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Electrophoretic Evaluation of Various Procedures for Solubilizing Erythrocyte Membranes[1] (38354)

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Of the various experimental approaches to the study of erythrocyte membranes, the analysis of constituent parts is one to which considerable attention has been devoted (1–6). Although it has been shown that such an approach by itself will not yield complete information on functional membrane structure (7–11), the information to be gained from disaggregation of these complex structures into individual constitutent molecules is essential to a full knowledge of erythrocyte membranes; therefore, a wide variety of techniques has been developed to disrupt them and prepare their constituent molecules. Maddy and co-workers (12–14) have compared several procedures for the extraction of proteins from erythrocyte ghosts and found that all yielded soluble membrane components with similar characteristics. The fact that proteins with similar properties can be extracted by different methods suggests that the solubilized materials represent distinct molecular species rather than aggregations produced during the isolation procedures, and one goal of the present research was to test this possibility by comparison of additional preparative techniques on erythrocytes of several species.

Our interest in this subject stemmed also from a desire to determine whether certain physiological stresses might induce changes in any of the constituent proteins of erythrocyte membranes and we sought, therefore, to develop techniques that would permit such an assessment. This paper describes (i) a qualitative and semiquantitative gel electrophoretic characterization of proteins in both hypotonic wash and membrane fractions, following each step in lysis and extraction; (ii) a comparative study of procedures for extracting constituent proteins of erythrocyte ghosts with dodecyl sulfate (DS), Triton X-100 (TX), butanol, pyridine, EDTA, and water; and (iii) a comparative study of these procedures applied to human, bovine, equine and rabbit erythrocytes.

Materials and Methods. Preparation of ghosts and wash (cytoplasmic) fractions. Blood samples from human, bovine, equine and rabbit subjects were collected and prepared as described by Hanahan and co-workers (15, 16); fractions collected after each wash step were lyophilized, resuspended and dialyzed overnight against hypotonic buffer.

Solubilization of proteins of erythrocyte ghosts. Membrane proteins were solubilized by the procedures of previous workers as follows: extraction by water and EDTA (14, 17, 18); butanol extraction (19); pyridine extraction (20); treatment with nonionic detergent, TX (21, 22); and treatment with anionic detergent, DS (23, 24). A summary of the procedures by which the various samples of washings and membrane fractions were prepared for electrophoretic characterization in the presence of DS (DS-electrophoresis) and in its absence (standard electrophoresis) is shown in Fig. 1.

Treatment of ghosts with 1% DS in the presence of 10 m*M* dithiothreitol (DTT), 1 m*M* EDTA and 10% sucrose resulted in complete solubilization. In contrast, treatment with TX (over a range of concentrations from 0.2 to 5%/mg of protein), with or without added 10 m*M* DTT plus 1 m*M* EDTA, caused the highly

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viscous suspension of ghosts to become visually clear but solubilized only 30–40% of the membrane proteins (Fig. 1), as Capaldi (22) has shown. Therefore, this suspended mixture was allowed to stand for 1 hr to ensure maximum disruption of the membrane structure, then was centrifuged at 39,000*g* for 30 min to remove insoluble residues.

Following extraction treatments in which butanol, pyridine, EDTA or water were employed, TX was added to the soluble fraction before standard electrophoresis was run in order that the patterns developed would be comparable to those obtained when TX was used to solubilize the membranes. Butanol extraction of the ghosts yielded no recoverable insoluble residue. but insoluble material was separated after extraction with pyridine, EDTA or water; these were divided into two portions for treatment with DS and TX, respectively (Fig. 1). The portion subjected to the anionic detergent was completely solubilized by treatment for 20–30 min at 37° with 1% DS in the presence of 10 m*M* DTT, 1 m*M* EDTA and 10% sucrose. The other portion, which was to be treated with TX, was further divided into two parts; to one, TX was added in the presence of 10 m*M* DTT and 1 m*M* EDTA, and the other portion was treated with TX alone. Neither treatment fully solubilized the membrane constitutents, so the preparations were centrifuged at 39,000*g* for 30 min to remove insoluble residues.

