SOLUBILIZATION AND ELECTROPHORESIS OF HUMAN RED CELL STROMA

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Access to a structural study of the lipid-protein complex comprising the membrane of the human red blood cell has been restricted by this material's insolubility. A method for solubilizing human red cell stroma is herein presented which permits electrophoretic demonstration of á number of protein components on polyacrylamide gel as well as histochemical identification of lipid and alkaline phosphatase. Stroma isolated from hematologically normal individuals yields a characteristic pattern of protein bands. This technique may make possible the elucidation of the membrane defects postulated for such genetically determined red cell disorders as Hereditary Spherocytosis and Hereditary Elliptocytosis (Jacob and Jandl, 1961; Murphy, 1965).

Two reports within the past year have presented methods for solubilizing the major portion of red cell stroma in the human (Azer et al., 1965) and ox (Maddy, 1964). The present technique offers the advantage of complete solubilization of this material as evidenced by the absence of any detectible protein at the origin following electrophoresis. This method differs from those previously described in that it enables one to solubilize whole red cell stroma without subjecting it first to efforts at lipid extraction with organic solvents. Electrophoresis of the solubilized stroma on polyacrylamide gel resolves over a dozen protein bands in the normal individual.

Method. Red cell stroma was prepared from 20 ml of freshly obtained blood which was defibrinated and subjected to gradual hemolysis by means of stepwise

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dialysis against increasingly hypotonic tris-buffered saline (Schrier & Doak, 1963). Schrier has further modified this method so that dialysis of the cells for several hours through each buffer change is no longer necessary. Instead the successive changes of buffered saline are achieved by vigorously shaking the red cells until they are well suspended in cold dialysate, centrifuging them, decanting the old buffer, then resuspending the cells as before in newly added buffer. At the end of the sequence the hemoglobin depleted red cells were suspended and centrifuged in 1.65×10^{-4} M tris buffer at pH 7.4. Only the clear white precipitate surrounding the small central buff-colored portion of the button was aspirated to make a suspension with protein concentration of 5 mg/ml. Examination of this suspension by phase contrast microscopy revealed characteristic red cell "ghosts" without any evidence of other contaminating cellular material. This suspension was stored overnight at 0°C, thawed, then frozen and thawed two additional times prior to solubilization and electrophoresis the following day. Aliquots of the suspension were also lyophilized. Solubilization and electrophoresis yielded essentially similar patterns. Lyophilized samples showed greater stability of their electrophoretic pattern with storage.

Solubilization was achieved by adding 50 µl of frozen-thawed stroma (or .3 mg lyophilized stroma) to a mixture of 50 µl of 5% Triton X-100 in 8 M urea and 50 µl of 0.1 M beta-mercaptoethanol. The suspension clarified immediately on shaking.

Electrophoresis was carried out on the polyacrylamide gel system of Ornstein and Davis (1962) with the following buffers:* Upper reservoir: .0509 M glycine and .053 M tris (pH 8.9); Lower reservoir: 0.5 M HCl and 0.1 M tris (pH 8.1). The small pore gel was made up in 8 M urea and poured to a column height of 4 cm within 6.5 cm x 0.5 cm glass tubes. Fifty µl of solubilized stroma was layered into the gel column through the upper buffer. Electrophoresis was carried out at room temperature first for a period of 10 minutes at 2 ma/tube to prevent sample loss by convection, then for an additional 10 minutes at 4.5 ma/tube. Migration

