

## TWO-DIMENSIONAL ELECTROPHORETIC MAPPING OF HUMAN ERYTHROCYTE PROTEINS

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### Introduction

The human erythrocyte has been extensively studied because of its apparent homogeneity as a cell type, its ready availability, and the many advantages which it offers for genetic, enzymatic, and clinical investigations. Electrophoretic and kinetic variants of both cytoplasmic and membrane bound red blood cell proteins have been described (1, 2) and may account for many hereditary defects. Defects in the molecular anatomy of the erythrocyte may be due to a number of causes including abnormal amount of a protein gene product; molecular instability of a protein resulting from structural modifications which may affect function; or the abnormal posttranslational modification of a protein which alters its stability, catalytic properties, or structural function. The clinical interest in these modifications is the association of specific variants with disease states while theoretical interest is based on the elucidation of functional impairments directly related to molecular alterations.

Several specific problems are associated with the study of both cytoplasmic soluble proteins and membrane bound proteins of the human red cell. Analysis of soluble proteins, either by isolation of particular enzymes or by studies on the whole cell, is complicated by the presence of vast quantities of hemoglobin which may mask or interfere with minor proteins known to be present. Both one-dimensional electrophoretic techniques and enzyme assays for kinetic variants can be dramatically affected by the presence of such an overwhelming amount of one protein.

Problems associated with the study of erythrocyte membrane proteins by conventional one-dimensional electrophoretic methods include the apparent heterogeneous population of proteins, as has been demonstrated by Mueller et al. (3) in their analysis of the sialoglycoproteins of normal erythrocytes. Another problem is the complication caused by the aggregation of polypeptides following solubilization by conventional methods. Progress in the analysis of nonhemoglobin erythrocyte proteins, especially membrane proteins, has been seriously limited by the lack of suitable solubilization methods and by analytical systems capable of resolving the complete protein population of the cell.

The combination of solubilization in sodium dodecyl sulfate (SDS) and the separation of proteins based upon two biophysically unrelated parameters, namely charge and molecular weight, has proved to be a powerful tool for the study of complex mixtures of proteins. This form of two-dimensional analysis of proteins, perfected to a high resolution technique by O'Farrell (4) has been modified by Anderson and Anderson (5, 6) to allow large numbers of samples to be run in parallel (ISO-DALT system). Utilizing the ISO-DALT system, Edwards et al. (7) have recently demonstrated that over 250 discrete proteins or protein subunits can be resolved from the human erythrocyte lysate. Recently, the proteins of whole (nonselectively solubilized) erythrocyte membranes were mapped by two-dimensional electrophoresis by Harell and Morrison (8) and by Rubin and Milikowski (9).

Two-dimensional mapping of the proteins of the human erythrocyte provides a new and valuable approach to the search for genetic variants which will aid in the elucidation of the relationships which exist between the molecular structure and the function of enzymes and structural components of the cell. This approach is essential for the detection of possible molecular aberrations associated with dysfunctional conditions. In this paper we present the results of studies designed to resolve the protein components of soluble and membrane fractions of human erythrocytes and to identify some of the major enzymes and structural proteins.

## Experimental Procedures

**Materials.** Whenever possible, all chemicals employed were of reagent grade quality. All materials and equipment for this study have been previously described (7).

**Preparation of erythrocyte lysate and purified enzymes.** The preparation of human erythrocyte lysate proteins and the purification and mapping of a number of erythrocyte enzymes have been previously described by Edwards et al. (7).

**Preparation and treatment of erythrocyte membranes.** Fresh blood samples were drawn into evacuated tubes containing 1.4 mg/ml ethylenediaminetetraacetic acid (EDTA) as anticoagulant. The erythrocytes were washed five times with equal volumes of phosphate-buffered saline, pH 7.4. During aspiration of the supernatant following each wash, care was taken not to disturb any of the packed cells. To remove contaminating leukocytes and platelets the cell suspension was passed through a column of microcellulose/ $\alpha$ -cellulose (Sigma Chem. Co.) equilibrated with phosphate-buffered saline, pH 7.4, according to the procedure of Beutler et al. (10). The cells were lysed by adding five volumes of glass-distilled water and stirring gently for 1 hour at 4°C. The membrane fraction was collected by centrifugation at 50,000  $\times g$  for 20 minutes and freed from contaminating cytoplasmic proteins by five washes with cold hypotonic (5 mM) phosphate buffer, pH 8.0.

