, using an intermediate gel containing 300 μl of anti-membrane antibodies at a concentration of 30 $\mu l \cdot cm^{-2}$ (0.8 $mg \cdot cm^{-2}$) no change was observed in the α-1- or β-lipoprotein immunoprecipitate in the upper reference gel developed with specific antibodies against the lipoproteins.

Common antigenic constituents among the major dodeyl sulfate bands of erythrocyte membrane proteins

All bands were examined for possible cross-reactions by means of crossed-line immunoelectrophoresis [29]. Control experiments with blank intermediate gels were

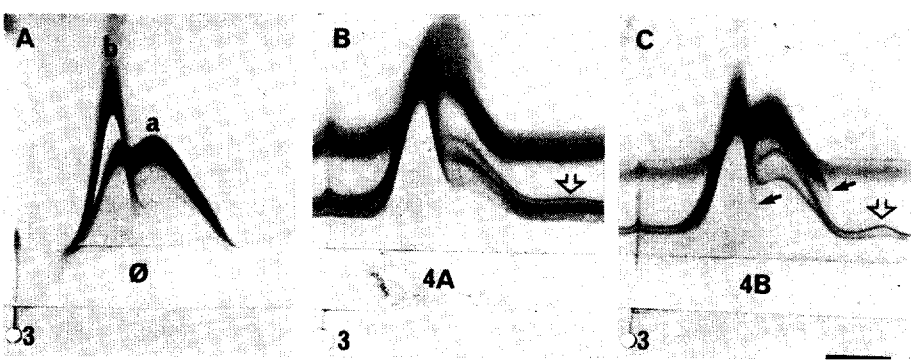

Fig. 6. Crossed-line immunoelectrophoresis of band 3 (7 μl). (A) Control with blank intermediate gel (Ø). (B) 100 μl of band 4A in the intermediate gel. (C) 100 μl of band 4B in the intermediate gel. All plates contained 10 μl anti-membrane antibody per cm^2. Filled arrows indicate precipitates that do not fuse with horizontal precipitation lines. Open arrows indicate elevation of the horizontal lines that do not correspond to any precipitates. Experimental conditions are otherwise as in Fig. 5.

carried out for every crossed-line immunoelectrophoresis. Cross-reactions are established when a precipitation arc is elevated in comparison to the control, or when a precipitation arc is fused on to a line precipitate [30]. The cross-reactions between the antigenic components of bands 1, 2 and 2.1 have been described in a previous report [16]. The same cross reactions were found in our present studies, but no cross-reactions with the other 7 bands were observed. Bands 3, 4A, 4B and 5 were also shown to have antigenic components in common. The components of precipitate a and b were common for bands 3, 4A and 4B, seen by the reaction of identity between the precipitates in crossed-line experiments (Fig. 6). Compared to the control (Fig. 6A), the position of precipitates a and b for band 3 were elevated by the uniform incorporation of bands 4A and 4B in the intermediate gel, respectively (Figs 6B and 6C). Fusing of the precipitation lines of band 3 with the horizontal line-precipitates for bands 4A and 4B is clearly observable. However, not all precipitation arcs show this behavior. The arrows in Fig. 6C indicate non-fused precipitation arcs, the finding of which shows that band 4B is deficient in antigenic determinants components present in band 3. The components giving rise to peak a for band 5 also cross-reacted with a from band 3. In crossed-line immunoelectrophoresis with band 5 in the intermediate gel, precipitate a for band 3 was elevated and some of the distinct precipitation lines of peak a showed the formation of outward feet analogous to the formation of inward feet described in ref. 28, whereas horizontal line-precipitates were not definitively established, possibly due to the relative faintness of the precipitates for band 5. The precipitates for bands 6, 7 and 8 were too faint to give clear results about possible cross-reactions. Precipitates c for bands 4A and 4B and d for band 5 represented other antigens. Thus precipitate c was still seen when antibodies absorbed with intact erythrocytes were used (see the section below). The antigens of c and d were not present in the other bands and did not cross-react with MN-glycoprotein. Furthermore, non-precipitating antigenic material was found in band 3, observable through

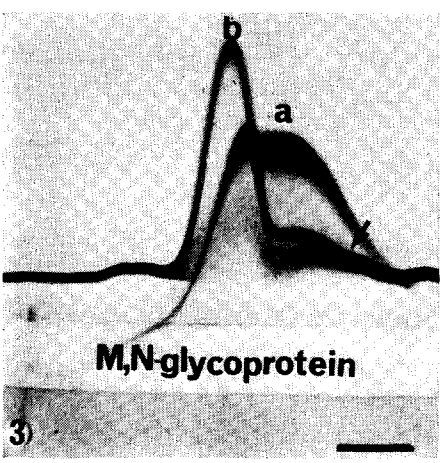

Fig. 7. Crossed-line immunoelectrophoresis of band 3 (7 μl) with 2.5 μg MN-glycoprotein in the intermediate gel. 8 $\mu l \cdot cm^{-2}$ anti-membrane antibody. Precipitate b fuses with the horizontal line-precipitate of MN-glycoprotein which also consists of three distinct narrow precipitation lines (arrow). Precipitate a appears unaffected. Experimental conditions and figure designations are otherwise as in Fig. 4.

elevation of the line-precipitate on the anodic side of precipitate *a* (Figs 6B and 6C, open arrow). This elevation did not correspond to any precipitate for band 3 (Fig. 6A).

