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THE IMMUNOCHEMICAL APPROACH TO THE CHARACTERIZATION OF MEMBRANE PROTEINS

HUMAN ERYTHROCYTE MEMBRANE PROTEINS ANALYSED AS A MODEL SYSTEM

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SUMMARY

1. Crossed immunoelectrophoresis was used for extensive characterization of individual proteins of human erythrocyte membranes solubilized in non-ionic detergent.

2. The precipitates were assigned to extrinsic or intrinsic proteins.

3. Four glycoproteins were identified by their lectin binding behaviour, whilst five proteins were affected by neuraminidase, indicating them to be sialoglycoproteins.

4. Enzymatic activity is retained in the solubilized system and the presence of acetylcholinesterase and an ATPase was demonstrated. The formation of phosphorylated membrane proteins on incubation with [^{32}P]ATP was demonstrated by autoradiography on the immunoelectrophoresis plates.

5. Five proteins located on the outer cell surface were identified by antibody binding to intact cells. These same proteins were degraded by proteolytic enzymes in intact cells but only three of them were labelled by lactoperoxidase-catalysed ^{125}I-iodination.

6. Analysis of erythrocyte membrane proteins using quantitive immunoelectrophoresis yields results concordant with those obtained by dodecyl sulfate-polyacrylamide gel electrophoresis.

INTRODUCTION

Solubilisation of membrane proteins for the investigation of their structural and functional properties is at the present time best achieved using non-ionic detergents. It is believed that this is because the membrane proteins are then present in a state closely resembling their natural state in the membrane [1–3]. Normally, however, analysis of the solubilized proteins in the presence of the non-ionic detergent has proved difficult because of the lack of a simple analytical system comparable to that

provided by dodecyl sulfate-gel electrophoresis. However, immunoelectrophoretic analysis in the presence of such detergents is possible because the antigenicity of most membrane proteins is retained after solubilization with non-ionic detergent. This is probably due to selective binding of the non-ionic detergent to the hydrophobic part of the membrane proteins [3, 4] leaving the antigenic determinants unaffected and free to react with antibodies.

We have previously demonstrated the use of quantitative immunoelectrophoresis for analysis of erythrocyte membrane proteins [5, 6] and the method has recently been extended to other membrane systems [7–13]. In the present communication we describe further studies on erythrocyte membrane proteins together with techniques for the characterization of membrane proteins on molecular basis without prior purification. In addition, we employ a new kind of "molecular amplification" whereby membrane proteins carrying single antigenic determinants on the outer surface can be identified.

A similar model study involving characterization of membrane proteins of bovine milk fat globules will be published later.

MATERIALS AND METHODS

Membrane material. Erythrocyte membranes were prepared according to Dodge et al. [14], as elaborated in ref. 15. The protein concentration was determined by measuring $E_{280\ nm}$ after addition of sodium dodecyl sulfate [16]. The membranes were solubilized to a final protein concentration of 2 mg/ml in a buffer of 0.010 M glycine, 0.0038 M Tris (pH 9.2, 4 °C) containing 1 % (w/v) of the non-ionic detergent Berol EMU-043 (MoDoKemi, Stenungsund, Sweden) by sonicating the ice-cold solution for 3×5 s at 20 000 Hz (Branson Sonifier B12 with microtip). The supernatants obtained after centrifugation for $6 \cdot 10^6 \times g_{av} \cdot$ min were divided into small aliquots and stored at −20 °C for subsequent immunoelectrophoretic analysis.

Salt extraction of erythrocyte membranes was performed with 0.154 M NaCl, 0.008 M phosphate buffer (pH 7.4) by gentle stirring for 30 min at 4 °C (final protein concentration 3 mg/ml). The supernatant obtained after centrifugation for $3 \cdot 10^6 \times g_{av} \cdot$ min was used directly for immunoelectrophoresis. Dodecyl sulfate-polyacrylamide gel electrophoresis showed the presence of band VI (glyceraldehyde-3-phosphate dehydrogenase) in the extract [17].

EDTA extraction of erythrocyte membranes was performed with 0.5 mM EDTA, 0.5 mM NaN_3 (pH 8.0) by dialysis against 500 volumes of the same buffer overnight at 20 °C (final protein concentration 2 mg/ml.). The supernatant obtained by centrifugation for $6 \cdot 10^6 \times g_{av} \cdot$ min was used directly for immunoelectrophoresis. Crude spectrin was isolated from the EDTA extract by gel filtration on Sephadex G-200 [18].

Membrane-depleted lysate was obtained by lysis of washed (five times) erythrocytes in four volumes of 0.008 M phosphate buffer (pH 7.4) and centrifugation for for $6 \cdot 10^6 \times g_{av} \cdot$ min.

Protein was determined by the method of Lowry et al. [19] with bovine albumin as standard, as elaborated in ref. 6.

Antibodies. Rabbit antibodies against code 100 SF human serum proteins, albumin and haptoglobin, and swine antibodies against rabbit IgG were obtained

from Dakopatts A/S, Copenhagen. Rabbit antibodies against human erythrocyte membrane material were produced and purified as described previously [6]. Immunoglobulin concentration was 24 mg/ml. Aprotinin (see next section) was added to the antibodies to a concentration of 1000 K.I.E./ml, so as to inhibit plasmin present in the antibody preparation [20]. Aprotinin does not affect the antigen-antibody reaction [21]. Crossed immunoelectrophoresis of serum with anti-membrane antibodies in the intermediate gel [22] revealed the presence of precipitating antibodies against serum albumin and haptoglobin in the anti-membrane antibody preparation. No absorption was performed. Anti-membrane antibodies similar to those used in the work described here are available from Dakopatts A/S. Rabbit antibodies against membrane-depleted lysate of human erythrocytes were prepared as described in ref. 23.

Enzymes and inhibitors. Trypsin (crystalline, 4200 Anson units/g) and α-chymotrypsin (crystalline, 1085 NF/mg) were obtained from NOVO A/S, Copenhagen, pronase (B grade, 45 000 P.U.K./g) from Calbiochem, neuraminidase (V. Comma, 500 units/ml) from Behringswerke AG, soya bean trypsin inhibitor (Type I S) from Sigma, and Aprotinin (10 000 K.I.E./ml) from Novo GmbH, Mainz).

Lectins. The following lectins were employed: Free concanavalin A, concanavalin A coupled to Sepharose, leucoagglutinin (all from Pharmacia Fine Chemicals), *Ricinus communis* lectin (a gift from Dr. J. P. Susz); its activity was tested by the interaction with serum proteins containing galactose [24], wheat germ agglutinin (code 79-016), fucose binding protein (code 79-014), soya bean agglutinin (code 79-018) (all from Miles-Yeda Ltd.), pokeweed mitogen (code 536, Gibco, Grand Island, N.Y.), and phytohaemagglutinin P (code 3110-56, Difco).

Immunoelectrophoretic methods. Electrophoresis conditions were as described previously [6]. The agarose employed (AGS192) was from Litex A/S, Glostrup, Denmark. Crossed, crossed-line [25] tandem-crossed, and crossed immunoelectrophoresis with an intermediate gel were performed as described in refs. 6, 22, 26 and 22, respectively. Normally, 20 μg of solubilized membrane protein was electrophoresed at 10 V/cm for 60 min in the first dimension gel and at 2 V/cm for 18 h in the second dimension gel. The immunoglobulin concentration was 6.5 $\mu l/cm^2$. Crossed immuno-affinoelectrophoresis with agarose-bound lectin in the intermediate gel and free lectin in the first dimension gel was performed as described in refs. 27 and 28. Staining for protein [23] and lipoprotein [29] was performed with Coomassie brilliant blue R (Gurr, Searle) and Sudan black B (Merck), respectively.

Staining for enzymatic activity. Acetylcholinesterase activity was demonstrated as in ref. 15. Peroxidase activity of immunoprecipitated haemoglobin was identified by the blue colour reaction which takes place on incubation (15 min, 20 °C) with a freshly prepared staining solution consisting of benzidine (1 %, w/v) (Sigma), H_2O_2 (2 %, w/v) and acetic acid (20 %, w/v). ATPase activity was demonstrated by histochemical staining according to Gomori [30]. The washed and dried immunoplate was incubated overnight at 37 °C in a solution containing 0.002 M ATP (grade II, Sigma), 0.01 M $MgSO_4$, 0.002 M Pb $(NO_3)_2$ and 0.07 M Tris · HCl (pH 7.2). The plate was then rinsed in water and incubated with 0.4 M $(NH_4)_2S$ solution. Precipitated PbS appeared brownish yellow in the gel. No staining occurred in controls lacking ATP.

Formation of phosphorylated membrane proteins was demonstrated according to Guthrow et al. [31], using radioactive phosphate. The assay mixture (total volume 0.4 ml) contained erythrocyte membrane material corresponding to 0.35 mg of

protein together with 3 μmol of sodium phosphate buffer (pH 6.5), 3.5 μmol of $MgCl_2$, 0.15 μmol EGTA (ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid) and 2 nmol of [^{32}P]ATP (20 μCi, New England Nuclear). After incubation for 5 min at room temperature, 0.6 ml of ice-cold 0.008 mM sodium phosphate buffer (pH 7.4) was added and the mixture centrifuged for $6 \cdot 10^5 \times g_{av} \cdot$ min at 4 °C. The washing was repeated once with 1 ml of the same buffer. The pellet was solubilized as described above and used directly for immunoelectrophoresis. After washing, drying and staining of the immunoplates, autoradiography (4 days exposure) was performed (RPX-omat film, Kodak).

