Acrylamide Gel Electrophoresis Studies of Human Erythrocyte Membrane

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A sensitive system has been presented for the isolation of erythrocyte membrane and the separation of its solubilized components by acrylamide gel electrophoresis. Comparisons were made between the membrane from both normal patients and patients with hereditary spherocytosis. While variation in a specific band on electrophoresis could be correlated with the disease state, no such variation existed among the normal membrane samples. The possible reasons for these observations are discussed.

Because of the increasing interest in the structure and function of biomembrane systems, it is imperative to establish rapid, efficient methods of membrane preparation as well as sensitive, simple means of separating solubilized membrane components. This will allow a study of the components of normal cell membranes and expedite the comparative study of the components in normal and disease states. In this paper, we present a method of membrane preparation which is fast and efficient. In addition, a method of analysis of solubilized membrane components, i.e., polyacrylamide gel electrophoresis, is described which is sensitive and reproducible.

It has been suggested by Jandl that membrane protein may be important in the etiology of hereditary spherocytosis. We have compared the electrophoretic patterns of solubilized normal human erythrocyte membrane preparations with those of patients with hereditary spherocytosis (H.S.).

## Materials and Methods

Erythrocyte membrane derived from the A, B, and O systems, all Rh (–), was examined as a control and consisted of recently outdated packed cells from the Medical College of Virginia Blood Bank preserved in acid citrate dextrose (ACD). Fifteen to 50 ml. of blood from patients with H.S. were collected in citrate alone. Nine fresh samples from normal subjects were drawn in citrate alone and used as a control for the observations made on Blood Bank blood drawn into ACD.

After removal of the buffy coat following centrifugation for 20 minutes at 1500 g., 25 ml. of packed cells were added to 250 ml. of cold 30 mOsm buffer (16.8 Gm. NaHCO₃, 7.4 mg. ethylene-diamine-tetraacetic acid (EDTA) and 3.6 Gm. NaCl diluted to 20 L. with distilled water and adjusted to pH 7.5–8.0). The hemolystate was centrifuged (17,000 RPM, rotor 21, Spinco model L preparative ultracentrifuge, 1 hour). The supernatant was discarded and the membrane pellet resuspended by gentle swirling in the original buffer. This process was repeated, discarding the small red button which appeared below...
the white membrane. After six buffer washes, the supernatant was colorless. With the preparation free of hemoglobin, the pearly white pellets were combined with an equal volume of distilled water, homogenized in a ground glass homogenizer, and centrifuged. This was repeated four additional times using water.

Aliquots of the membrane preparation were taken after the first, third, and fifth buffer washes and after the first, third, and fifth water homogenization. These samples were subjected to amino acid, phosphorus, carbohydrate and cholesterol analysis in order to determine the relative amount of protein, phospholipid, carbohydrate, and cholesterol in the membrane. The anthrone reaction was used to determine total neutral sugars while phosphorus and cholesterol were determined by the methods of King and Rosenthal et al., respectively. Amino acid analysis was done using a Beckman Spinco model 120-C amino acid analyzer on samples which had been hydrolyzed for 20 hours in 6N HCl under vacuum.

The final membrane pellet was suspended in an equal volume of a solution which was 0.2M in sucrose and 0.2M in EDTA. The addition of a phenol:urea:acetic acid:water solvent (2:1.2:1:1/W:W:V:V) (PUA) in a volume equal to the volume of the above membrane suspension yielded a clear “solution.” Residue was not obtained at the bottom of centrifuge tubes following centrifugation at 50,000 RPM for 45 minutes (Spinco model 50 rotor). This preparation was used as a sample for gel electrophoresis as described below. In four cases a supersaturated solution of normal membrane in PUA was prepared and the unsolubilized membrane was isolated by centrifugation at 50,000 RPM for 30 minutes (Spinco model 50 rotor). The supernatant was saved and the pellet was solubilized by adding more PUA. Both preparations were electrophoresed simultaneously to rule out differential solubilization of membrane components by the PUA.

