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TWO-DIMENSIONAL ELECTROPHORETIC ANALYSIS OF HUMAN ERYTHROCYTE MEMBRANE PROTEINS

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The study of the structure of the human erythrocyte membrane, and in particular, of its constituent proteins, is of some urgency because various hereditary diseases (spherocytic anemias, muscular dystrophies, certain diseases of the CNS) are characterized by disturbances of the protein component of the erythrocyte membrane and, as a result, by disturbance of its structural and functional organization. In connection with the systematic study of products of the expression of human genes using a molecular-anatomical approach, which has now begun, it is extremely important to determine the number and principal properties of the polypeptides forming the erythrocyte membrane and to compile a catalog of proteins. Opportunities for research of this kind, and also the search for the primary biochemical defect in inherited anomalies increased considerably after the widespread introduction of two-dimensional polyacrylamide gel (PAG) electrophoresis, between coordinates of isoelectric point and molecular weight [2, 9]. Nevertheless, so far as we know, there have been very few attempts at two-dimensional electrophoretic study of erythrocyte membrane proteins and their results have proved contradictory [5, 7, 11, 12].

The aim of this investigation was to obtain a reproducible two-dimensional gel by electrophoresis of human erythrocyte membrane proteins and to draw a provisional protein map as the first step toward creation of a catalog of the polypeptides constituting this cell structure.

EXPERIMENTAL METHOD

Packed erythrocytes were obtained by passing heparinized blood (about 20 ml) through a column with HBS-cellulose, after which the erythrocytes were allowed to stand in a 3% solution of 500,000 dextran in 0.15 M NaCl twice, for 45 min each time. Erythrocyte membranes were obtained from erythrocytes by lysis in 5 mM Na-phosphate buffer, pH 8.0, containing 1 μM phenyl-

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methylsulfofluoride (PMSF) by the method in [4]. Erythrocyte ghosts were washed three times more in lytic buffer, after which the membrane residue was lyophilized. Linear electrophoresis in PAG was carried out in the presence of sodium dodecylsulfate (SDS) by the method in [8] in a block with 5-25% acrylamide gradient. Two-dimensional protein electrophoresis was carried out by Anderson's method [3] with modifications. The first direction, namely isoelectric focusing (IEF), was done in glass tubes 2 mm in diameter and 180 mm long, at 200 V for 80 min and at 600 V for 2 h. Samples for IEF were prepared in two stages: in the first, lyophilized membranes were dissolved in solution I, containing 9.5 M urea, deionized on amberlite MB-2 (Serva, West Germany), 1% SDS, 5% 2-mercaptoethanol, 1% glycine, 0.2% ampholines 3.5-10 (LKB, Sweden), and 1 μM PMSF. After incubation for 20 min at 25°C the membrane lysate was treated with an equal volume of solution II: 9.5 M urea, 6% Triton X-100, 5% 2-mercaptoethanol, 0.2% ampholines 3.5-10, 1% glycine, and 1 μM PMSF.

The samples thus obtained were centrifuged, before application to the gel, at 30,000 rpm on an L2-65 ultracentrifuge (Beckman, USA), in an SW65 K rotor for 15 min. The samples were applied to the gel in two ways: from the anode and from the cathode. After the end of IEF the gels were removed and placed for 45 min in 5 ml of specimen buffer (Laemmli system). The gel columns thus obtained can be kept at −70°C for 2 weeks without visible change.

Separation in the second direction was done in the Laemmli system in a 5-25% PAG gradient. The equilibrated column of gel was placed on the top end of the PAG plate and fixed with 1% agarose in the corresponding buffer. The gel plates were stained by two methods: 1) in a mixture of isopropanol—acetic acid—water (25:10:65), in Coomassie R-250 in 0.05% concentration at 65°C, followed by rinsing in 7% acetic acid at 90°C; 2) by a modified silver impregnation technique [1, 10]. The gel stained with Coomassie R-250 can be effectively stained with silver virtually without any background, if kept in water about 3 days. The pH gradient in the gels in the first direction was measured with Orion contact microelectrodes (LKB) with appropriate correction for urea concentration.