Lectin binding. Aliquots of solubilized membrane proteins (1.3 mg/ml) were mixed for 20 min at 20 °C with the following lectins dissolved in a 0.0038 M Tris/0.01 M glycine buffer (pH 8.7) (final lectin concentration 1.0 mg/ml): concanavalin A, *R. communis* lectin, wheat germ agglutinin, fucose binding lectin, soya bean agglutinin, leucoagglutinin, phytohaemagglutinin P and pokeweed mitogen. After centrifugation for $3 \cdot 10^6 \times g_{av} \cdot$ min the supernatants were subjected to crossed immunoelectrophoresis. Controls lacking lectin were run in parallel. In the precipitation patterns which showed changes the specificity of the lectin-glycoprotein interaction was examined by inhibition studies. Sugars which are known to bind to the respective lectins [32] were mixed with the lectins to a concentration of 5 % (w/v) before the addition to the membrane proteins, and the first dimension electrophoresis was performed with 5 % (w/v) of sugar in the gel according to Bøg-Hansen et al. [33]. Restoration or partial restoration of the original patterns was taken to indicate specific lectin binding. Crossed immuno-affinoelectrophoresis was performed with 75 μg of lectin per cm^2 of gel [27, 28].

^{125}I labelling. Peroxidase-catalysed ^{125}I iodination of intact erythrocytes was performed exactly as described by Morrison [34], using lactoperoxidase from milk (57 units/mg; Sigma). Preparation of membranes, solubilization and autoradiography (for 3 weeks) were performed as described above.

Enzymatic degradation. Aliquots of washed intact erythrocytes [15] compacted for $3750 \times g_{av} \cdot$ min were incubated with gentle agitation for 1 h at 37 °C with equal volumes of isotonic phosphate buffer (pH 7.4) containing trypsin (0.1–10 mg/ml), α-chymotrypsin (0.3–20 mg/ml) and pronase (0.01–10 mg/ml), respectively. In the case of the first two enzymes the reaction was stopped by washing the cells twice with five volumes of isotonic phosphate buffer containing trypsin inhibitor (0.05–8 mg/ml) and aprotinin (2 %, w/v). With pronase, four washings with isotonic phosphate buffer containing 0.5 % (w/v) of human albumin (reinst, Behringswerke) were performed [35]. Haemolysis was negligible. The membranes were prepared and solubilized as described above, the only difference being the addition of 0.2 % (w/v) of aprotinin. Incubation of the erythrocytes with neuraminidase (25–400 units/ml) was performed according to the same scheme except for the last washing and incubation, which were performed in 0.030 M acetate buffer (pH 5.5) containing 0.125 M NaCl and 0.01 M $CaCl_2$. The cells, showing slight hemolysis were washed twice with isotonic phosphate buffer before lysis.

Isolated membranes in 0.008 M phosphate buffer (protein concentration 10 mg/ml) were subjected to enzymatic degradation as described above, using trypsin (0.04–5 mg/ml) and α-chymotrypsin (0.04–5 mg/ml), respectively. The reaction was stopped by washing with 0.008 M phosphate buffer containing the above-mentioned

inhibitors. Incubation with neuraminidase (150–500 units/ml) was performed as described above.

Solubilized membrane proteins (2 mg/ml) were incubated as described for the membranes, using equal volumes of trypsin (0.008–1 mg/ml) and α-chymotrypsin (0.04–5 mg/ml), respectively. The reaction was stopped by addition of trypsin inhibitor (0.016–10 mg/ml) and aprotinin (1000 K.I.E./ml).

Antibody binding to intact cells. Absorption of the anti-membrane antibody preparation with intact erythrocytes was performed as described in ref. 15. That the correct dilution of the control antibody was obtained was checked in the immunoelectrophoresis by comparing the heights of a rocket precipitate for purified spectrin placed in the absorbed and the control antibody-containing gels [36]. Binding of antibodies to the cells was achieved by mixing 1 ml of compacted, washed erythrocytes with 1 ml of the antibody preparation and 4 ml of 0.154 M NaCl, and incubating for 30 min at 4 °C. The partly agglutinated erythrocytes were washed with 5 × 10 ml of 0.154 M NaCl. A control was prepared and solubilized as described above.

RESULTS

The precipitation pattern obtained by crossed immunoelectrophoresis of human erythrocyte membrane proteins solubilized in non-ionic detergent Berol

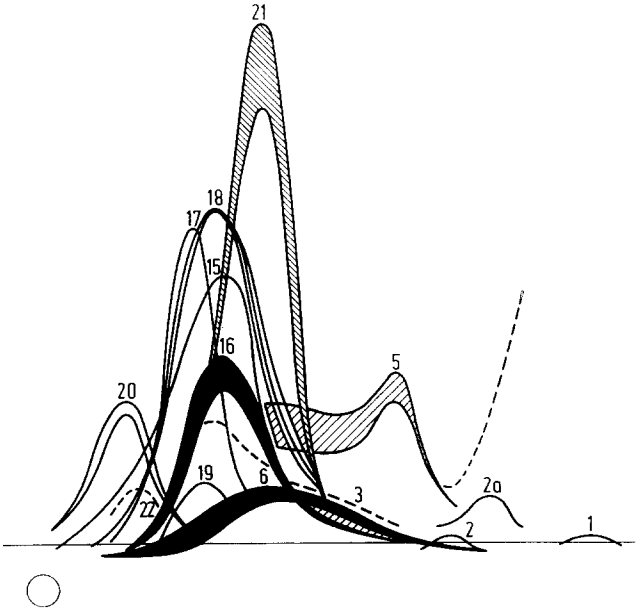

Fig. 1. A drawing, based on results obtained with 10 membrane preparations, of the crossed immunoelectrophoresis precipitation pattern for human erythrocyte membrane proteins solubilized with the non-ionic detergent Berol EMU-043, where secondary degradation has been avoided by addition of aprotinin to the antibody preparation. "Erratic" precipitates are shown as dotted lines. First dimension electrophoresis was performed for 60 min at 10 V/cm. Anode to the right. Second dimension electrophoresis was performed at 3 V/cm for 18 h. Anode at the top. The plates were stained for esterase (1-naphthyl acetate, Fast red TR) and protein (Coomassie brilliant Blue R).

EMU-043 is drawn in Fig. 1. The precipitates are numbered in accordance with previously published patterns [6, 15]. The pattern shows no obvious signs of protein degradation because the proteolytic enzyme plasmin which contaminates the isolated immunoglobulin fraction of the antibodies has been inhibited by addition of aprotinin [20]. This inhibition resulted in the disappearance of the degradation products of spectrin (precipitates 7–12), and led to formation of more distinct precipitates delimiting relatively smaller areas (Nos. 5, 12, 16, 18, and 21). Other changes in the pattern and the appearance of the new precipitates 2a and 22 can be explained by the fact that a new pool of antibodies was used.

The drawing is based on the results obtained for 10 different membrane preparations. The full lines represent precipitates seen with all the preparations, whereas the dotted lines represent those which appeared more irregularly. No. 1 is an endogenous degradation product of protein 16 [5, 6] (cf. Fig. 2C). When present precipitate 3 can vary considerably in shape, only the descending leg being seen in some cases (cf. Fig. 7). Where precipitate 3 crosses precipitate 16 a dislocation is observed, indicating a weak reaction of partial identity [37] (cf. Fig. 5). No. 5 also shows partial identity with No. 16 since the cathodic leg stops at No. 16. However, instead of direct fusion a clear zone is observed as indicated on Fig. 1; this is an example of the so-called "sink" effect [38]. Sometimes No. 5 may show a doubling of precipitation lines and an anodic "flying" precipitate (cf. Figs. 2c and 9). The latter is probably a result of degradation, since it is also seen after exposure to proteolytic enzymes (cf. Fig. 10). Precipitate 6 conceals two other immunochemically different precipitates, both of which are related to spectrin since they are seen with preparations of certain antibodies raised against spectrin preparations [23]. Slight degradation can also reveal their presence, as can be seen for instance in Fig. 8. No. 18, which is composed of several fine distinct precipitation lines (cf. Figs. 8 and 10), is not seen in fresh unfrozen preparations of membranes of freshly drawn erythrocytes (cf. Figs. 6 and 7). However, with fresh preparations of outdated bank blood and with frozen preparations the precipitate is observed.

Four parameters were used to recognize the individual precipitates: (1) The electrophoretic migration relative to serum albumin (No. 2) measured horizontally from peak to peak of the precipitates. The mean values obtained for 10 different membrane preparations are given in Table I. (2) The staining intensity and sharpness of the precipitates (see Table I). Some precipitates (Nos. 5, 6, 18 and 21) are composed of several distinct lines. (3) The shape of the precipitates (see Fig. 1). Some are asymmetric (Nos. 3, 5, 6 and 16). (4) The relative area below the precipitates. An impression of the variation of this parameter is obtained by comparing Figs. 2 and 4–11, which correspond to different membrane preparations.

The modified solubilization procedure described in the present work is just as efficient as that described previously [6]. Sonication of the stated intensity and duration did not change the precipitation pattern to any appreciable extent. Results concerning the stability and reproducibility of the solubilized material have been reported previously [6].