The washed membranes were prepared for two-dimensional electrophoresis by a combination of solubilization steps. The loosely packed membrane pellet was made 2% (w/v) with SDS and 5% (v/v) with mercaptoethanol. The sample was heated on a boiling water bath for seven minutes then centrifuged at 200,000  $\times g$  for 1 hour. No pellet could be observed following centrifugation indicating that solubilization of the membranes was complete. Following dialysis versus glass distilled/deionized water to remove excess SDS, the sample was lyophilized to dryness. Resolubilization of the sample to a protein concentration of approximately 10 mg/ml was accomplished by the addition of buffer containing 9.5 M urea/5% mercaptoethanol/8% Nonidet P-40/1% wide range ampholytes as a modification of the

procedure described by Harell and Morrison (8). Samples containing 100 to 300 micrograms of membrane protein were used for mapping by two-dimensional electrophoresis.

**Spectrin extraction from washed membranes.** Spectrin was extracted from the washed erythrocyte membrane ghosts by dilution of the packed membranes with five volumes of glass distilled/deionized water. After allowing the mixture to stand at room temperature for two hours, the sample was centrifuged at  $50,000 \times g$  for 30 minutes. The supernatant containing extracted water-soluble protein was dialyzed overnight at 4°C against 100 volumes of water. After lyophilizing the sample to dryness, the powder was resolubilized to approximately 5 mg/ml in buffer containing 50 mM CHES (cyclohexylaminoethane sulfonic acid)/2% SDS/5% dithiothreitol/10% glycerol, pH 9.2. The sample was heated for 5 minutes at 95°C prior to mapping by two-dimensional electrophoresis. Samples containing 5-25 micrograms of protein were routinely mapped.

**Two-dimensional electrophoresis.** The two-dimensional mapping of human erythrocyte lysate proteins and purified erythrocyte enzymes utilizing the ISO-DALT system of Anderson and Anderson (5, 6) has been described in detail (7). Minor modifications of this procedure were made in order to map the protein components of the membrane. The first dimension electrophoretic separation (isoelectric focusing) of membrane proteins was essentially as described for lysate proteins with the exception that the samples were layered beneath 10 microliters of a solution containing 5 M urea/2% mercaptoethanol/3% Nonidet P-40/0.5% wide range ampholytes. Electro-focusing was begun at 200 volts for 2.5 hours, then increased to 700 volts for 15 hours for a total of 11,000 volt-hours. Following electrofocusing the gels were equilibrated in buffer containing 0.0625 M Tris-Cl, pH 6.8/2.3% SDS/5% mercaptoethanol/10% glycerol as described by Harell and Morrison (8). Electrophoresis on slab gels (second dimension) in the presence of SDS was performed with the DALT apparatus. For the electrophoresis of solubilized whole membranes gradients of polyacrylamide ranging from 5-20% and containing 2.7% bis-acrylamide as crosslinker were used. The second dimension electrophoresis of purified spectrin was performed on slab gels of 4-10% polyacrylamide with 2.7% bis-acrylamide as crosslinker.

Electrophoresis of both solubilized whole membrane and purified spectrin was at 120 mA constant current per gel. Fixation and staining with Coomassie Brilliant Blue R250 and subsequent destaining and photography were as described (7).

## Results and Discussion

The two-dimensional electrophoretic map shown in Figure 1 reveals that the soluble fraction of the human erythrocyte can be resolved into over 250 discrete proteins or protein subunits even in the presence of hemoglobin. The denatured protein units separated by this technique range in molecular weight from approximately 10,000 to 100,000 daltons. The map positions of five enzymes or their subunits, identified by co-electrophoresis of purified samples, are also shown. These enzymes were purified by conventional procedures as described in (7) and a summary of their purification is shown in Table 1. Of the enzymes purified, only lactate dehydrogenase is in such low quantity that the map position could not be determined with certainty when whole lysate samples were run. However, lactate dehydrogenase has been mapped in hemoglobin-free lysates (7).