EXPERIMENTAL RESULTS

The main problems arising during fractionation of erythrocyte membrane proteins by IEF and two-way electrophoresis are linked with proteolysis, due primarily to incomplete purification of the packed erythrocytes from leukocytes and aggregation of high-molecular-weight proteins: spectrins, and proteins 2.1 and 3. Among the methods of isolation of membranes tested, the list of those described in "Experimental Method" proved to satisfy the criterion of greatest resistance of the preparations to proteolysis most closely, as was demonstrated by one-way PAG electrophoresis in the presence of SDS (Fig. 1). The principal criterion of the accuracy of two-way electrophoresis of erythrocyte membrane proteins, in our opinion, was that it is possible to identify by this method, in particular, polypeptides whose molecular weight corresponded to those characterized and classified by one-way electrophoresis in the presence of SDS [6]. The method of preparation of the sample for IEF (in the first direction) and the details of the IEF procedure proved to be extremely important for successful protein fractionation. Addition of 1% glycine to the lytic solutions, increasing the Triton X-100 concentration to 3%, and in particular, conducting IEF at low voltage (about 200 V) enabled many of the difficulties connected with aggregation of membrane proteins to be overcome, and two-dimensional separation to be obtained by electrophoresis in the presence of high-molecular-weight polypeptides. A two-dimensional gel obtained by electrophoresis of erythrocyte membrane proteins, when the specimen was applied from the anode and the gel stained with Coomassie R-250 and silver impregnation, is illustrated in Fig. 2. The corresponding two-dimensional protein map in coordinates of molecular weight and mobility during IEF is shown in Fig. 2c. All the main membrane proteins identified by molecular weight, incuding spectrins, ankirin, and band 3 protein, could be identified on it. However, even under the conditions specified above, the latter do not stand up sufficiently well to IEF. It will be clear from Fig. 2a, b that these proteins focused in one narrow pH zone, yet according to data in the literature, the isoelectric points of these proteins, although close together, are nevertheless different.

In our view this phenomenon is due to partial aggregation during the IEF process. When the sample is applied from the anode it becomes acid at the very beginning of IEF, the buffer capacity of the ampholines is insufficient, and because of the large hydrophobic domains the proteins aggregate.

The higher the voltage used for IEF, the more strongly this phenomenon is exhibited, so that even at 400-600 V, the voltages usually used and recommended for IEF, aggregation is so serious that it spreads mechanically to other proteins; consequently only a few low-molecular-

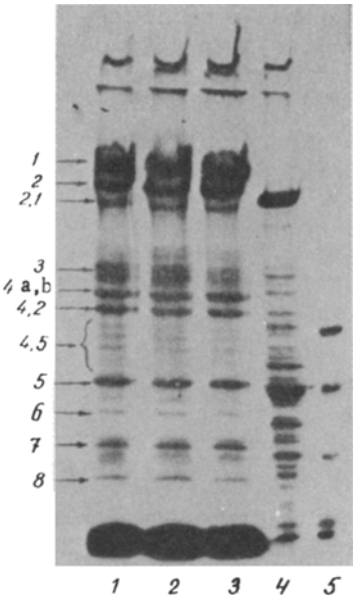
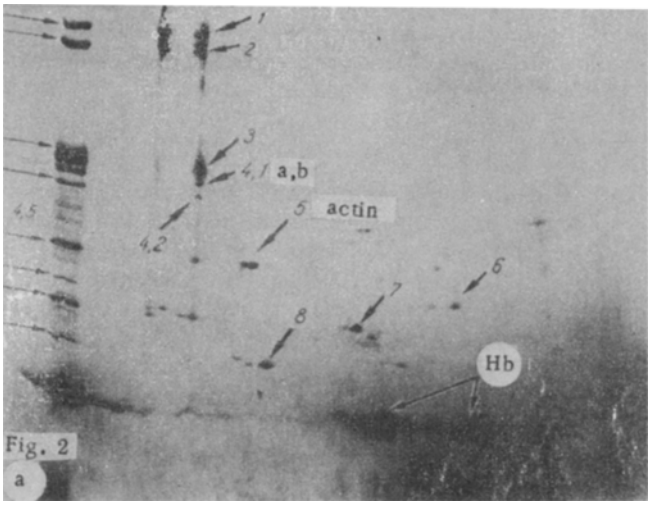
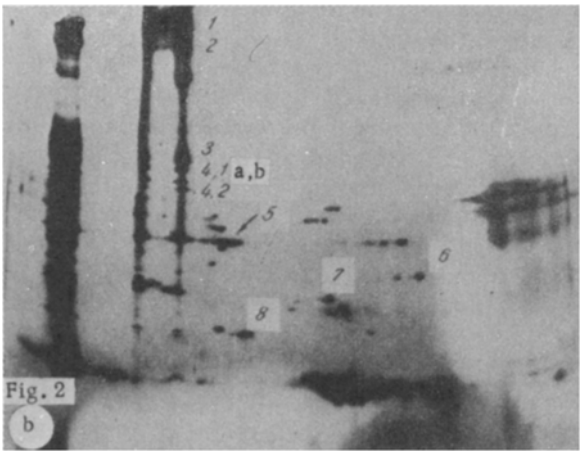