That all the immunoprecipitates are those of proteins is shown by the fact that digestion of the membrane proteins with proteolytic enzymes resulted in disappearance of the precipitates (see section below: Proteolytic degradation). Staining with Sudan black did not reveal the presence of any lipid in the precipitates. However, the

TABLE I

SOME CHARACTERISTICS OF THE PRECIPITATES AND THEIR CORRESPONDING HUMAN ERYTHROCYTE MEMBRANE PROTEINS OBSERVED IN CROSSED IMMUNOELECTROPHORESIS

The electrophoreses were performed in 0.100 M glycine, 0.038 M Tris buffer (pH 8.9, 16 °C) containing 1 % (w/v) of Berol EMU-043. Protein numbering is according to Fig. 1. Relative migration velocity is measured horizontally from the peak of the relevant precipitate to the peak of the serum albumin precipitate (No. 2) using the front edge of the application well as zero point. "Stain" refers to the visual dye uptake of the precipitate (+++, heavy; +, faint). "Sharpness" refers to the morphology of the precipitation line (+++, distinct; +, blurred). The asterix denotes an irregularly occurring precipitate.

Protein No.	Relative migration velocity ±S.D. %	Stain	Sharpness	Sialoglyco-protein	Exposure on the outer surface	Enzymatic activity	Name
2	1.00	+	+++				serum albumin
2a	1.09±0.02	+	++				
3*	–	+	+				
5	0.74±0.12	++	+				
6	0.61±0.05	+	+				spectrin(s)
15	0.39±0.04	++	+			peroxidase	haemoglobin
16	0.43±0.02	+++	++	+	+	ATPase	major "intrinsic" protein
17	0.32±0.03	+	+				
18	0.38±0.03	+	+	+	+		major "intrinsic" protein
19	0.32±0.03	+	+	+	+		
20	0.15±0.02			+	+	esterase	acetylcholinesterase
21	0.46±0.03	+	+	+	+		MN-glycoprotein
22*	–	+	+				

presence of lipids was easily demonstrated after the first dimension electrophoresis, where they follow the proteins.

There exist techniques which make it possible to directly identify the immunoprecipitates obtained in quantitative immunoelectrophoresis [22, 25, 26], provided that characterized, purified proteins and/or mono-specific antibodies are available. Thus, using purified spectrin as the line in crossed-line immunoelectrophoresis [25] a reaction of full immunochemical identity was found between precipitate 6 and the line, showing that precipitate 6 corresponds to spectrin. In an earlier investigation the same technique was used to show that precipitate 21 corresponds to the major MN-glycoprotein (glycophorin) [15]. In the sections below we describe how further characterization of the proteins can be achieved.

Membrane specificity of precipitates

The anti-membrane antibodies for this investigation were non-absorbed. That the precipitated proteins were of membrane origin was therefore checked by crossed immunoelectrophoresis of Berol-solubilized membrane material using an intermediate gel containing (a) serum and (b) membrane-depleted lysate and by employing antibodies against first (a) then (b) in each case. Only one precipitate (No. 2) corresponds to a serum protein (albumin). In the lysate/antilysate system protein 15 was dominant and it was identified as haemoglobin by its peroxidase activity (see below). Membrane-depleted lysate also contained traces of protein 6 and 17. A detailed description of experimental methodology and the interpretation of the precipitation patterns obtained is to be found in ref. 23.

Extrinsic and intrinsic proteins

Proteins extracted from the erythrocyte membranes by media of low and high ionic strength were examined by crossed immunoelectrophoresis. The pattern observed for an EDTA extract of human erythrocyte membranes is shown in Fig. 2A. The six precipitates observed were numbered in relation to the reference pattern (Fig. 1) via a crossed-line immunoelectrophoresis with the EDTA extract in the intermediate gel (Figs. 2B and 2C). This EDTA extract contained proteins 1, 2, 6, 21 and x but none or only very slight amounts of proteins 5, 16, 17, 18, 19 and 20. Haemoglobin (No. 15) was not present because white ghosts were used for this experiment. The pattern shows signs of proteolytic degradation indicated by the small anodic peak and splitting of precipitate 6 [40] the presence of precipitate 1 [6] and the reaction of partial identity between precipitate x and 21 (Fig. 2A, arrow) [37]. Degradation was difficult to avoid completely but in non-degraded samples Nos. 1, 21 and x disappeared. The electrophoresis pattern for an extract prepared using 0.154 M NaCl (pH. 7.4) is shown in Fig. 3A. With the exception of one irregularly shaped precipitate the pattern is similar to that for a control extract prepared with 0.008 M phosphate buffer (pH 7.4) (Fig. 3B). The shape of the precipitate was variable and in crossed-line immunoelectrophoresis it could not be correlated to any of the precipitates of the reference pattern (Fig. 1).

This classification of membrane proteins was also correlated to the extent to which precipitates were formed subsequent to removal of detergent from the gels (Fig. 4). Without detergent in the gels aggregation of some of the proteins took place, giving rise to higher migration velocity and the appearance of irregular, blurred

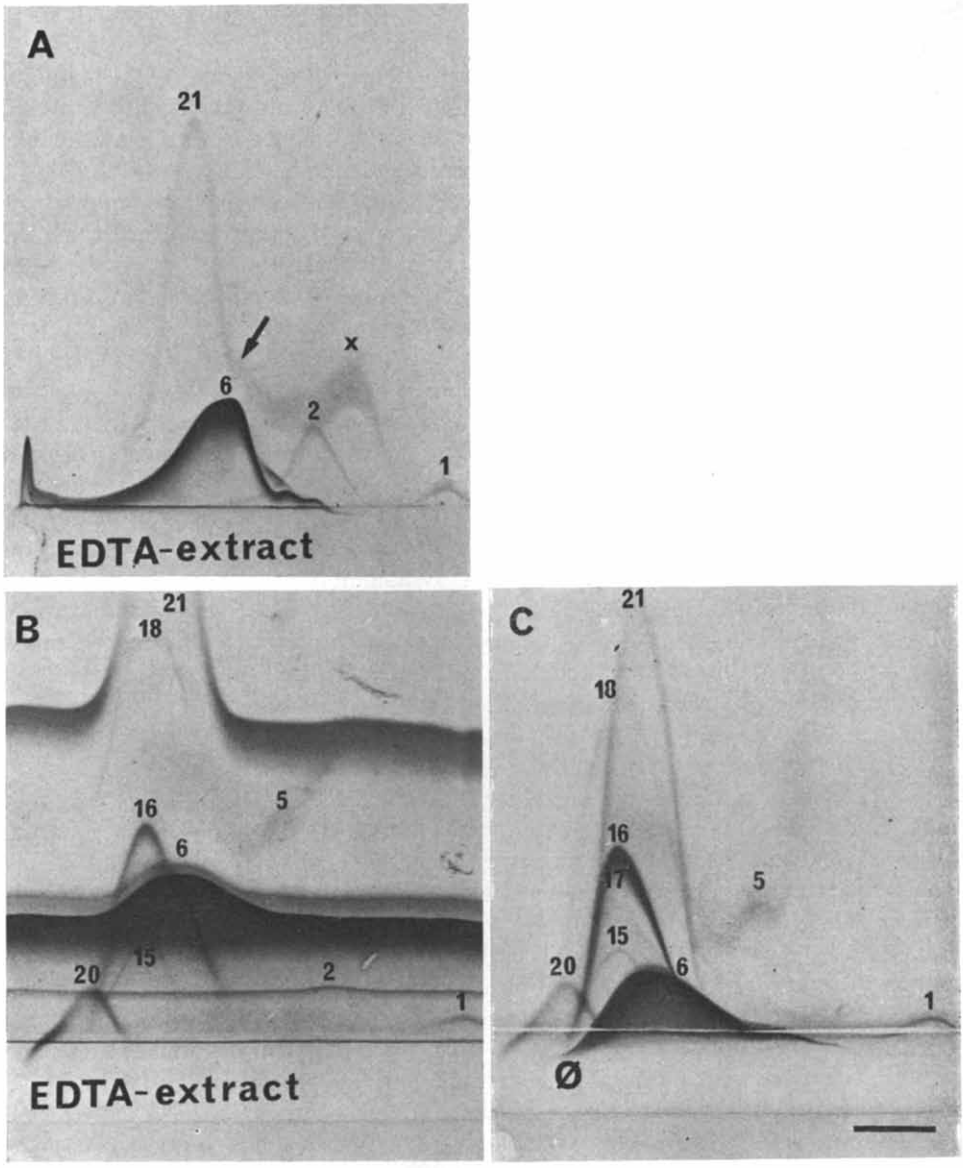

Fig. 2. Crossed immunoelectrophoresis of 8 μg (25 μl) of an EDTA-extract of human erythrocyte membranes (A). Identification of the precipitates was achieved by comparing the crossed-line immunoelectrophoresis pattern for 20 μg of Berol-solubilized erythrocyte membrane proteins obtained with 93 μg (300 μl) of EDTA extract in the intermediate gel (B) with that of a control (C). Elevated precipitates indicate the presence of the corresponding proteins in the extracts. The arrow indicates a reaction of partial identity. Antibody content of the gels: A, 3.5 μl/cm^2, B and C, 6.1 μl/cm^2. Experimental conditions were as for the experiment of Fig. 1. The bar represents 1 cm.

precipitates consisting of multiple lines. The area delimited by the precipitates also increased. Furthermore, in another experiment the activity of the intrinsic membrane protein acetylcholinesterase [39] was found to be associated with several precipitates,