A number of the proteins display a significant degree of charge heterogeneity which possibly represents either in vivo or in vitro deamidation (Figure 1). The possibility that deamidation was occurring in vitro was tested by the analysis of both heated and nonheated samples. In both cases, the spot patterns of these protein units remained the same suggesting that the charge heterogeneity observed is an in vivo situation and is not a preparation artifact.

The charge heterogeneity observed in our two-dimensional patterns confirms the findings of others (11, 12) that multiple electrophoretic forms of some human erythrocyte enzymes do occur. Figure 2 shows the two-dimensional electrophoretic separation of the seven human erythrocyte proteins which we have purified and demonstrates the charge heterogeneity found in these

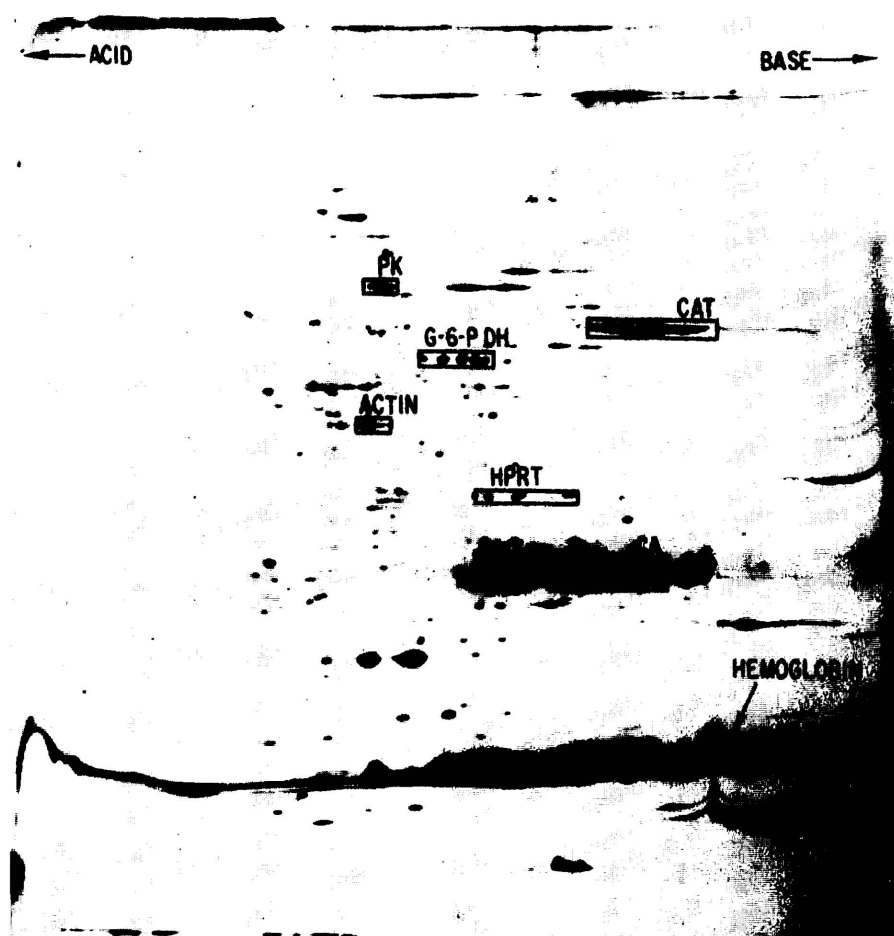


Fig. 1. Two-dimensional separation of human erythrocyte lysate proteins. Isoelectric focusing was from right to left, and molecular weight separation from top to bottom. Abbreviations: CAT = catalase; PK = pyruvate kinase; G-6-P DH = glucose-6-phosphate dehydrogenase; HPRT = hypoxanthine phosphoribosyltransferase; CA = carbonic anhydrase. Hemoglobin and actin are also labeled. (Reprinted by permission of Blood 53, 1121-1132, 1979).

protein units. In one case (Figure 2F) the well documented differences in molecular weight (approximately 1,000) in the isozymes of the monomeric enzyme carbonic anhydrase can be easily seen. A discussion of our findings relating to these purified proteins has been previously reported (7).