Fig. 1. One-way electrophoresis of human erythrocyte membrane proteins in the presence of SDS, after Laemmli [8]. Lanes 1-3: lysates of erythrocyte membranes isolated as described in "Experimental Method"; 4) mouse heart lysate proteins used as molecular weight markers; 5) marker proteins (from Serva, West Germany). The classification of erythrocyte membrane proteins according to Fairbanks et al. [6] is used to describe the gel.





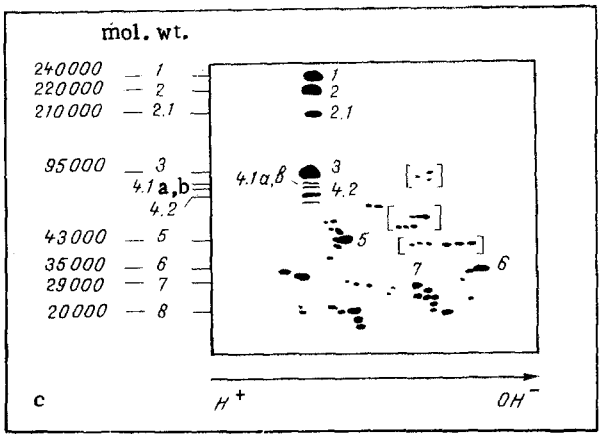


Fig. 2. Two-way electrophoresis of human erythrocyte membrane proteins. Abscissa, mobility during IEF. Specimen applied and IEF carried out from anode (on left in figure). Ordinate, molecular weight. Numbers denote principal membrane proteins according to Fairbanks' nomenclature: a) Stained with Coomassie R-250, b) stained with silver, c) two-dimensional map of erythrocyte membrane proteins.

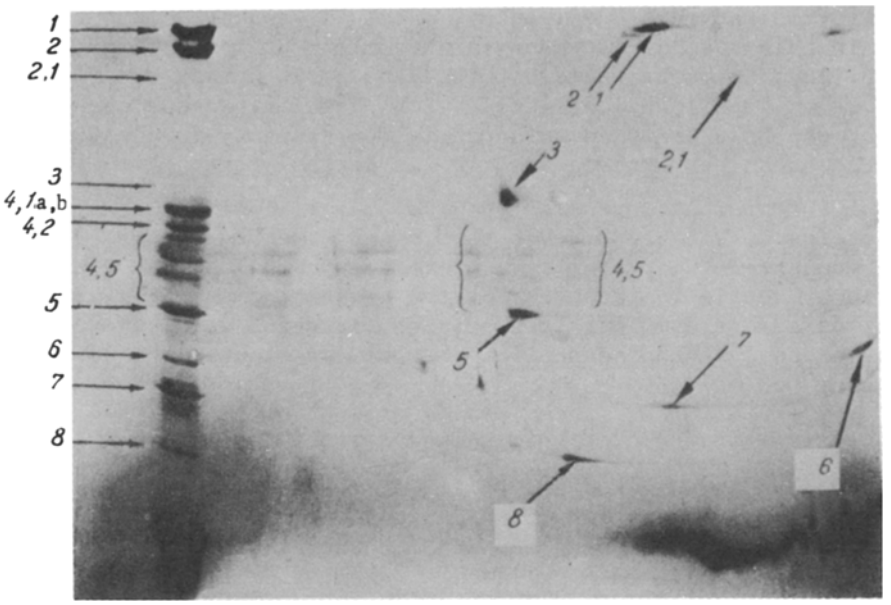


Fig. 3. Two-way electrophoresis of human erythrocyte membrane proteins. Abscissa, mobility during IEF. Application of specimen and direction of IEF from cathode (on right in the figure). Ordinate, molecular weight.

weight polypeptides are amenable to IEF. As a result, the two-way gels obtained by electrophoresis are not reproducible, and contain only some proteins of low molecular weight (data not given).