Line immunoelectrophoresis of purified MN-glycoprotein, prepared without use of detergent, gave rise to 3 lines, similar to the lower line of Fig. 6B. Crossed-line immunoelectrophoresis of the different dodecyl sulfate bands with MN-glycoprotein in the intermediate gel gave rise to elevation of precipitate b for bands 3–5 together with a complete reaction of identity. The horizontal line-precipitate of MN-glycoprotein consisting of three narrow distinct precipitation lines fuse with the lines of precipitate b (Fig. 7, arrow). Precipitate a appeared unaffected.

Exposed and hidden antigenic determinants of the erythrocyte membrane

Immunoelectrophoretic analysis using an antibody solution which has been absorbed with intact erythrocytes allowed us to detect antigens exposed on the outside of the cells [21], because the intact cells remove antibodies directed against the antigenic determinants exposed on the outside of the erythrocytes only. Rocket immunoelectrophoresis of the isolated dodecyl sulfate bands employing such a depleted antibody solution shows that some precipitates were unaffected (bands 1, 2 and 2.1), other (bands 3–5) did not develop and finally other precipitates increased in height (bands 3–5, 7) (compare Figs 3A and 3B). The rockets of bands 6 and 8 were faint and the height ill-defined so that no conclusions could be drawn. Crossed immunoelectrophoresis of band 3 shows that precipitate b disappeared (Fig. 4). Precipitate a appeared more blurred but lacked the depression where peak b should have crossed. Furthermore, the area below precipitate a of Fig. 4B was larger than that of Fig. 4A, where a non-depleted antibody solution diluted to the same extent as that used in Fig. 4B was used. This increase in area is due to a corresponding titre decrease of the absorbed antibody preparation, indicating that some antigenic determinants of the antigen corresponding to precipitate a are situated on the outer cell surface.

When antibodies were absorbed with an excess of isolated membranes, no precipitates were seen at all, indicating that the antigenic determinants observed are components of the outer and/or inner surfaces, not of the membrane core.

DISCUSSION

Dodecyl sulfate electrophoresis allows high resolution fractionation of proteins in numerous cellular membranes. However, the procedure causes denaturation of the membrane proteins and generally interferes with the assignment of function or immunochemical specificity to the separated bands.

Our present studies show (1) that extensive immunochemical characterisation of membrane proteins can be achieved in non-ionic detergents, (2) that small amounts of sodium dodecyl sulfate do not interfere with the immunoelectrophoretic analysis of several well characterized soluble proteins in the presence of non-ionic detergents and (3) that membrane proteins separated by dodecyl sulfate electrophoresis can be well characterized by immunoelectrophoresis in non-ionic detergents. Formation of mixed micelles between the two types of detergents, whereby the critical micellar concentration of the ionic detergent is reduced to the level of the non-ionic detergent [34],

explains the effect of non-ionic detergent. Also, ionic and non-ionic detergents might compete for the same binding sites on solubilized protein [36, 37].

Non-ionic detergents do not generally affect antigen-antibody precipitation [2, 37], except in the case of certain serum lipoproteins [33]. Thus, the α-1-lipoprotein and β-lipoprotein of human serum fail to give immunoprecipitates when analysed in the presence of 1 % of non-ionic detergent.

Under our conditions, artifacts due to the presence of dodecyl sulfate are unlikely. The precipitation patterns of model proteins upon immunoelectrophoresis in 1 % Berol are normal in the presence of up to 100 nmol of dodecyl sulfate. This amount of dodecyl sulfate is 10 times more than that applied with membrane fractions isolated by preparative polyacrylamide electrophoresis in dodecyl sulfate. Moreover, in two-dimensional immunoelectrophoresis, the amount of dodecyl sulfate associated with membrane proteins is further reduced during electrophoresis in the first dimension, i.e. prior to immunoprecipitation.

Our studies are an extension of previous work, and indicate that many of the various components separated by dodecyl sulfate-polyacrylamide gel electrophoresis constitute more than one molecular entity. Thus, in the case of proteins from human erythrocyte membrane, multiple N-terminals have been found for bands 1, 2, 3, and 5 [25, 38, 39]. Glycoproteins are present near bands 3, 5 and 7 [1]. Moreover Steck and Dawson [40] have demonstrated the existence of another glycoprotein between band 4 and 5, and Tanner et al. [41] found a glycoprotein in the region of band 4. A recent study of EDTA- and Triton X-100-extractable membrane proteins, employing isoelectric focusing followed by dodecyl sulfate-polyacrylamide gel electrophoresis, revealed the presence of two major and three minor components in the region of band 3, one major and two minor non-glycosylated components in the region of band 5, and two components in the region of band 6 [42–44]. These results are in agreement with our present immunoelectrophoretic findings.

