Protein and Glycolipid Components of Human Erythrocyte Membranes*

John Lenard†

ABSTRACT: Procedures are described by which human red cell membrane proteins completely penetrate acrylamide gels and are separated into molecular weight classes by electrophoresis in the presence of sodium dodecyl sulfate. The entire membrane is used in this procedure, without any prior extraction or fractionation. Proteins of fourteen different molecular weight classes were identified, and these could not be interconverted by treatment with detergent, alkali or urea, suggesting that they are genuinely different polypeptide chains rather than noncovalently bound aggregates of smaller units. The four most intensely staining bands are shown by comparison of electrophoresis with gel filtration to be the major protein components of the red cell membrane, accounting for approximately 60–65% of the total membrane protein. The molecular weights of these four proteins are estimated to be about 255,000, 240,000, 108,000, and 86,000. Specific staining of the sodium dodecyl sulfate–acrylamide gels for carbohydrate suggests that only the 108,000 molecular weight component contains substantial amounts of carbohydrate, although small amounts may be present in other bands. A second major carbohydrate-containing component migrated through the gel more rapidly than any of the protein bands. This material was identified as a glycolipid, and some of its chemical properties are described.

The protein components of red blood cell membranes and of other biological membranes have until very recently resisted characterization because of their insolubility and close association with lipids. The technique of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate has been shown to separate protein mixtures into their component molecular weight classes (Shapiro *et al.*, 1967; Shapiro and Maizel, 1969). Since intact membranes or their extracted proteins can generally be made to dissolve in sodium dodecyl sulfate solutions, this technique provides a powerful tool for preliminary characterization of all the proteins in a membrane. This report describes procedures which permit this approach to be applied to unfractionated, unextracted red cell membranes, and the initial results which have been obtained.

The protein portion of human red cell membranes was found to be a complex mixture of fourteen different molecular weight classes of proteins, of which four accounted for over half of the total protein. This finding is consistent with reports of heterogeneity of red cell proteins from other laboratories (Azen *et al.*, 1965; Rosenberg and Guidotti, 1968, 1969; Zwaal, and Van Deenen, 1968; Poulik and Lauf, 1969).

Materials and Methods

Hemoglobin-free human red cell membranes were obtained from outdated blood, 3-weeks old or older, by hypotonic lysis of the washed cells in 0.008 M sodium phosphate buffer (pH 7.4) (Dodge *et al.*, 1963). The membranes were than dialyzed exhaustively against 5 mM EDTA–5 mM 2-mercaptoethanol, (pH 7.5) at 4° (Marchesi *et al.*, 1970). To the unfractionated contents of the dialysis bag or an aliquot was then added sufficient 10% sodium dodecyl sulfate solution to make a final sodium dodecyl sulfate concentration of 2–3%. This sample was then heated at 100° for 3 min to complete the protein disaggregation, and applied directly to a Sephadex G-200 column or to polyacrylamide gel.

Sodium dodecyl sulfate–acrylamide gel electrophoresis (Shapiro *et al.*, 1967) was performed in 1% sodium dodecyl sulfate–0.1 M sodium phosphate buffer, (pH 7.0). The gels were 6 mm in diameter and 10 cm long, 5% cross-linked, and contained the same concentrations of sodium dodecyl sulfate and sodium phosphate buffer as used in the medium. They were prerun for at least 1 hr before applying the sample. Urea or sucrose was added to the sample to permit layering between the buffer and the top of the gel. The gels were run at 10 mA/gel for 4 hr. They were fixed in 50% trichloroacetic acid overnight, stained for 2.5 hr in 0.25% coomassie blue in 20% trichloroacetic acid,[1] and then rinsed repeatedly over a period of 2–3 days with 7% acetic acid to remove background color.[2] The procedure of fixing and staining described by Shapiro *et al.* (1967) for gels run in 0.1% sodium dodecyl sulfate did not give visible bands when gels contained 1% sodium dodecyl sulfate.

Separate gels were stained for glycoproteins and glycolipids using a periodic acid–Schiff stain as described by Zacharias *et al.* (1969). These gels were destained with 7% acetic

* From the Division of Endocrinology, Sloan-Kettering Institute for Cancer Research, New York, New York 10021. *Received October 28, 1969.*

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[1] This staining procedure was developed in collaboration with Dr. Thomas Terry.