Table 1. Summary of Purifications of Six Human Erythrocyte Enzymes

Enzyme	Lysate* (1:2)	Final Product	Overall Purification
Catalase	Slope/mg = 0.053	Slope/mg = 2.72	51.3
Carbonic Anhydrase	0.0009 $\mu$ M/min/mg	1.75 $\mu$ M/min/mg	194.4
G-6-P-DH	0.005 "	39.66 "	7932.0
HPRT	0.0024 "	23.33 "	9720.8
LDH	0.043 "	15.69 "	364.9
Pyruvate Kinase	0.016 "	79.38 "	4961.3

\* Total protein of lysate based upon a hemoglobin concentration of 300 mg/ml of packed cells. (Reprinted by permission of Blood 53, 1121-1132, 1979).

Analysis of the human erythrocyte membrane by two-dimensional electrophoresis has been complicated by the aggregation of some membrane components (particularly spectrin and actin) when solubilized in SDS. Other cell types have been solubilized by SDS and the membranes subsequently mapped with excellent results (4, 13). These investigators showed that addition of an excess of nonionic detergent over SDS was required to be added to the sample prior to isoelectric focusing. Although this requirement held for the erythrocyte membrane, some aggregation of the spectrin-actin complex frequently occurred resulting in both vertical and horizontal streaking in our gels. The aggregation problem was not solved until we used the method of Harell and Morrison (8), which involved extensive heating of the sample. Figure 3 shows the results of our efforts to use this solubilization procedure and apply it to the separation of erythrocyte membranes utilizing the ISO-DALT system. Many of the spots seen in the map can be directly related to the bands seen on conventional one-dimensional gels. A total of about 75 spots can be seen with only moderate loading of the sample. The broad diffuse region labeled band 3 in one-dimensional systems is also not well defined in the two-dimensional map, indicating as previously suggested, that band 3 is composed of a very heterogeneous population of protein units (2).

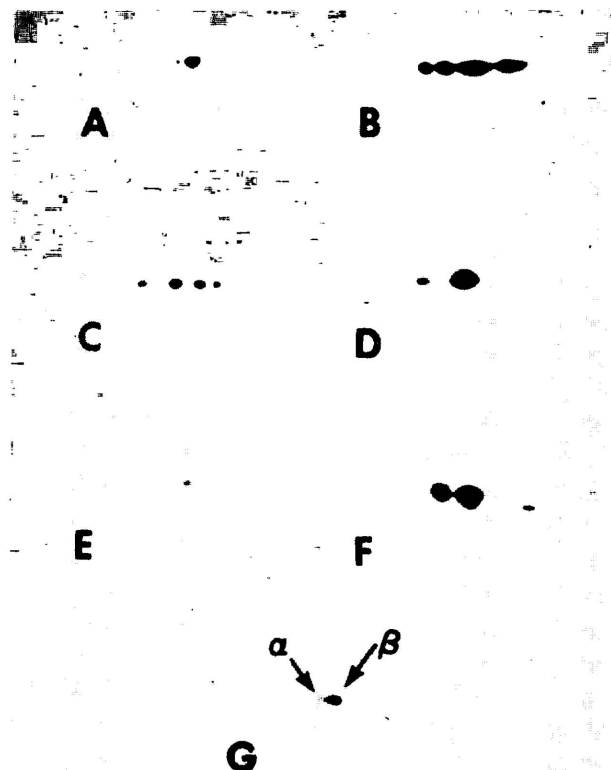


Fig. 2. Sections from two-dimensional gels showing the protein subunit patterns of seven purified human erythrocyte soluble proteins. A, pyruvate kinase; B, catalase; C, glucose-6-phosphate dehydrogenase; D, lactate dehydrogenase; E, hypoxanthine phosphoribosyltransferase; F, carbonic anhydrase; G, actin. (Reprinted by permission of Blood 53, 1121-1132, 1979).

Erythrocyte spectrin is currently the focus of much attention because of its role in maintaining membrane shape, because it may contain the attachment sites for membrane protein and interact with band 5 (erythrocyte actin), and because of its heterogeneity as determined by isoelectric focusing and N-terminal analyses (14, 15). Figure 4 shows the charge heterogeneity commonly seen in two-dimensional separations of this protein. One possible explanation for the lack of clearly defined resolution of spectrin is the lack of solubility at its isoelectric point in the focusing gel (15).