Procedures for standard- and DS-electrophoresis. Simple modifications of the procedures of Steward *et al.* (25) and Cherry *et al.* (26) permitted their use in the present study. Electrophoresis in the presence of DS was accomplished by substituting 0.4 *M* sodium phosphate buffer (pH 7.1) in the system and adding 0.1% DS to the running gel and bath mixture

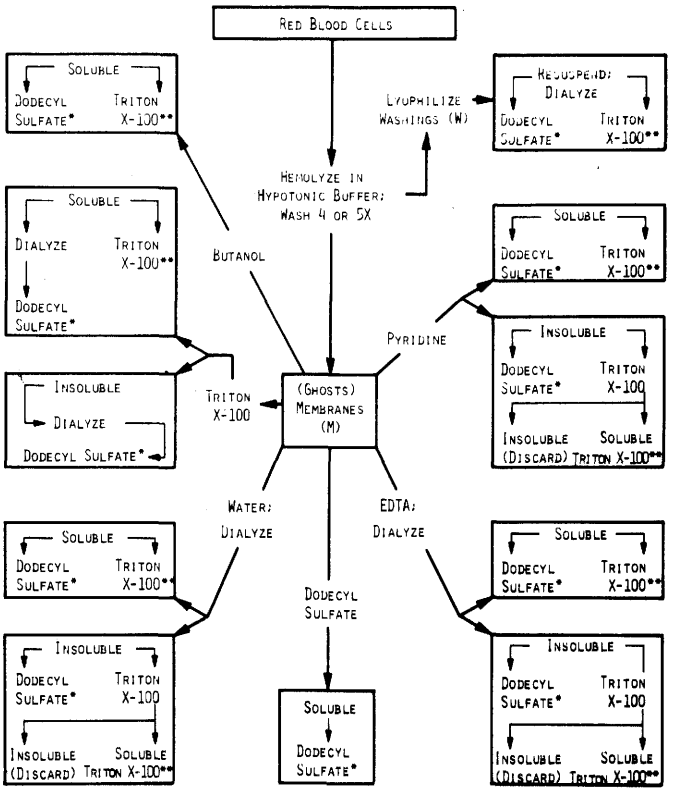

FIG. 1. Flow chart showing the procedures for hemolyzing erythrocytes, washing to produce membrane ghosts, and solubilizing membrane proteins with DS, TX, water, EDTA, pyridine, and butanol. Details and rationales of the procedures are given in the text. *Dodecyl Sulfate: samples contain 1% DS, 10 m*M* DTT, 1 m*M* EDTA and 10% sucrose; subjected to DS-gel electrophoresis. **Triton X-100: samples contain 2.5% TX, with or without 10 m*M* DTT, 1 m*M* EDTA; subjected to standard-gel electrophoresis.

(24). DS-gels were fixed overnight in 15% trichloroacetic acid–25% isopropanol at 0–4°, then stained with 1% amido black in 7% acetic acid (23). Standard gels were fixed only in the solution of 1% amido black in 7% acetic acid. Lipoproteins were located by means of the oil red O lipid staining procedure described by Smithies (27) and glycoproteins by the procedure of Zacharius *et al.* (28). Molecular weights were estimated by gel electrophoresis in the presence of DS by the procedures of Shapiro *et al.* (24), Weber and Osborn (29) and Dunker and Rueckert (30).