[2] It has been found earlier by Dr. O. O. Blumenfeld (personal communication) that phospholipid and cholesterol could be extracted from sodium dodecyl sulfate solubilized erythrocyte membranes under similar conditions.

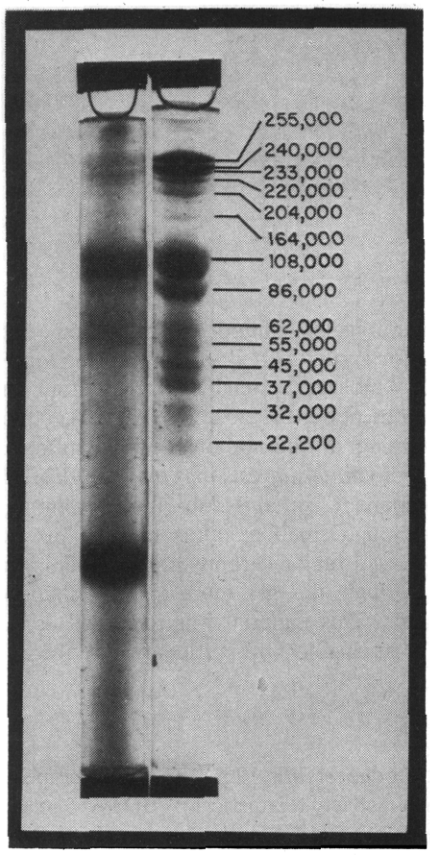

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis patterns of proteins from unfractionated human red cell membranes. Left gel (*ca.* 300 μg of protein) was stained for carbohydrates with a periodic acid–Schiff stain. Right gel (*ca.* 50 μg of protein) was stained with coomassie blue. Molecular weights were estimated by comparison with a standard curve. Details in text.

acid. Destaining with water does not remove the background from gels made with 1% sodium dodecyl sulfate.

Molecular weights were calculated from the migration of several proteins of known molecular weight subjected to electrophoresis in parallel with the red cell membrane preparation. Chymotrypsinogen, ovalbumin and its dimer, bovine serum albumin and its dimer, and immunoglobulin G (all from Mann Biochemicals) were each subjected to electrophoresis in the presence of myoglobin. The position of each band relative to myoglobin was measured after staining, and a plot of the logarithm of molecular weight *vs.* migration (Shapiro *et al.*, 1967) was fitted using the method of least squares. The molecular weights of the red cell membrane proteins were then determined from this curve. Molecular weights higher than that of immunoglobulin G (160,000) were estimated by extrapolation of the plot.

Gel filtration was performed on a Sephadex G-200 column equilibrated with 1% sodium dodecyl sulfate–0.05 M ammonium bicarbonate.

Ninhydrin analysis after alkaline hydrolysis was performed as described by Moore (1968). Total phosphorus was analyzed by the procedure of Bartlett (1959). Cholesterol was measured using *o*-phthalaldehyde according to the method of Zlatkis and Zak (1969).

Results

The patterns shown in Figure 1 were obtained from sodium dodecyl sulfate–polyacrylamide gel electropherograms of unfractionated human red cell membranes solubilized as described above. Comparison of migration rates of the coomassie blue stained bands (right gel) with those of known proteins resulted in the molecular weight estimates shown. Fourteen bands are consistently seen. Of these, four bands (molecular weights about 255,000, 240,000, 108,000, and 86,000) always stain most intensely, while the four between 240,000 and 108,000 appear from their staining intensities to be very minor components.

Staining the gels with periodic acid–Schiff stain (Zacharias *et al.*, 1969) showed two major carbohydrate-containing bands (left gel, Figure 1). The slower of the two major bands (closer to the top of the gel) corresponds to the 108,000 molecular weight band. Several other protein bands were lightly stained with this reagent.

The faster moving of the two major periodic acid–Schiff staining bands (left gel, Figure 1) was not visualized in the corresponding coomassie blue stained gel (right gel, Figure 1). This component does stain with coomassie blue when larger amounts (containing over 100 μg of protein) of solubilized membrane are applied to the gel, in which case it appears as a band of somewhat different color from the protein bands. It is visible as the most rapidly migrating band in Figure 2 (gel 6). This insensitivity to staining by coomassie blue may be partially due to the fact that the glycolipid is washed from the gel during the long time of fixing (in contrast to the short time required for the PAS stain) and may also reflect the different chemical properties of this material. It was identified as glycolipid on the basis of evidence detailed below.