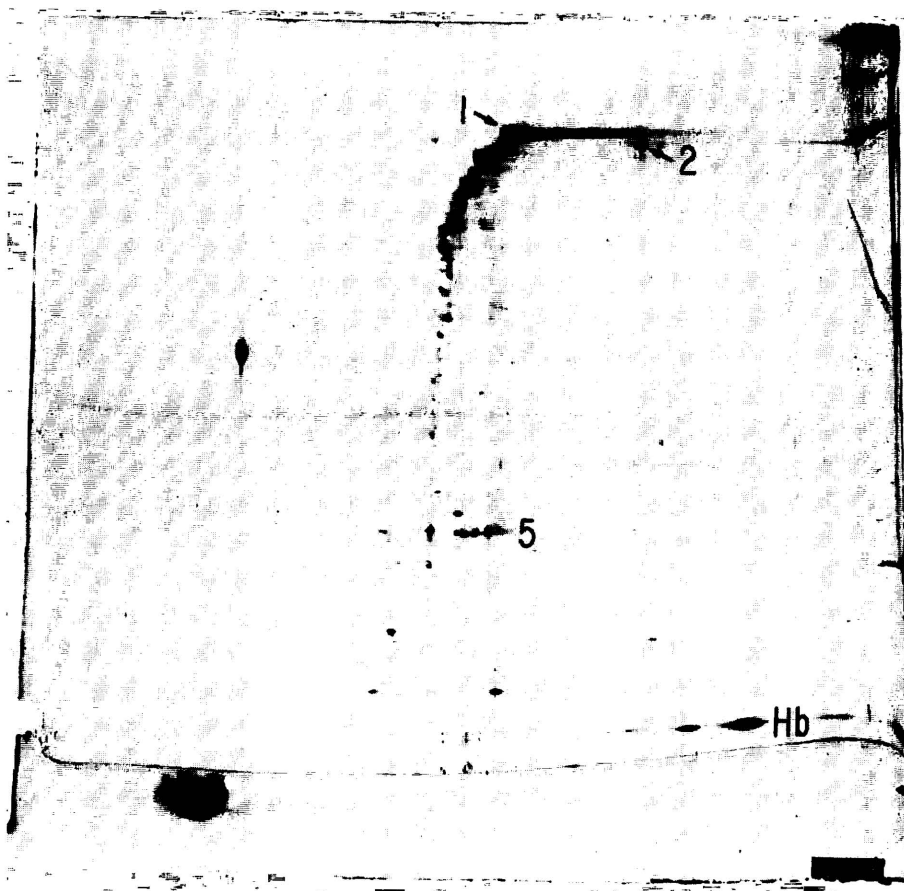


Fig. 3. Two-dimensional separation of human erythrocyte membrane proteins. Isoelectric focusing with wide range ampholytes (Brinkman 2-11) was from right to left and molecular weight separation from top to bottom. Numbers correspond to the designation of proteins in one-dimensional patterns as adopted by Fairbanks et al. (16).

Another possible explanation for the lack of resolution in the first dimension separation may be due to degradation caused by residual protease activity inherent to the red cell membrane or from contaminating leukocytes. This problem is currently being investigated.

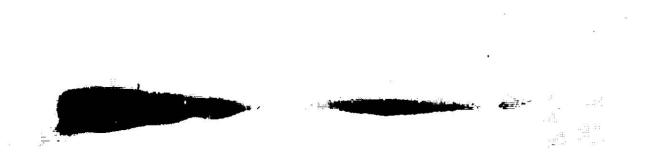


Fig. 4. Two-dimensional separation of human erythrocyte spectrin. (Band 1)

#### Conclusions

This paper demonstrates the feasibility of using two-dimensional electrophoresis as an analytical method for mapping both the soluble and the membrane bound proteins of the human erythrocyte. The resolution of these proteins obtained by this method extends the already valuable use of the human erythrocyte as a tool for the study of many metabolic disorders. Furthermore, the use of the ISO-DALT system, with its multisample capability, provides a method for the full utilization of the erythrocyte for genetic screening purposes.

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