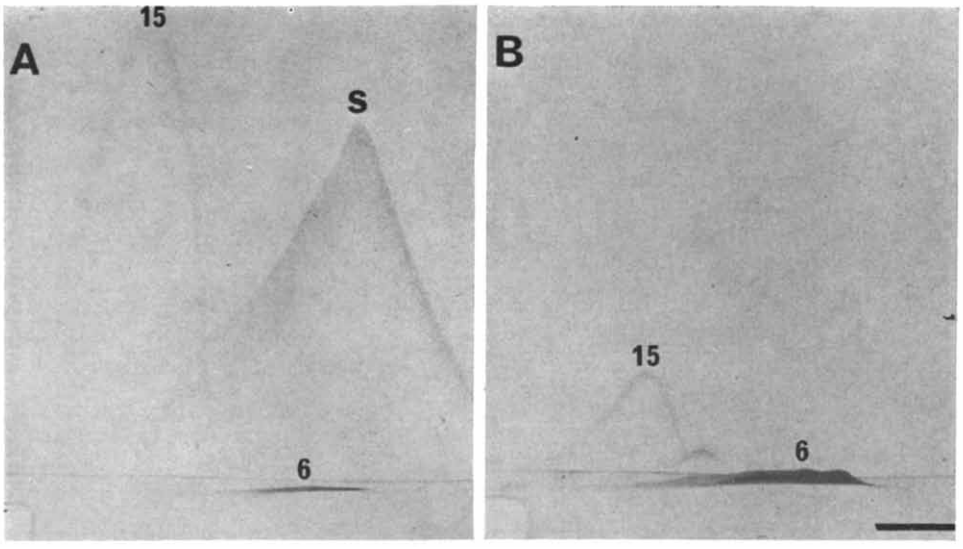

Fig. 3. Crossed immunoelectrophoresis of 3.5 μg (50 μl) of a salt extract (0.154 M NaCl, pH 7.4) of (A) human erythrocyte membranes and (B) a control extract with 0.008 M phosphate buffer (pH 7.4). Note the irregularly shaped precipitates in A, probably corresponding to glyceraldehyde-3-phosphate dehydrogenase. Antibody content of the gels: 5.3 μl/cm^2. Experimental conditions were as for the experiment of Fig. 1; esterase staining has not been performed. The bar represents 1 cm.

in contrast with its localization in one well-defined precipitate (No. 20) in the presence of detergent. Since hydrophilic proteins do not normally bind to non-ionic detergent [3, 4] and they are not affected by the presence of non-ionic detergent in immunoelectrophoresis [5] (as also found to be the case for the EDTA-extracted proteins), those precipitates which shows changes probably correspond to membrane proteins which bind detergents i.e. intrinsic proteins. Comparison of A and B of Fig. 4 shows that precipitates 2, 6, 15 and 17 remained bellshaped (extrinsic proteins), whereas precipitates 2a, 5, 16, 21 and 22 were changed (intrinsic proteins). Other experiments show that proteins 18, 19 and 20 also belong to the latter group. Thus, with the exception of protein 17 agreement between the results obtained by extraction and by detergent binding was found.

Enzymatic activity

The occurrence of several types of enzymatic activity was demonstrated by histochemical methods. Acetylcholinesterase appeared as precipitate 20 (see Fig. 2C) [15]. The peroxidase activity of haemoglobin correlated this protein to precipitate 15. Liberation of phosphate on incubation with ATP [30] revealed ATP-ase activity of one precipitate (Fig. 5A) which after Coomassie staining was identified as No. 16 (Fig. 5B). This enzyme required Mg^{2+} for activity and was not inhibited by Ca^{2+}. Formation of phosphorylated membrane proteins was established by autoradiography of crossed immunoelectrophoresis plates for solubilized erythrocyte membranes which had been incubated with [^{32}P]ATP (Fig. 6A). Three proteins were phosphorylated and were identified as Nos. 5, 6, and 16 by comparison with the Coomassie-stained plate (Fig. 6B). The presence of 10 mM Ca^{2+} strongly inhibited the phosphorylation as noted in ref. 31 and caused precipitate 5 to disappear from

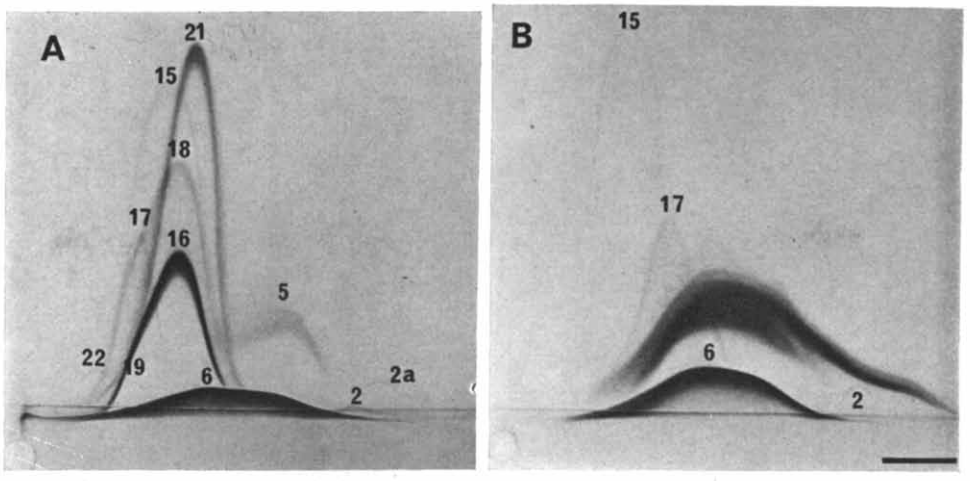

Fig. 4. Crossed immunoelectrophoresis of 20 μg (10 μl) Berol-solubilized human erythrocyte membrane proteins, (A) in agarose gel containing 1 % (w/v) of Berol EMU 043 and (B) in detergent-free gel. Removal of the detergent reduced the interpretability of the pattern. Experimental conditions and other figure designations were as for the experiments of Fig. 1; esterase staining has not been performed. The bar represents 1 cm.

the precipitation pattern. A more detailed study of the ATPases and the phosphorylated erythrocyte membrane proteins using these methods is in progress.

Sialoglycoproteins

The negative charge of sialic acid groups contributes significantly to the mobility of sialoglycoproteins at pH 8.7 [41]. As a result removal of the sialic acid residues by neuraminidase treatment results in reduced migration velocity of these glycoproteins. The effect on the precipitation pattern for Berol-solubilized membrane proteins which results from treatment of intact erythrocytes in this manner can be seen in Fig. 7. A series of experiments employing a range of enzyme concentrations was performed to ensure that all accessible groups were removed. The pattern for the neuraminidase-treated sample (Fig. 7A) shows changes in the precipitates of five proteins relative to that of a control (Fig. 7B). No. 19 has disappeared and proteins 16, 20, and 21 show migration velocities of 78, 40 and 11 %, respectively, of those of the same proteins in the control. More haemoglobin was retained in the neuraminidase-treated membranes than in the control, as revealed by the greater area below precipitate 15 for the treated sample. The precipitates of desialated proteins No. 21 (MN-glycoprotein) (identified by tandem-crossed immunoelectrophoresis) and No. 20 (cholinesterase, identified by its colour reaction) appear fainter and delimit a smaller area than those of their untreated counter parts. This is an effect resulting primarily from the reduced migration velocity of the proteins (which show partial cathodic migration (see Fig. 7, arrows)) [41]. Furthermore loss of antigenic determinants was observed for the MN-glycoprotein but not acetylcholinesterase. By means of an immunochemical quantification technique which does not involve electrophoresis, viz. single radial immunodiffusion, neuraminidase-treated MN-glycoprotein was found to give rise to a smaller and fainter precipitation ring than a non-treated control [42, 50].

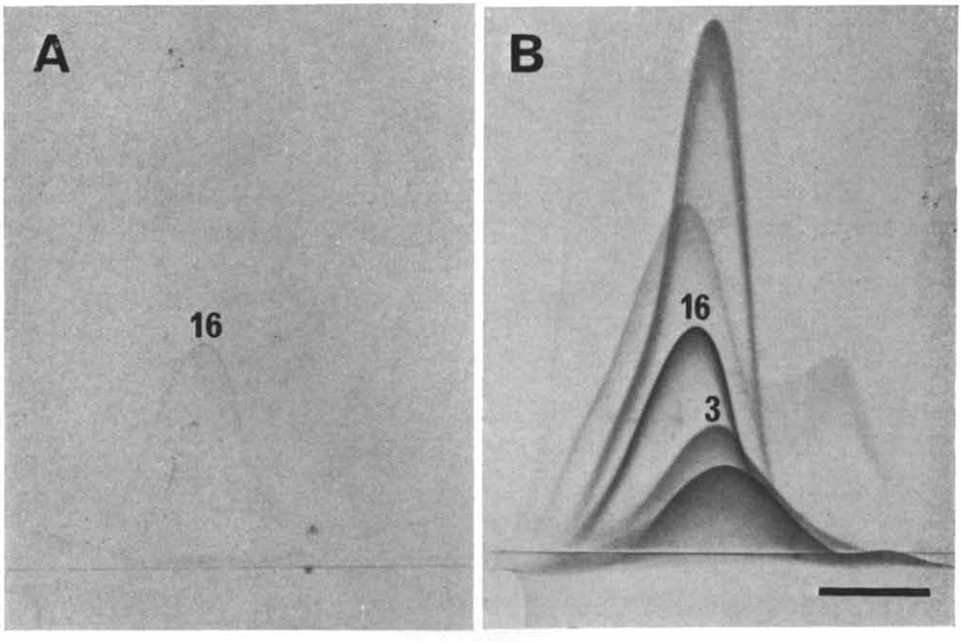

Fig. 5. Crossed immunoelectrophoresis of Berol-solubilized human erythrocyte membrane proteins. (A) is stained for ATPase activity (after incubation with ATP liberated phosphate has been precipitated with Pb^{2+} and made visible with sulfide). (B) is the same plate after Coomassie brilliant blue staining. The yellow-brown stain corresponds precisely to precipitate 16 (major intrinsic protein). Note the appearance of precipitate 3 in this preparation (B). Experimental conditions were as for the experiment of Fig. 1. The bar represents 1 cm.