The method employed here to achieve optimum resolution of bands is a modification of several methods: Cotman, Takayama, as modified by Baum. Gels were made by using solution A (acrylamide 6 Gm.; N, N' methylene bisacrylamide 0.16 Gm.; urea 12 Gm.; glacial acetic acid 28 ml.; distilled water to 60 ml.) and solution B (ammonium persulfate 0.4 Gm.; urea 12 Gm.; and distilled water to 20 ml.), in a ratio of 2:1 (6.6 per cent acrylamide). N, N', N' Tetramethylethylenediamine (TEMED) was added after the A and B stock solutions were mixed to give a concentration of 0.5 µL. per milliliter of final gel. Gels, poured to a depth of 60 mm. in 5-mm. I.D. glass tubes, layered gently with 10 per cent acetic acid, and polymerized in a 60°C oven for 30 minutes were soaked in the PUA solvent for a period of three days without removing them from the glass tubes. Fifty µL. of the solubilized membrane were layered under 10 per cent acetic acid onto gels, current was applied at 5 mA./tube for two hr., and the gels were stained with Coomassie blue.

The electrophoretic runs were made in a Buchler Polyanalyst apparatus capable of holding 12 gels. Eight to 10 gels were run simultaneously and the remaining two to four positions were blocked with glass rods. Each electrophoresis of H.S. membrane was done in duplicate and always simultaneously accompanied by duplicate electrophoresis of normal membrane or membrane from patients with other diseases as a control. In this manner, direct comparisons of the electrophoretic patterns were always available among the membranes derived from normals, H.S. patients and patients with other diseases.

RESULTS

Table 1 shows the results of the chemical analyses of the membrane sampled during the successive washings. The amount of protein, phospholipid, carbohydrate and cholesterol is expressed as per cent of dry weight of membrane residue recovered after the washings. The per cent protein, lipid, and carbohydrate changed during the successive washings: The per cent protein decreased by 10 per cent from 60 to 50 per cent; the per cent phospholipid

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*Buchler Instruments, Inc., Fort Lee, N. Y.
Table 1.—Effect of Successive Washings on the Relative Amount of Protein, Lipid and Carbohydrates in Erythrocyte Membrane Preparation

| Successive Washings | Protein* | Phospholipid † | Cholesterol Percent | Carbohydrate ‡%
|---------------------|----------|----------------|---------------------|----------------
| Buffer              |          |                |                     |                |
| 1                   | 59.6     | 25.9           | 10.4                | 8.1            |
| 3                   | 55.3     | 25.7           | 10.0                | 7.8            |
| 5                   | 55.1     | 25.7           | 9.9                 | 7.8            |
| Water Homogenization|          |                |                     |                |
| 6                   | 53.4     | 25.8           | 9.9                 | 7.7            |
| 8                   | 50.5     | 27.7           | 9.6                 | 7.7            |
| 10                  | 50.2     | 27.8           | 9.6                 | 7.6            |

* Protein as determined by Amino Acid Analysis.
† Phospholipid as determined by weight of phosphorus multiplied by a conversion factor of 25.
‡ Anthrone-positive neutral sugars as an indication of carbohydrate.

increased from 26 to 28 per cent, while the per cent cholesterol and carbohydrate decreased slightly. The relatively large decrease in protein indicated that loosely adsorbed protein, especially hemoglobin, was removed by the repetitive buffer and water washes. The early buffer washes removed more protein than the later washes, and the protein content of the remaining membrane changed only slightly during the last three washes. The final membrane preparation consisted of 50% protein, 37% lipid, and 8% carbohydrate.