Results of gel filtration of the solubilized membrane preparation on Sephadex G-200 are shown in Figure 2. Individual fractions from the column were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (gels 2–6, Figure 2). The pattern from unfractionated membranes is shown for comparison (gel 1, Figure 2). It can be seen that the retardation of the proteins by Sephadex G-200 is consistent throughout the elution profile with the rate of migration of the protein during gel electrophoresis.

The fractions from Sephadex G-200, the electrophoretic patterns of which are shown in Figure 2 (gels 2–6), were then treated with a variety of denaturing agents. The following procedures were without any visible effect on the electrophoretic pattern obtained from each fraction: (1) treatment with 1 M piperidine, 1 min, 100°; (2) treatment with 6 M urea, 3 min, 100°; (3) dialysis to remove most of the sodium dodecyl sulfate, lyophilization, addition of 2% sodium dodecyl sulfate to dry sample, and heating at 100° for 3 min.

Since these very severe denaturing conditions did not visibly alter any of the bands from any of the fractions tested, it was concluded that the bands seen on electrophoresis represent genuinely different polypeptide chains, which do not arise by noncovalent aggregation of smaller chains.

As mentioned above, the four most intensely staining bands in the gels correspond to molecular weights of 255,000, 240,000, 108,000, and 86,000. These four bands were found to be present almost exclusively in that part of the G-200 elution profile represented by gels 2 and 3 (Figure 2). If

it is assumed that the total area under the ninhydrin curve in Figure 2 is proportional to the total protein in the membrane, and further that the area represented by gels 2 and 3 represents specifically the proteins comprising the four major bands, it can be estimated that these major bands account for about 60–65% of the total protein in the membrane.

The glycolipid identified in Figure 1 by its reaction with periodic acid–Schiff stain is seen in Figure 2 (gel 6) to be eluted from Sephadex G-200 with the phospholipids and cholesterol. Its identification as a glycolipid is based on the following observations.

(1) It stained strongly with periodic acid–Schiff stain, showing that it contains carbohydrate.

(2) The ability of this material to be stained by coomassie blue (and the characteristic color and migration rate of this stain) was used as an assay in studying its extraction behavior. By this criterion, it was quantitatively extracted from the lipid-containing portion of the Sephadex G-200 eluate (Figure 2) into 1-butanol. About 70% of the phospholipid was simultaneously extracted into the 1-butanol phase. In the butanol phase the ratio of ninhydrin-positive material after alkaline hydrolysis to total phosphorus was 0.6:1. This ratio is expected from the published composition of red cell membrane lipids (Rouser *et al.*, 1968) and suggests that little if any protein is extracted into the butanol phase. Thus, the carbohydrate-containing material cannot contain appreciable protein, and demonstrates lipid-like solubility properties.

Other noteworthy observations were made on the glycolipid, using its migration in the sodium dodecyl sulfate–polyacrylamide gels as a qualitative assay.

(1) The glycolipid was never seen in polyacrylamide gels run in 0.1% sodium dodecyl sulfate. On the other hand, it was consistently demonstrated in gels run in 1% sodium dodecyl sulfate, provided sufficient material was applied to the gel.

(2) The glycolipid was not removed from dried red cell membranes by repeated extraction with chloroform–methanol (3:1), conditions which extracted virtually all of the lipid phosphorus. Phospholipids and gangliosides were found not to stain with coomassie blue.

(3) The glycolipid could be extracted with 1-butanol from the unfractionated membrane preparation, solubilized as described above.