Results and Discussion. Protein components shown by DS-gel electrophoresis. Figures 2 and 3 show the results of DS-gel electrophoresis of protein fractions from ghosts of human, bovine, equine and rabbit erythrocytes after solubilization with DS. The effects of sequential washings are shown, as are the consequences of adding DTT. DS-gel patterns of washings and membranes from the four species included in this study were generally similar (Fig. 3); variations among species were noted mainly in the relative amounts of proteins, including hemoglobin, in region 0.60–0.95. Each washing step during hypotonic hemolysis resulted in a decrease in the amount of protein in region 0.60–0.95 and in both qualitative and quantitative increases in proteins in region 0–0.6 (Fig. 2; cf. patterns Mw_{1-4} and M). Similar changes in the protein patterns of hypotonic washings were also noted (Fig. 2; cf. patterns W_{1-5} and M); proteins with corresponding mobilities were observed in the gel patterns of both washings and membranes. In the membrane fraction, two glycoproteins were clearly resolved in region 0.25–0.30. The presence of DTT generally improved the overall resolution of the DS-gels (Fig. 2).

Protein components shown by standard-gel electrophoresis. Preparations of membrane and wash proteins treated with TX, rather than DS, and examined on standard electrophoretic gels are illustrated in Fig. 4. These patterns also revealed that during the stepwise process of hemolysis and washing of erythrocytes, the concentration of proteins in region 1.5–5.0 cm, including hemoglobin, progressively decreased and simultaneously, those in region 0–1.5 cm increased in number and quantity. Aggregation of these proteins, solubilized by the treatment of ghosts with TX and examined on standard gels, was common; they migrated as diffuse bands in

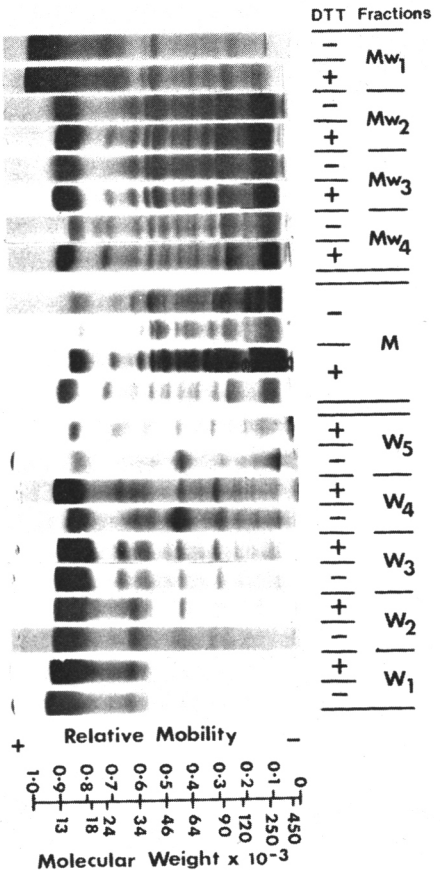

FIG 2. Patterns obtained from DS-gel electrophoresis of fractions from human erythrocytes after each step. The samples designated Mw_1, Mw_2 etc., consisted of the pelleted material collected after each wash; supernatant fluids from the washings are designated W_1, etc; M signifies the final membrane fraction, colorless or faint pink. Each sample was run with and without 10 m*M* DTT. Gels containing different quantities of protein are shown for the membrane fractions.

region 0–1.0 cm (Fig. 4). This area of the gel and a protein band in region 1.0–1.5 cm specifically stained as both lipoproteins and glycoproteins; in gels of the washings, hemoglobin (region 2.4–4.5 cm) and a band in region 1.0–1.5 cm showed affinity for the lipoprotein stain. The presence of DTT in these TX-soluble fractions generally decreased aggregation within the diffuse regions of the standard gels (Fig. 4), thus indicating that oxidation of sulfhydryl groups in isolated membrane extracts might be contributing to the capability of the proteins to aggregate (21, 23, 31–33). In contrast to the results of analysis by DS-gel electrophoresis, examination of wash and membrane proteins on standard gels