More than 200 electrophoretic patterns of erythrocyte membrane from 50 patients of types A, B, and O, all Rh (−), were studied. In addition, the nine fresh samples from normal subjects drawn directly into citrate alone were similarly examined. A representative example is shown in Fig. 1 (normal erythrocyte membrane, E.M.). Duplicate samples which were always run simultaneously gave identical patterns. The eight most prominent bands (A through G) always appeared in the same relative position and with the same relative intensity. As seen in the photographs, there were several faint bands, as indicated by the letter x. The C band was not only prominent, but also absolutely consistent among the normal samples. Neither the blood type nor the anticoagulant (ACD or citrate alone) had any effect on the band patterns produced. In addition, samples from two patients with dyskeratosis congenita with pancytopenia, from two patients with autoimmune hemolytic anemia, and from two patients with transient burr cell formation secondary to thermal burn all gave normal band patterns. Likewise, membrane from a patient with congenital nonspherocytic hemolytic anemia (G-6PD deficiency) gave a normal band pattern. The supernatant and solubilized pellet from the differential solubilization experiment outlined above were identical when run simultaneously.

Fifty electrophoreses were carried out on six patients with H.S. (Fig. 1, H.S.). Normal membrane was always electrophoresed simultaneously with H.S. membrane in order to provide a suitable control. Figure 1 represents such a situation as the gels pictured were run side by side. Band C was noted to be markedly reduced in some H.S. patients while being present in others. It is the variability of band C noted among the patients with H.S. as illustrated in
Fig. 1.—Representative acrylamide gel electrophoresis band patterns of normal erythrocyte membrane (E.M.) and of membrane from patients with hereditary spherocytosis (H.S.).

Fig. 2 that distinguishes these band patterns from those obtained using normal membrane. There was variation in the staining intensity of other bands among the gels obtained; although the relative intensity of the bands in each gel varied, only the variation in the C band could be correlated with disease. The five patients who were splenectomized all had reticulocyte counts below 1.5 per cent and hemoglobins above 10.5 Gm. per cent; nevertheless, the C band showed variation among these patients. One patient was not splenectomized, maintained a reticulocyte count above 12 per cent and had a hemoglobin of 10.5 Gm. per cent. A change in the C band was demonstrated when the patient's blood was resampled after a period of two months during which time her reticulocyte count did not change (See Fig. 2; G, G2).

Figure 2 summarizes the relative intensity of the C band for normal membrane of the various blood types tested and for membrane from the various
Fig. 2.—Relative C band intensity among the membrane preparations tested. Letters represent blood type of normal membrane tested and the initials of specific patients with hereditary spherocytosis. Each bar shown represents the mean intensity of eight repetitive determinations. The mean “C” band intensity of normal membrane has been assigned a value of 100 per cent. All bars had an insignificant variation about the mean when measured by scanning densitometry.

patients with H.S. The intensity of the C band in normal membrane was assigned a value of 100 per cent and the intensity of the bands in H.S. were estimated while viewing them together. Figure 2 demonstrates the consistency with which the C band was present in normal membrane as well as the variation encountered among H.S. patients.

DISCUSSION

The purpose of this investigation was to develop and simplify methods of membrane preparation and separation of membrane components, and to apply these methods to the study of membrane associated disease. For these purposes a clinically stable patient population is necessary. Therefore, all but one of the patients with H.S. who were chosen for study had been splenectomized and were clinically stable with normal hemoglobins and reticulocyte counts.

The method of membrane preparation presented here consists of exhaustive washing with aqueous solvents. Since membrane protein per se is not water soluble, it is membrane associated aqueous soluble proteins which are lost in the purification. Phospholipid apparently was a tightly bound membrane constituent, and this is reflected in the data as a small increase in the relative percentage of phospholipid.