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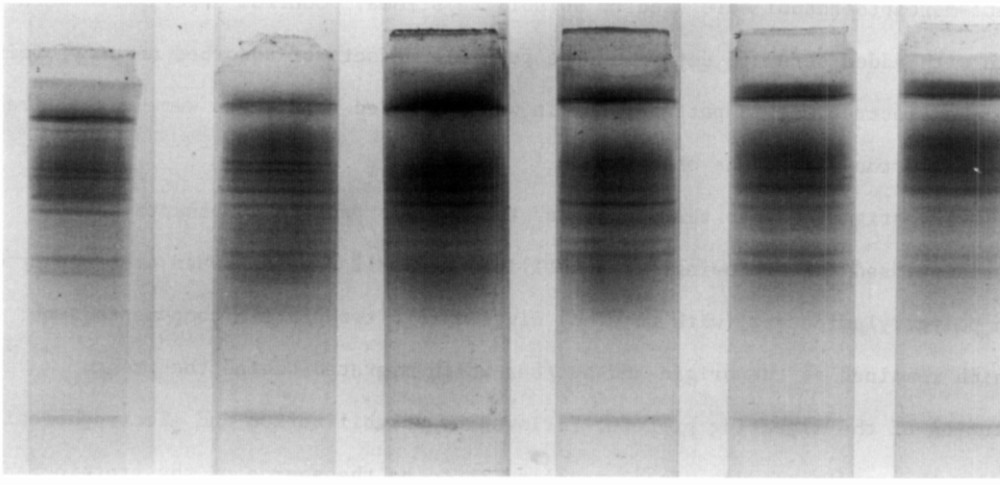
of the tracking bromphenol blue dye under these conditions was about 2.4 cm.

Results.

Origin (-)



Illustrated above are electrophoretic runs of stroma obtained from 6 normal individuals. Gels were stained with 1% Amido Black. Over forty normal samples have been run with patterns (and variations) similar to those pictured. Within the complex of bands a reproducible appearance of lightly staining and densely staining components characteristic of normal stroma could be observed. The fastest migrating band, coinciding with the front, was also present in control runs of serum and stroma-free hemolysate. Although it stained faintly positive to benzidine, its electrophoretic properties were not those of hemoglobin, suggesting therefore that the band might contain altered hemoglobin or other heme-containing components. The slowest migrating band was also the densest staining. This band stained positively for lipid with Oil Red O and showed alkaline phosphatase activity with alkaline buffered substrates, p-nitro phenyl phosphate and alpha naphthyl phosphate. Between these two bands lay zones of proteins of varying intensity. Gross deviations in these bands including loss of some of the more intensely staining components were observed in stroma derived from morphologically abnormal red cells. Present studies are aimed at exploring these differences further.

Experiments were done varying the proportions of stroma and solubilizing agents. These produced no significant alterations in the electrophoretic pattern. Up to 20% Triton X-100 and as much as three times the usual proportion of beta-mercaptoethanol were used to solubilize stroma. Control experiments were run with added serum to determine the possible effects of adsorbed serum proteins on the electrophoretic patterns and it was concluded that these were not a likely source of the variations observed.

Investigation into the sources of the various protein components have so far disclosed the following: Unsolubilized red cell "ghosts" when electrophoresed on polyacrylamide gel (with no urea) divided into two protein components, one which remained at the origin and another which migrated behind the front. Elution of the migrating protein, followed by solubilization and electrophoresis under the conditions described, revealed it to be the source of the protein bands of varying intensity. Similar treatment of the protein component which remained at the origin showed that it represented the slowest migrating densely staining protein with lipid and alkaline phosphatase activity.

The migrating component of unsolubilized red cell "ghosts" was thought to represent a protein portion of the membrane that is removable in an electric field, or possibly an adherent non-membrane protein. Conceivably it could be intracellular protein not removed with the hemoglobin during hemolysis. However, electrophoresis of unsolubilized "ghosts" following freeze-thaw lysis (i.e. stroma) revealed no increase in the migrating band and no new bands.

Preliminary observations suggest that this technique may also be adaptable for comparative studies of stroma from different animal species and possibly for genetically defined strains within species.

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Bibliography:

Azen, E. A., Orr, S., and Smithies, O., J. Lab & Clin. Med. 65:440, (1965).

Jacob, H.S., and Jandl, J. H., J. Clin. Invest. 43:1704, (1964).

Maddy, A. H., Biochem. Biophys. Acta, 88:448, (1964).

Murphy, J. R., J. Lab & Clin. Med. 65:756, (1965).

Ornstein, L. and Davis, B. J., "Disc Electrophoresis," Reprinted by Distillation Products Industries, Rochester, New York, (1962).

Schrier, S. L., and Doak, L. S., J. Clin. Invest. 42:756, (1963).