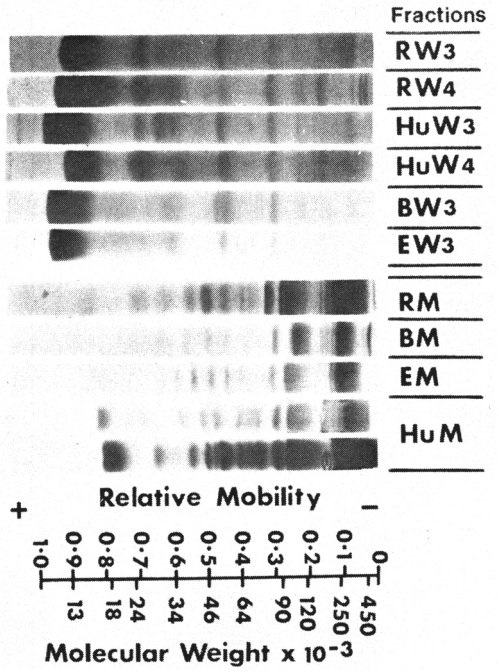

FIG. 3. Patterns of DS-gel electrophoresis of proteins from membranes (M) and cytoplasmic washings (W_3, W_4) of human (Hu), rabbit (R), bovine (B) and equine (E) erythrocytes during hypotonic hemolysis. All samples contained 10 m*M* DTT. Comparison of gels containing different quantities of protein is shown for the human membranes.

revealed variations among the species (cf. Figs. 2–4). This interspecies polymorphism may be attributable to the inability of TX to prevent the isolated membrane proteins from aggregating in various ways, then migrating in the standard gels as discrete bands. Such interactions are diminished in the presence of DS because of the charge-damping effect of this reagent.

As pointed out by other researchers (7–11), the basic structural integrity and composition of erythrocyte membranes is altered during the isolation procedures. Standard and DS-gels of washings and membranes from the four species included in this study revealed proteins with relative mobilities which were similar to one another, as the patterns in Figs. 2–4 show. Bovine and equine erythrocytes released hemoglobin more readily than those from humans and rabbits; four washing steps sufficed for bovine and equine erythrocytes, whereas five washings were necessary to achieve colorless or faint pink preparations of rabbit and human ghosts. This observation is in agreement with those of previous investigators (15, 16), who also showed that bovine erythrocytes were more susceptible to hypotonic lysis than those of humans. These data suggested that although hypotonic lysis readily removed hemoglobin and other intracellular components from erythrocytes at a rate dependent upon the species, some cytoplasmic proteins may be adsorbed to or entrapped in the isolated ghosts. Alternatively, those membrane-bound proteins that are readily sol-

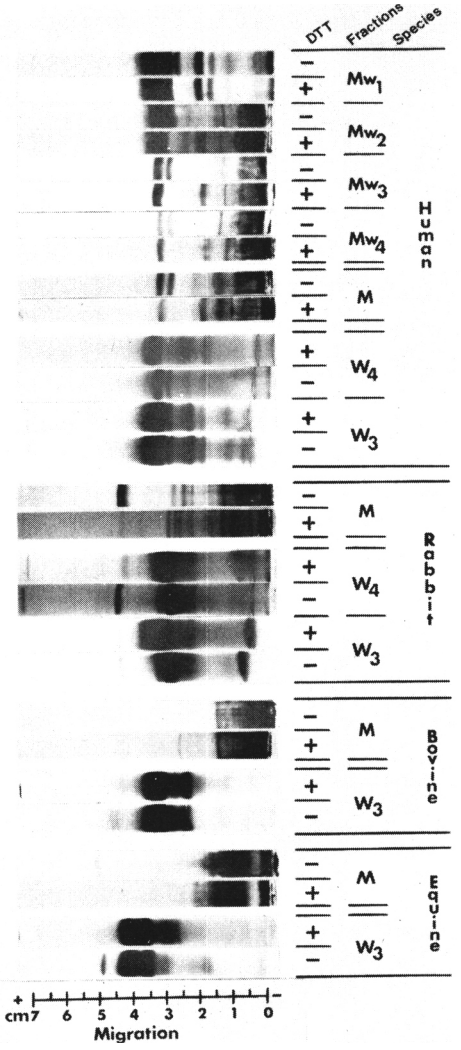

FIG. 4. Comparison of patterns obtained by standard-gel electrophoresis of fractions isolated from erythroyctes after each step in hypotonic hemolysis. Samples designated Mw_{1-4} were composed of the pelleted material collected after each wash; the supernatant fluid from the washings are represented by W_1, etc., and M signifies the final membrane preparations. The comparative effects of 10 m*M* DTT are shown.