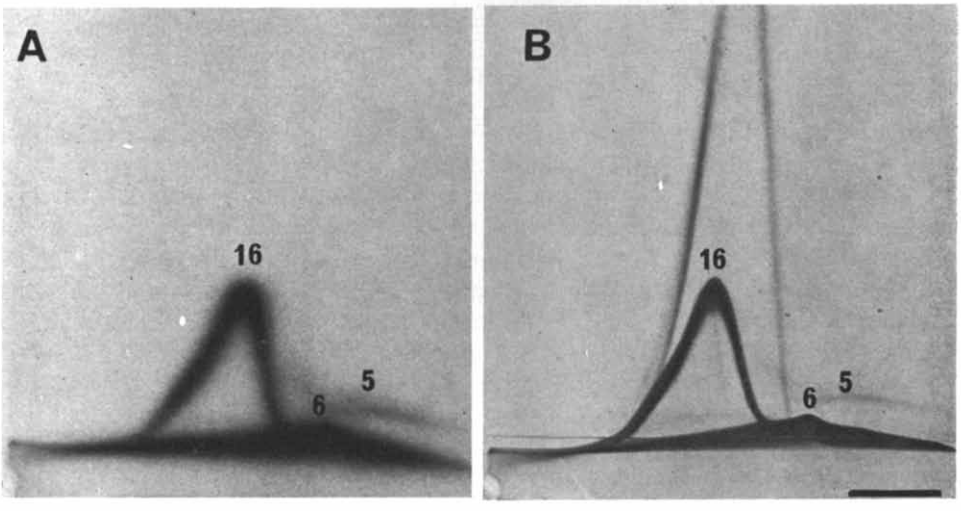

Fig. 6. Crossed immunoelectrophoresis of Berol-solubilized human erythrocyte membrane proteins which have been phosphorylated by incubation with [^{32}P]ATP. (A) Autoradiograph. (B) The same plate after Coomassie brilliant blue staining. Precipitates 5, 6, and 16 were radioactive. Antibody content of the gel: 5.3 μl/cm^2. Experimental conditions were as for the experiment of Fig. 1. The bar represents 1 cm.

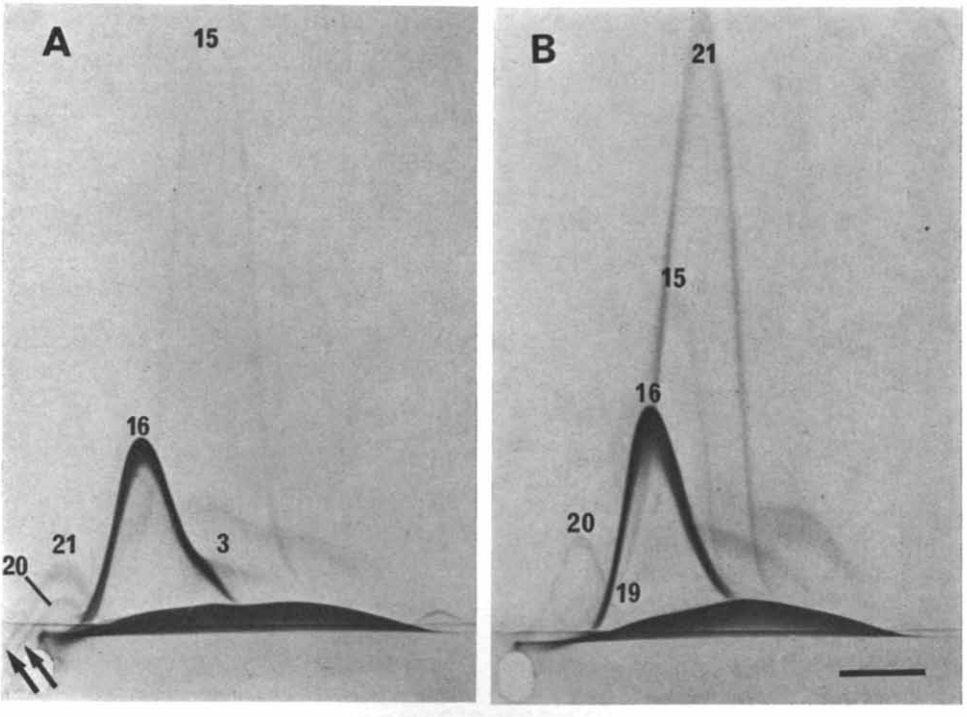

Fig. 7. Crossed immunoelectrophoresis of 30 μg of Berol-solubilized human erythrocyte membrane proteins of (A) erythrocytes treated with neuraminidase (125 units/ml) for 1 h at 37 °C and (B) untreated erythrocytes. Compared to B, pattern, A showed disappearance of No. 19, reduced migration velocity of Nos. 16, 20, and 21. Note the reduction in area below the precipitates for desialylated proteins 20 and 21 because of partial cathodic migration (arrows) and the increase in area below that for No. 15 (haemoglobin). Precipitate 3 is seen in this membrane preparation. Antibody content of the gel: 7.1 μl/cm^2. Experimental conditions were as for the experiment of Fig. 1. The bar represents 1 cm.

Precipitate No. 18 is not visible in Fig. 7, but other experiments showed that this protein migrated together with protein 16.

Neuraminidase treatment of isolated membranes gave rise to the same kind of changes as described above, there being no sign of proteolytic degradation of the membrane proteins. Precipitate 5 was not seen because Ca^{2+} was present in the incubation medium. Neuraminidase treatment of solubilized membrane proteins could not be performed since the membrane proteins aggregated at pH 5.5 and in the presence of Ca^{2+} [41].

Lectin binding proteins

The interaction of lectins with cell membranes has many applications in cell biology [32]. Since non-ionic detergents only react with the hydrophobic parts of membrane proteins, leaving the hydrophilic part including the carbohydrate moiety unchanged [3, 4], interaction between lectins and membrane proteins can be detected directly in immunoelectrophoresis because it results in changed precipitation patterns [23]. Information can be obtained about cell surface receptors and about the molecular composition of the carbohydrate part of the receptor if the binding specificity of

the lectin is known. With the identification of lectin binding proteins in mind we have screened the interaction between Berol-solubilized erythrocyte membrane proteins and a series of lectins (see Materials and Methods), performing the experiments in the simplest way by merely mixing the components before analysis. Significant changes in the precipitation pattern (Fig. 8) were only observed with wheat germ agglutinin (affecting Nos. 16, 18, and 21), fucose binding protein (affecting Nos. 16 and 18) and phytohaemagglutinin P (affecting Nos. 20 and 21). Precipitate 5 showed irregular minor changes with most of the tested lectins, but these were not regarded as being due to specific binding because of the lability of this protein. To take an example, the precipitation pattern changes produced by wheat germ agglutinin can be seen by comparing Fig. 8A with the pattern for an untreated control sample (Fig. 8C), when a reduction in the area below precipitates 16, 18, and 21 is apparent. The specificity of the interaction is illustrated in Fig. 8B, which shows the nearly unaltered pattern obtained after incorporation of 5 % (w/v) of *N*-acetylglucosamine in the sample and in the gel for the first dimension electrophoresis. However, the changes observed with the fucose binding protein were not inhibited by the presence of 5 % fucose, so that the interaction observed could be due to reactions with impurities present in the lectin preparation. Since the specificity of phytohaemagglutinin P is not known [32], the effect of inhibitors on the interaction of this lectin with membrane proteins could not be tested. Further studies on the binding of concanavalin A to solubilized membrane proteins were performed with crossed immuno-affinoelectrophoresis using free concanavalin A in the first dimension gel [28] or concanavalin A coupled to agarose in the intermediate gel [27]. Binding of acetylcholinesterase to concanavalin A-agarose was the only interaction observed. The same results were obtained when the detergent was omitted from the gel.

Topographical distribution of membrane proteins

Among the more important characteristics of membrane proteins is their localization within the membrane. Since all sialic acid residues of the erythrocyte are located on the outer membrane surface [43], identification of proteins as sialoglycoproteins provides proof of the external exposure of these proteins. The same is probably the case for lectin binding proteins. Three further methods for determination of the location of membrane proteins by quantitative immunoelectrophoresis are described below.