For the reasons given above, we postulated that high-molecular-weight proteins will focus better if the specimen is applied from the cathode, in the pH region remote from their isoelectric point (pI). In fact, as will be clear from Fig. 3, in this case the spectrins and protein 3 were much better resolved. Thus all the main erythrocyte membrane proteins classified by one-way electrophoresis could be identified on two-way gels, whether the specimen was applied from the anode or from the cathode.

Besides the principal protein spots, about 30 other protein spots were found on the gel after two-way electrophoresis with application from the anode and staining with Coomassie R-250. The use of the more sensitive silver impregnation technique revealed up to 50 polypeptides without overloading the gel (Fig. 2b). Among the minor fractions, resolved well by two-way electrophoresis under the conditions specified, we may mention about six polypeptides in the molecular weight zone of 45,000 daltons, corresponding to actin, but they were located nearer the cathode. Their "layout" on the gel suggests the presence of several isomers of one protein. Alongside actin, in a slightly more acid pH region with somewhat smaller molecular weight, a polypeptide also not previously identified was always present on the gels. The 4.5 group of proteins also was separated over a fairly wide pH range.

The results of this investigation are in close agreement with those obtained by other workers [5, 7], but the electrophoretic pattern obtained by the latter [7] could not be reproduced since they used a method of forming the pH gradient with "pH-isolytes" not available commercially. Workers who, like us, used ampholines from the firm LKB published results [12] which differ sharply from those described above. They obtained two-way gels by electrophoresis of erythrocyte membrane proteins, on which about 200 spots were discovered on staining with Coomassie R-250, and another group of investigators found up to 600 spots on their gels by staining with silver [11]. It is very important to note that in both the papers cited, proteins with mol. wt. of not more than 80,000 daltons were indicated on the protein maps. The authors of one of these papers [12] also state that the presence of spectrin and other high-molecular-weight proteins in the membrane preparations somehow "masks" the minor components of the membranes, making the gels difficult to work with and not reproducible. We have already pointed out above that the aggregating power of high-polymer membrane proteins interferes strongly with IEF. It is worth noting that, according to our data, with weak control over

proteolytic activity in the membrane lysates, spectrins and protein 3 very quickly undergo degradation, and in this case an increase in the number of spots is observed on the two-way gels. The possibility cannot be ruled out that the large number of polypeptides found by the workers cited above on two-dimensional gels, with the simultaneous absence of high-molecular-weight components, can be attributed to uncontrolled proteolysis. In fact, the number of proteins composing the human erythrocyte membrane is not so great.

The result of the present investigation was thus to obtain two-way gels of human erythrocyte membrane proteins by electrophoresis showing all the previously classified proteins and about 50 minor components. The preliminary protein map obtained can serve as the basis for further filling in of detail in the study of the protein composition of erythrocyte membranes and the compiling of a more complete catalog. The map which we obtained may perhaps also serve as a high-resolution tool in the search for the primary biochemical defect in corresponding hereditary diseases.

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CHANGES IN K^+, Na^+, and H^+ PERMEABILITY OF VESICLES FROM ISOLATED APICAL EPITHELIOCYTE MEMBRANES OF THE RABBIT SMALL INTESTINE DURING Ca-ACTIVATED LIPOLYSIS

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The system of membrane phospholipid metabolism in epitheliocytes of the mammalian small intestine contains Ca^{++}-activated phospholipases A_2 and A_1 (or lysophospholipase) [3]. No detailed information could be found on phospholipase compartmentalization in the literature. It can be postulated that these enzymes may participate in the regulation of epitheliocyte membrane permeability in the small intestine, at least under pathological conditions accompanies by de-energization of the cells, a raised Ca^{++} level in the cytosol, and impossibility of phospholipid resynthesis [1, 8].

With the above facts in mind it was interesting to study permeability of apical membranes of small intestinal epitheliocytes located on the boundary between the external and internal media of the body, and this was the aim of the present investigation.

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