ubilized in hypotonic buffer may form weak points in the membrane structure so that the flow of the cytoplasmic constituents from the cell forces their release. This could explain the observation that in both the wash and membrane fractions, there are lipoproteins having similar electrophoretic mobilities on standard gels. However, it is impossible with present techniques to distinguish between the true membrane components in the gel patterns and those adsorbed to, entrapped by or removed from the membrane during the stepwise process of hypotonic hemolysis.

Comparison of membrane proteins solubilized by different techniques. The present research was designed, in part, to extend the studies of Maddy and co-workers (12–14) by characterizing and comparing, on both standard and DS-gels, the membrane proteins solubilized in the presence of TX, DS, butanol, pyridine, EDTA and water, according to the scheme shown in Fig. 1.

Treatment of erythrocyte ghosts from all four species by use of TX never completely solubilized the ghosts (Fig. 1) but it did solubilize the glycoproteins in region 0.2–0.3 (Fig. 5) and a number of minor proteins (Fig. 5; regions 0–0.2 and 0.3–0.6) as shown by DS-gels. When the TX insoluble fraction was treated with DS and run in DS-gels, two major protein bands were observed in region 0–0.2 and several minor constituents were evident in region 0.2–0.6; some of the latter, including the glycoprotein, were observed as major and minor bands in gels of the soluble fraction. These data suggest that those proteins in the TX-soluble fraction that have electrophoretic mobilities resembling those in the insoluble preparations may be similar constituents in the intact membrane. However, the former may be extrinsically bound to the membrane and readily released by TX, whereas the latter probably are more firmly bound to the membrane structure. Comparison of DS-gels of the TX-soluble and TX-insoluble fractions with those obtained by extracting the ghosts only with DS showed that the latter had a banding pattern

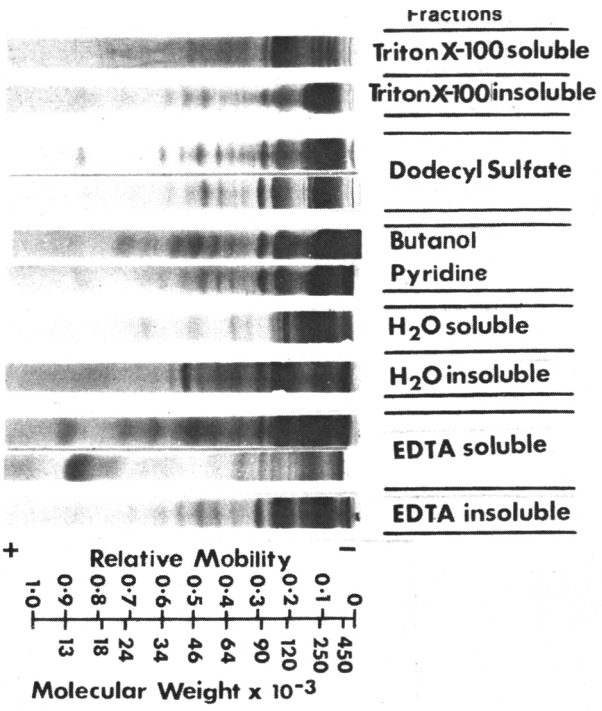

FIG. 5. Comparisons between DS-gels containing soluble and insoluble membrane proteins after treatment of human ghosts with TX, DS, butanol, pyridine, water or EDTA. Insoluble fractions remaining after treatment of the ghosts with TX, water or EDTA were solubilized by DS before electrophoresis; DTT was included in all samples. Comparisons of gels containing different quantities of protein are shown for the DS and EDTA-soluble fractions.

that contained constituents of both the TX-soluble and TX-insoluble preparations (Fig. 5). These same components from the TX and DS preparations appeared in region 0–2.5 cm of standard gels as a group of diffuse bands or aggregates of proteins, lipoproteins and glycoproteins (Fig. 4).