^{125}I labelling. Peroxidase-catalysed ^{125}I iodination is a well established method for labelling proteins exposed on the outer membrane surface of intact cells [44]. The autoradiograph of a crossed immunoelectrophoresis plate for Berol-solubilized membranes of intact erythrocytes which have been subjected to such labelling is shown in Fig. 9A. Three precipitates were radioactive and comparison with the Coomassie-stained plate (Fig. 9B) identified these as Nos. 16, 18, and 21. A part of the spectrin precipitate (No. 6) positioned solely below precipitates 16 and 21 was also radioactive. However, we assume this to be a non-specific reaction resulting from entrapment or co-precipitation of radioactive material from Nos. 16 and 21, since such entrapment has also been observed with certain enzymes detected by their enzymatic activity [12, 23, 45]. ^{125}I labelling of isolated membranes showed that all the Coomassie-stainable precipitates were radioactive, and no new precipitates were observed.

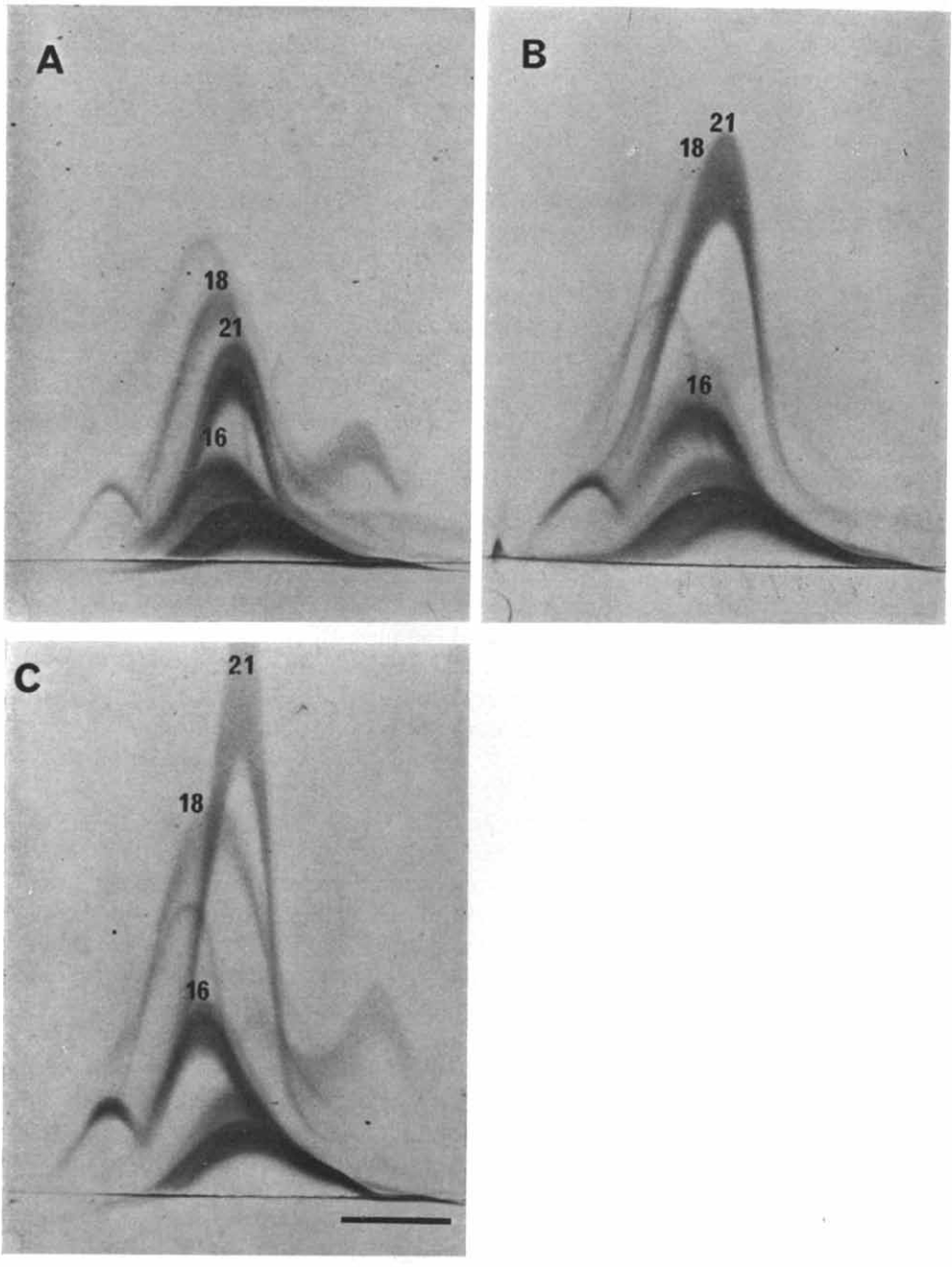

Fig. 8. Crossed immunoelectrophoresis of 10 μg of Berol-solubilized human erythrocyte membrane proteins mixed with (A) wheat germ agglutinin (1.6 mg/ml), (B) 5 % (w/v) of *N*-acetylglucosamine plus wheat germ agglutinin (1.6 mg/ml) and (C) 5 % (w/v) of *N*-acetylglucosamine. In addition the first dimension gel of B and C contained 5 % (w/v) of *N*-acetylglucosamine. Compared to C the areas below the precipitates for proteins 16, 18, and 21 are reduced in A and to lesser degree in B. Antibody content of the gels: 6.3 μl/cm^2. Experimental conditions for the experiment fo Fig. 1 except for the electrophoresis time of 45 min for the first dimension. The bar represents 1 cm.

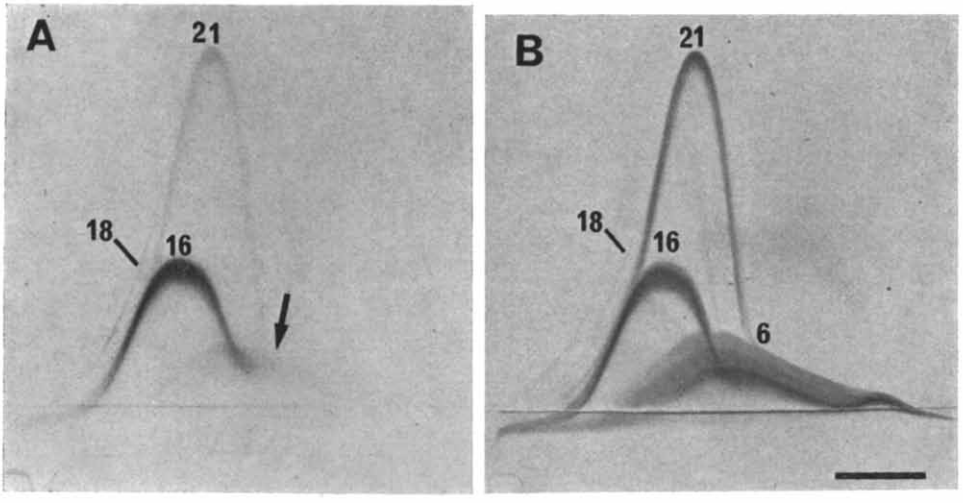

Fig. 9. Crossed immunoelectrophoresis of Berol-solubilized membrane proteins of human erythrocytes for which lactoperoxidase-catalysed ^{125}I iodination had been performed on the exposed proteins of the outer surface. (A) Autoradiograph. (B) The same plate after Coomassie brilliant blue staining. Precipitates 16, 18, and 21 were radioactive. The arrow indicates entrapment of radioactivity in precipitate 6. Antibody content of the gels: 7.1 $\mu l/cm^2$. The bar represents 1 cm.

Proteolytic degradation. Treatment of intact cells with proteolytic enzymes in order to determine exposed proteins is another well-known tool in membranology [46]. The effect of trypsin, α-chymotrypsin and pronase on erythrocytes was investigated by crossed immunoelectrophoresis and polyacrylamide gel electrophoresis. A representative precipitation pattern for erythrocyte membrane proteins after exposure of the intact cells to proteolytic enzymes is shown in Fig. 10A. In this experiment the cells were exposed to α-chymotrypsin (3 mg/ml) for 1 h at 37 °C. Comparison with a control (Fig. 10B) revealed changes in five precipitates: Nos. 16 and 18 showed a slight cathodic shift in position, No. 21 appeared as a "camel" precipitate (double peak), No. 5 became more blurred and fused with a "flying" (unending) precipitate, and Nos. 19 and 20 disappeared completely. The degraded membranes retained more haemoglobin than the control as can be seen from the increased area below No. 15. It should be noted that the degradation revealed by the pattern in Fig. 10A was not maximal. Increase of the enzyme concentration to 10 mg/ml under the given conditions resulted in reduction of the "camel" precipitate to a single peak corresponding to a protein with a relative migration velocity of 20 % of that of albumin.