By the criterion of immunochemical identity [30] we find that common antigenic determinants exist between the components of bands 1–2.1, on one hand, and those of bands 3–5, on the other. The cross-reactions of the components of bands 1–2.1 have been discussed [16]; they lack antigenic determinants common to the other bands. The immunochemical data strongly support the contention forwarded on the basis of identical N-termini [38] that bands 1 and 2 are molecularly closely related. On the other hand, they show that little molecular similarity exists between the spectrins and the major components of band 3, in spite of the similarity in N-termini of these bands [38]. Finally, the data do not support the findings of Green et al. [45], whose study of EDTA extracts of bovine erythrocytes suggests that band 5 has antigenic determinants in common with bands 1–2.1.

Precipitates a and b of band 3 correspond to the major intrinsic or core protein of the membranes and MN-glycoprotein, respectively; these two entities dominate the region of band 3. Precipitate b has been directly identified with MN-glycoprotein by crossed-line electrophoresis (Fig. 7) and precipitate a gives a reaction of identity with purified major intrinsic protein. (Bhakdi, S., Bjerrum, O. J. and Knüfermann, H., to be published). The correlation of precipitate a and b with these two proteins was further supported by the vectorial studies using antibody preparation absorbed with intact erythrocytes. The antigenic material associated with precipitate b is exclusively located on the outer side of the membranes, whereas the

antigenic material giving rise to precipitate a is located on both surfaces of the cells. The results are analogous to those of labeling studies of erythrocytes [46–48]. The reaction of partial identity observed between precipitate a and b for bands 3–4B (Figs 4 and 5) might indicate formation of complexes between the proteins of these precipitates under the given experimental conditions. That the observed precipitation pattern should result from residual sodium dodecyl sulfate bound to the proteins [33] was rendered improbable as it disappeared in the precipitation patterns obtained with absorbed antibodies (Fig. 4).

The occurrence of identity reactions between MN-glycoprotein and bands 3–5 is not surprising, since glycoproteins related to the MN-glycoprotein aggregate in dodecyl sulfate electrophoresis to varying degrees [49, 50]. The various glycoprotein multimers might also be present in non-ionic detergents and could be expected to form multiple immunoprecipitates [21], as observed in our system. Similarly, the multiple immunoprecipitates seen for precipitate a could reflect various aggregation states of the major intrinsic protein, as the decrease in staining intensity of the precipitate seems to indicate (compare Figs 4A, 4B, 4C and 4D). The presence of antigenic material of this protein in bands of lower molecular weight than 90 000 is an interesting finding, which is presently under further investigation. It may indicate that the protein of band 3 is a dodecyl sulfate-stable complex composed of proteins of lower molecular weight, as also found for the MN-glycoprotein in band 3.

Presence of serum α-1- and β-lipoproteins in the erythrocyte membrane

Langdon [51] has recently suggested that about half of the membrane proteins of human erythrocyte membranes corresponds to the apo-proteins of serum α-1- and β-lipoproteins. This conclusion was based on (1) immunoprecipitation in double diffusion of dodecyl sulfate-solubilized membrane proteins with commercial goat antisera directed against either human serum α-1- or β-lipoprotein, and (2) similarities in NH_2-terminal and COOH-terminal amino acids. Green et al. [52] have recently shown that samples containing sodium dodecyl sulfate gave rise to artifactual precipitation lines in double diffusion experiments with whole rabbit antiserum. We also obtained these precipitates in double-diffusion experiments using rabbit antibodies against human α-1- and β-lipoproteins from another commercial supplier. However, these precipitates were not formed in corresponding immunoelectrophoresis experiments. This is possibly due to the applied current during the immunoprecipitation and use of purified antibodies. Similarly, we found no immunoprecipitates with the proteins isolated by the dodecyl sulfate-polyacrylamide gel electrophoresis or with crude EDTA extracts using either double immunodiffusion or immunoelectrophoresis. Furthermore, we detected no reaction between our anti-erythrocyte membrane antibodies [4] and serum α-1- and β-lipoproteins, using the sensitive technique of crossed immunoelectrophoresis with an intermediate gel containing the anti-membrane antibodies. Antibodies against serum lipoproteins did not affect the precipitation pattern of dodecyl sulfate-solubilized erythrocyte membrane proteins.

Since we have already shown that our anti-membrane antibody preparation precipitates nearly all membrane proteins [3], Langdon's suggestion [51] that the apoproteins constitute half of the membrane proteins cannot be correct. Only small amounts, if any, of serum α-1- and β-lipoproteins not reacting with our antibodies can be present in the erythrocyte membrane. The immunoprecipitates observed by

Langdon might be an artifact due to the presence of dodecyl sulfate [52] or might be due to contamination of the commercial antisera by anti-membrane antibody.

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