Previous investigators (19, 20) have shown that proteins of membranes solubilized by treatment with organic solvents readily aggregate and fail to separate into distinct bands when subjected to standard-gel electrophoresis. Our experiments showed similar results; solubilization of proteins by butanol or pyridine yielded components which remained near the origin on standard gels and stained as both glycoprotein and lipoprotein. When subjected to electrophoresis in the presence of DS, however, some of these components disaggregated and migrated as individual proteins (Fig. 5), forming patterns that resembled those obtained by solubilization with DS. The insoluble residue which was collected after treating ghosts with pyridine, when electrophoresed in the presence of DS, showed one major band near the origin and a few minor components located throughout the gel.

Proteins held in association with membranes by ionic interactions can be solubilized by treatment with EDTA solutions or by manipulations of either pH or ionic strength (14, 17, 18). The majority of these water-soluble proteins have large molecular weights, aggregate readily and contain little or no lipid, as was indicated in our experiments by their failure to separate into distinct components and stain as either glycoproteins or lipoproteins on standard gels. The presence of DS during electrophoresis reduced the amount of aggregation (Fig. 5) compared with that in standard gels. The patterns obtained by DS-gel electrophoresis of the water-insoluble fraction showed general similarity to the patterns of all three types of proteins solubilized by TX (Fig. 5). EDTA extraction resulted in an increased number of protein components solubilized from ghosts and produced DS-gel patterns resembling those resulting after treatment with DS, butanol or pyridine (Fig. 5). Electrophoresis of these samples in standard gels resulted in proteins remaining near the origin as diffuse bands. Moreover, bands in the DS-gels of water-soluble and EDTA-soluble fractions were also evident in the DS-gels of their insoluble preparations (Fig. 5). These data suggested that polypeptide components similar to those observed in the soluble fractions may be intrinsically bound in the membrane structure (i.e., lie buried in the hydrophobic regions of the membrane) and, therefore, are not available for solubilization by water or EDTA.

From the above, it is evident that similar components are shown by DS-gel electrophoresis of membrane proteins solubilized from ghosts by treatment with vastly different reagents, viz., TX, DS, butanol, pyridine, EDTA and water. Since proteins with similar electrophoretic characteristics were extracted from membranes by different techniques, it seems likely that these entities were not artifacts resulting from the methods used in solubilization. Each extraction procedure may disrupt the membrane into heterogenous constituents (e.g., lipoprotein, glycoprotein and protein complexes) that are further dissociated into their fundamental protein constituents by DS, which also prevents reaggregation, prior to and during, gel electrophoresis. In the absence of DS, these isolated components of the membrane structure tend to aggregate, perhaps as a consequence of hydrophobicity, into complexes which pass into standard gels with difficulty and form the basis of the electrophoretic variability observed among species.

Summary. Polyacrylamide gel electrophoresis in DS revealed certain similarities in the patterns for protein components of solubilized erythrocyte membranes and their cytoplasmic washings. Gels run without the addition of DS also showed similarities for membrane components and cytoplasmic washings, but they differed markedly from those obtained with DS-electrophoresis. Identical observations were made with bovine, equine, human and rabbit erythrocytes.

Electrophoretic patterns of erythrocyte constituents run in the presence of DS were similar for all four species, but in the absence of this anionic detergent standard-gel patterns of proteins showed considerable interspecies variation.

Proteins solubilized from ghosts by extraction with DS, butanol, pyridine, water, EDTA or TX had similar mobilities when electrophoresis was conducted in the presence of DS. The absence of DS during electrophoresis permitted many of the membrane constituents solubilized by these different procedures to form aggregates

and migrate as diffuse bands, rendering their comparison difficult.

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