The degradation produced by pronase was almost identical to that produced by α-chymotrypsin, there being a slightly greater effect on precipitate 5. However, the effect of trypsin was not so pronounced; thus, protein 16 was unaffected and the acetylcholinesterase activity was not completely destroyed within the range of trypsin concentration examined. This is in accordance with data given in ref. 47. Table II summarizes progressive changes observed in the precipitation pattern obtained in crossed immunoelectrophoresis after treatment with the three enzymes in increasing concentrations. The cleavage products released in the incubation medium did not give rise to any immunoprecipitates in crossed immunoelectrophoresis. Solubilization

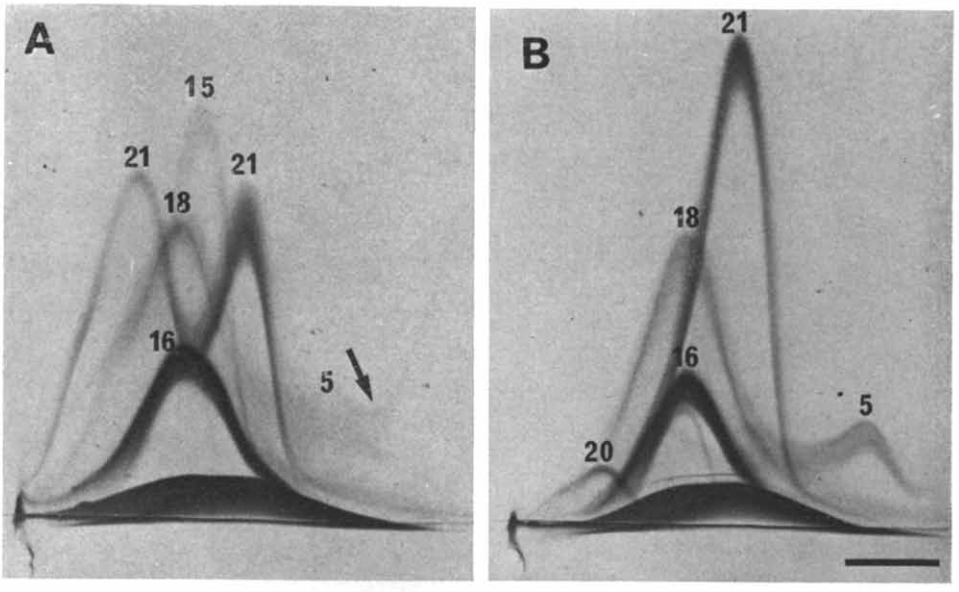

Fig. 10. Crossed immunoelectrophoresis of Berol-solubilized membrane proteins of (A) human erythrocytes treated with α-chymotrypsin (1.5 mg/ml) for 1 h at 37 °C and (B) untreated erythrocytes. Compared to B, pattern A showed disappearance of precipitates 19 and 20, reduced migration velocity of proteins 16 and 18, formation of a "camel" precipitate for protein 21, and blurring and splitting of the precipitate for protein 5 (arrow). Note the increase in area under precipitate No. 15 (haemoglobin). Antibody content of the gel: 8.0 μl/cm^2. Experimental conditions were as for the experiment of Fig. 1. The bar represents 1 cm.

of isolated membranes which had been incubated with trypsin or α-chymotrypsin and analysed by crossed immunoelectrophoresis revealed a progressive degradation resulting finally in a total disappearance of precipitates of which those corresponding to haemoglobin and acetylcholinesterase were most resistant to degradation. The degradation shows that most antigenic determinants are exposed and not hidden within the lipid core. Degradation of solubilized membrane material was even faster and the cleavage product gave rise to new precipitates. The non-ionic detergent therefore did not protect any antigenic structures detectable with immunoelectrophoresis. Haemoglobin and acetylcholinesterase were still relatively resistant under these conditions. Comparable precipitation patterns observed after pronase degradation have been published elsewhere [40].

Antibody binding. The immunochemical approach per se also provides the possibility of determining the location of proteins in the membranes [10, 12, 15, 36]. Treatment of the anti-membrane antibody preparation with a surplus of intact erythrocytes will remove the antibodies which are directed against proteins exposed on the outer cell surface. In crossed immunoelectrophoresis with such a depleted antibody preparation precipitates corresponding to proteins located solely on the outer surface will disappear, precipitates due to proteins penetrating the membrane (with antigenic determinants on both sides) will appear with an increased area and precipitates of proteins located solely on the inner surface will remain unchanged. A re-investigation with such absorbed antibodies, this time containing aprotinin, verified previously

TABLE II

THE EFFECT OF ENZYMATIC DEGRADATION OF MEMBRANE PROTEINS OF INTACT ERYTHROCYTES ON INDIVIDUAL PRECIPITATES OBTAINED IN CROSSED IMMUNOELECTROPHORESIS AFTER SOLUBILIZATION OF THE MEMBRANES WITH 1 % BEROL EMU-043

The progressive changes in precipitates observed with increasing enzyme concentration are indicated by the following numbers: 1, faintness of staining intensity; 2, blurring; 3, increase in area; 4, decrease in area; 5, movement to a more cathodic position; 6, "Camel" precipitate formation; 7, "Flying" precipitate formation; 8, disappearance.

Protein No.	Enzyme		
	Pronase	α-Chymotrypsin	Trypsin
2	–	–	–
2a	–	–	–
3	–	1	1
5	1, 4, 8	1, 2, 3, 7	1, 3, 7
6	–	–	–
15	3	3	3
16	5	5	–
17	–	–	–
18	1, 5	1, 5	–
19	8	8	8
20	1, 8	1, 5, 8	1, 4
21	4, 5	5, 4, 6	5, 4, 6

published results [15] concerning the external location of proteins 19, 20 and 21. In addition an increase in area below precipitate 18 was found, indicating that the corresponding protein spans the membrane. Precipitate 16 was unaffected, a finding which conflicts with the external location of this protein which is indicated by other methods used in this investigation. However, this behaviour would be expected if relatively few antigenic determinants of protein 16 were located on the outside rather than the inside of the membrane and if the inside-located determinants determine the position of the precipitate in crossed immunoelectrophoresis.

The existence of externally located antigenic determinants of protein No. 16 was demonstrated using swine antibodies against rabbit IgG as a "molecular amplifier" [48]. After binding antimembrane antibodies to intact erythrocytes, the cells were washed and the membranes isolated. In crossed immunoelectrophoresis of the solubilized membrane material with an intermediate gel containing swine antibodies against rabbit IgG the membrane proteins carrying bound rabbit IgG will be precipitated in the lower gel (Fig. 11A). Demonstration of acetylcholinesterase activity in the swine anti-rabbit IgG precipitate (Fig. 11A, arrow) indicates directly the retention of membrane proteins in this precipitate. The areas below precipitates 16, 18, 20 and 21 were reduced relative to the control (Fig. 11B). The area below the precipitate for the internally located [49] proteins spectrin (No. 6) was unaffected. Precipitate 19 is not visible in Fig. 11, but in other experiments this precipitate was also diminished. The antibody-treated membrane in this case also contained more haemoglobin than the control, as seen by comparison of the area below precipitate 15 in A and B of Fig. 11.

The reason for the failure of the precipitates for the exposed proteins to com-

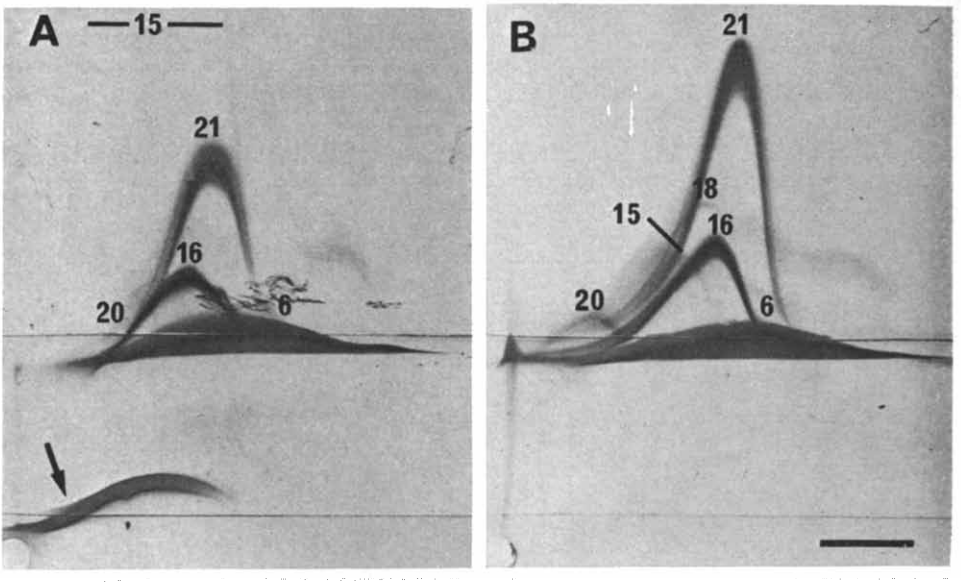

Fig. 11. Crossed immunoelectrophoresis of Berol-solubilized membrane proteins of (A) human erythrocytes treated with rabbit anti-membrane antibodies and (B) untreated erythrocytes. The intermediate gel contained in each case 10 μl/cm^2 of swine anti-rabbit IgG and 10 μl/cm^2 of aprotinin. Compared to B, pattern A showed a reduction in the area under precipitates 16, 18, 20, and 21. The arrow indicates the location of the acetylcholinesterase activity in the swine anti-rabbit IgG precipitate. The cloudy spots are due to impurities in the agarose gel. No line-precipitate formed between the rabbit and the swine antibodies is seen because of the antigen excess of the upper gel. Antibody content of the gels: 6.2 μl/cm^2. Experimental conditions were as for the experiment of Fig. 1. The bar represents 1 cm.

pletely disappear from the reference pattern is probably that some of the proteins are shielded by agglutination before they have taken up antibody molecules. A prerequisite for a correct interpretation is that all antibodies which are bound non-specifically are washed away from the cell, since such remaining antibodies will be able to react with proteins of the inner membrane surface during lysis.