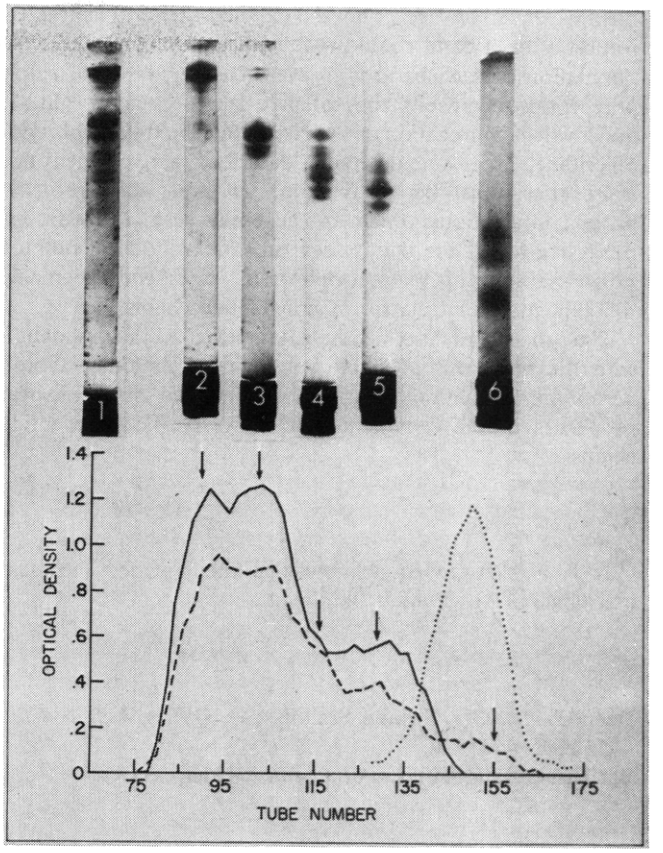

FIGURE 2: Sephadex G-200 gel filtration (2.5 × 90 cm column) of unfractionated human red cell membranes (*ca.* 150 mg of membrane protein) solubilized as described in text. Fractions (100 drop) were collected: (——) OD_{280}; (-----) OD_{570} from ninhydrin after alkaline hydrolysis; (· · · · ·) OD_{830} from phosphorus analysis. Cholesterol was eluted with the phospholipid. Gels 2–6 are sodium dodecyl sulfate–polyacrylamide gels of single fractions taken at the corresponding arrows. Gel 1 is the electrophoresis pattern of the unfractionated red cell membrane shown for comparison.

Discussion

The procedure described in this paper for the solubilization of red cell membranes offers a number of advantages over previously published procedures (Azen *et al.*, 1965; Rosenberg and Guidotti, 1968; Zwaal and Van Deenen, 1968; Poulik and Lauf, 1969). Perhaps most important is the elimination of the need to remove lipids, a procedure which is generally time consuming and which sets lower limits on the amounts of material which can conveniently be handled. The present procedure is limited only by the sensitivity of the staining of the sodium dodecyl sulfate–polyacrylamide gels. Thus, any preparation which yields about 100 μg (dry weight) of membranes can readily be studied by the techniques described.

The precise role of the dialysis of the intact membranes into EDTA–mercaptoethanol prior to solubilization with sodium dodecyl sulfate is not clear. If this step is omitted, the protein does not completely penetrate the polyacrylamide gel. Dialysis into EDTA–mercaptoethanol causes the membranes to fragment, and results in solubilization of part of the membrane protein (Marchesi and Steers, 1968; Marchesi *et al.*, 1970), although it should be emphasized that the soluble and insoluble components are not separated in the course of the procedure described in this paper. Although about 1% of the amino acid residues of red cell membrane protein is half-cystine (Rosenberg and Guidotti, 1968; Blumenfeld, 1968), it does not seem likely that disulfide-bond cleavage plays a major role in the disaggregation. This is suggested by the fact that the electrophoretic patterns shown by individual fractions eluted from Sephadex G-200 (Figure 2) did not change with time or with denaturation by various agents even though no mercaptoethanol was present in the eluting buffer or in the other denaturants employed.

The present study shows that no single protein chain present in red cell membranes has a larger molecular weight than about 255,000. It has been impossible to degrade this chain or any of the smaller chains which have been seen in the polyacrylamide gels into still smaller units using severe dena-

turing agents. However, only the detailed chemical characterization of each band could prove without ambiguity that the population of each band is in every case made up of single polypeptide chains of the calculated molecular weight. If noncovalent aggregation does persist after the treatments with piperidine, urea, and detergent described above, then this aggregation must be vastly more tenacious than protein–protein interactions which are generally seen. It seems far more likely that the bands seen on sodium dodecyl sulfate–polyacrylamide gel electrophoresis represent genuinely different molecular classes of polypeptide chains.

The procedures described in this paper, used in conjunction with the numerous available gel slicing techniques, should permit the association of various chemical and immunological properties of the cell membrane with specific polypeptide chains.

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