Conclusion. Nearly concordant results regarding the exposure of proteins 16, 18, 19, 20 and 21 on the outer surface of the cell were obtained by the three direct methods and by the indirect methods such as lectin binding and determination of sialoglycoproteins. Radioactive labelling with ^{125}I seemed to be the least sensitive method since labelling of two proteins Nos. 19 and 20, was not observed. The conclusion that acetylcholinesterase (No. 20) and the MN-glycoprotein (No. 21) are exposed on the outer surface agrees with previous work [15, 36, 44, 46, 47, 49] and requires that the major precipitate 16 corresponds to the major intrinsic protein (band III protein) [2, 36, 49] in agreement with its classification as intrinsic protein. Precipitate 18 was of irregular occurrence, appearing only for aged membrane preparations. When it occurred it exhibited behaviour similar to that of protein 16 as regards neuraminidase treatment, lectin binding and proteolytic degradation, and it may well represent some form of cleavage product of No. 16. Results obtained recently by Yu and Steck [2] show that the major intrinsic protein exists as a complex with other

membrane proteins both in the membrane and after solubilization with non-ionic detergent. Precipitates 16 and 18 may thus correspond to different types of complexes. Furthermore, purified major intrinsic protein was found to cross-react with protein 16 and 18 (Bjerrum, O. J. and Bhakdi, S., unpublished). The only indication of external location of protein 5 was provided by the proteolytic degradation behaviour. However the possibility of secondary degradation after lysis cannot be completely excluded.

DISCUSSION

The well-characterized human erythrocyte membrane proteins [49] provide an excellent model system for evaluation of the utility of the immunochemical approach to the analysis of membrane proteins. Attempts have been made to characterize this membrane system by double immunodiffusion and immunoelectrophoretic analysis according to Grabar [50, 51] but due to incomplete solubilization and difficulties encountered in identifying the precipitation lines obtained, little progress was made. It proved especially difficult to compare the results obtained by different investigators. However the efficient solubilization achieved with non-ionic detergents [52] and the high resolution obtained in crossed immunoelectrophoresis by incorporation of the same type of detergent in the gels [5, 6] makes it possible to establish a reference pattern with which it is easy to recognize precipitates (see Table I). Furthermore, an antibody preparation against human erythrocyte membrane material is now commercially available. Other membrane systems, including liver cell membranes [7, 11], lymphocyte membranes [9], milk fat globule membranes [53], rat brain synaptosome membranes [8] and bacterial cell membranes [10, 12] have also been investigated by means of crossed immunoelectrophoresis.

Although our understanding of the physicochemical behaviour of single membrane proteins in non-ionic detergent solution is increasing [3, 4], a detailed knowledge of the protein-protein interactions which take place when many membrane proteins (e.g. from an entire membrane) are present, is lacking for the moment. Furthermore, little is known concerning the way in which the binding of individual antibodies influences such protein-protein interactions [54]. An important question which has to be answered first, however, is whether each individual precipitate observed in the immunoelectrophoresis of membrane proteins corresponds to a single protein entity? The high reproducibility of the immunoelectrophoretic precipitation patterns shows that the disintegration of the membrane during the solubilization process takes place in a reproducible manner. Isolated membrane proteins such as spectrin and MN-glycoprotein each show reactions of immunochemical identity with only one precipitate in the precipitation pattern. The finding that acetylcholinesterase activity is associated with a single distinct precipitate, together with the well-defined location in the precipitation pattern of the radioactively labelled proteins (Fig. 9) supports the notion that one membrane protein gives rise to one precipitate. However, the occurrence of membrane protein complexes in one single precipitate has been reported [11]. These were complexes of membrane-bound enzymes which are known to be tightly associated in the membrane. Our demonstration that ATPase activity is associated with the precipitate of the major intrinsic protein (No. 16) (Fig. 5) also indicates the presence of protein complexes in the precipitation pattern.

In conclusion, these observations seem to indicate that some of the membrane proteins are present as single entitles after solubilization with non-ionic detergent and give rise to individual precipitates. However, in cases where proteins are complexed in situ in the membrane such structural units may persist during the immunoelectrophoretic analysis. In order to clarify this problem further, direct analysis of the polypeptide content of individual immunoprecipitates is in progress. After immunoelectrophoresis of radioactively labelled membrane proteins the immunoprecipitates can be cut out of the wet agarose gels and subjected to dodecyl sulfate-polyacrylamide gel electrophoresis after reduction of S-S bonds and solubilization. Autoradiography of the gel reveals directly the number of polypeptides present in the precipitate (Bjerrum, O. J., Norrild, B. and Bhakdi S., unpublished).

One of the functional properties of membrane proteins which is retained after solubilization with non-ionic detergent is enzymatic activity [7, 11, 12]. Antibody binding seldom completely prevents enzymatic analysis and the immunoelectrophoretic method allows a much more direct correlation of function to individual membrane protein than, for example, dodecyl sulfate gel electrophoresis. In the case of immunoelectrophoresis the enzymatic activity must follow a precipitate in two dimensions as a prerequisite for assigning the relevant enzyme function to the protein. However, in dodecyl sulfate gel electrophoresis the correlation is indicated only by a one-dimensional R_f value. Illustrative examples are the finding of ATPase activity associated with the complex containing the major intrinsic protein (No. 16) (Fig. 5), and the demonstration of the phosphorylation of spectrin (No. 6) and of the complex containing the major intrinsic protein (No. 16) in Fig. 6. These observations are consistent with our present knowledge of these proteins [31, 55, 56]. The heavy uptake of ^{32}P by protein 5 (Fig. 6), together with the disappearance of this protein from the precipitation pattern upon exposure to Ca^{2+}, sheds light on its function and makes an association with the band 4 region in dodecyl sulfate-gel electrophoresis feasible [56, 57].

Identification of glycoproteins in immunoelectrophoresis can be achieved by staining techniques [50], but such identification is difficult because immunoglobulins contain carbohydrates. Identification of sialoglycoproteins by their change in migration velocity after neuraminidase treatment is a more sensitive method which can detect a sialic acid content of as little as 0.2–0.3 % [41]. Besides the well known major sialoglycoproteins, the MN-glycoproteins [49] together with acetylcholinesterase [39] and the major intrinsic protein were shown to contain sialic acid (Fig. 7) [36]. Binding of lectins to membrane glycoproteins can also be detected by immunoelectrophoresis, only small amounts of membrane material (50 μg) and lectin being required. Moreover, inhibition studies can be performed directly in the gels [33], although a rather high sugar concentration (5–10 %) is necessary. Most of our positive evidence from simple immunoelectrophoretic analysis of lectin binding to erythrocyte membrane proteins is in agreement with previous work [58–60], but our finding of a specific interaction between wheat germ agglutinin and the proteins of precipitate 16 (Fig. 8) is new. The negative results obtained in our screening could be artifacts since weak binding would not be detected. With our experimental set-up we did not detect the expected binding of major intrinsic protein and MN-glycoprotein to *R. communis* lectin [59]. Neither was concanavalin A found to interact with the major intrinsic protein, in contrast with earlier work [60, 61], even using the more sensitive crossed immuno-affinoelectrophoresis method. This finding agrees with results

obtained for the purified protein [36]. However, the interaction, reported previously [60, 61], is not so well established since only part of the protein material present in band III from dodecyl sulfate gel electrophoresis shows interaction [61] and the binding is located at the front edge of band III [60].

Several methods have been devised for locating the positions of proteins within membranes [44, 46] and as shown in the present work they can easily be combined with immunoelectrophoresis. The progressive degradation of the MN-glycoprotein and acetylcholinesterase was much easier to follow by immunoelectrophoresis [46, 47]. Under the given conditions it was found that relatively high concentrations of enzymes were necessary to obtain complete cleavage of the exposed proteins. The remaining peptide of the MN-glycoprotein still gave rise to an immunoprecipitate, demonstrating that not all of the antigenic determinants were split off. In contrast, the major intrinsic protein showed surprisingly small changes after enzymatic degradation of intact cells with α-chymotrypsin and pronase (Fig. 10), indicating either the presence of relatively few antigenic determinants on this 30 000 molecular weight peptide or the presence of a pseudo-intact molecule, which is not dissociated by Berol after the enzymatic cleavage.

The possibility that relatively few antigenic determinants of the major intrinsic protein are exposed on the outer surface was supported by the fact that their presence could not be demonstrated directly by the use of absorbed antibody preparations [15]. However, with purified major intrinsic protein their presence was clearly demonstrated [36]. This is possibly due to a different ratio between the numbers of antigenic determinants of the inner and outer surface in the purified protein because of its exposure to sodium dodecyl sulfate during the preparation. Furthermore, by employing the one layer sandwich principle, known from indirect histological staining in immunoelectrophoresis, it was possible to demonstrate the exposure of antigenic determinants on the outer surface of protein 16 via the binding of specific rabbit IgG antibody molecules in intact cells.

We regard such molecular amplification by swine antibodies against rabbit IgG as a means for increasing the sensitivity of the immunoelectrophoretic technique. In principle, one single antigenic determinant on the outer membrane surface should be detectable in this way. A prerequisite being the appearance of the protein in the reference pattern. Thus we find an increased potential of immunoelectrophoretic methods in providing detailed descriptions of antigenic determinants at the molecular level and expect these methods to prove very useful in the study of membrane phenomena.

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