The methods for solubilization and electrophoresis were adapted from methods originally designed for use with the membranes of intracellular organelles, e.g., mitochondria,6,7 and synaptic vesicles.5 The PUA solvent gives solubilization of the red cell membrane without irreversible binding as occurs with detergents.8 The band patterns obtained with membrane solubilized in
PUA were reproducible. PUA solubilized membrane gives best results when used within one week of the date of solubilization. PUA did not give differential solubilization of membrane components. The electrophoresis method is reliable and reproducible if care is taken when layering the gels with 10 per cent acetic acid prior to polymerization. If the gels are not soaked in the PUA solvent for the correct length of time, resolution will be poor. Two to five days of soaking is desirable. In addition to the above precautions, attention must be paid to the concentration of acrylamide employed. It was found that the optimum concentration for best resolution was 6.6 per cent. A variation of plus or minus one per cent would either prevent entry of the protein into the gels at seven per cent, or produce only diffuse bands at five per cent.

The multiplicity of bands obtained in the work described herein would support the theory that all membranes including the red cell membrane are composed of a number of protein components. Other workers using a variety of technics have also contributed support to this theory. Red cell membrane from patients with H.S. has been compared to normal red cell membrane by a variety of investigators using different technics. The glycolytic pathway associated with the inner surface of the membrane has been shown to be intact in hereditary spherocytic red cells. Zail and Joubert have shown gross protein content to be equivalent to that found in normal red cells. These authors subjected solubilized red cell membrane to starch gel electrophoresis and could demonstrate no difference between H.S. membrane and normal membrane. Vulpis obtained anti-S protein antibody by immunizing rabbits with S protein from normal red cell membrane as well as from membrane from patients with H.S. He used Ouchterlony agar diffusion to demonstrate serologic identity between the S proteins. It is known that spherocytes in H.S. show both a greater pump activity as well as a greater inward leak of sodium than do normal cells. Wiley has measured the sodium pump activity (efflux) as well as sodium leak (influx) in both normal red cells and red cells from patients with H.S. Using ouabain incubation to inhibit the leak, he has shown that the increased sodium pump activity in H.S. was due to an increase in the number of pump sites in the membrane rather than an increase in the Na⁺ turnover rate of each site. A greater sodium leak (influx) is present to maintain a normal intracellular sodium concentration. Wiley stressed the fact that pump and leak activity varied in magnitude among those H.S. patients tested. In addition, some patients had normal pump activity and increased leak while in others the reverse was true.

Our results cannot be ascribed to variation in the reticulocyte count, the severity of the anemia, the blood type, the anticoagulant used, or the length of storage. The Blood Bank blood used as normal control samples came from patients who had not been splenectomized. All but one of the H.S. patients had no spleen. However, one of these splenectomized H.S. patients consistently had a normal electrophoretic pattern. If the presence or absence of the spleen were important in determining the band pattern, one would expect this patient’s band pattern to be altered. It seems unlikely that the presence or absence of a spleen influences the band pattern on electrophoresis. The patient who had not been splenectomized showed a change in her “C”
band when resampled two months after her initial examination. She had not changed clinically in this time nor had her hemoglobin or reticulocyte count varied. The reason for this change is unknown.

Membrane lipids must also be considered as an etiology for the observed results. The variations in reported lipid composition in hereditary spherocytosis may be a reflection of the same factors which produced variation in the band patterns among our patients with H.S. Although membrane lipid is known to be in equilibrium with serum lipid, the lipid in association with the membrane may be influenced by changes in membrane protein in a fashion analogous to the enzyme–protein interactions described by Woodward and Munkres.

The removal of aqueous soluble proteins during the preparation raises the question of the differential removal of component protein among the H.S. membrane samples. It is possible that the decrease in the “C” band in some of the H.S. patients could be due to loss of the “C” band in the membrane preparation. No such variation or differential loss was observed among the normal samples. Thus, the differential loss of membrane component protein would represent a valid but at present unexplainable difference between H.S. and normal membrane. Alternatively, the decrease in the “C” band could have been present in vivo.

Our findings would indicate that there is an abnormality in a specific membrane component of H.S. membrane and that this abnormality is present to a variable degree among H.S. patients as reflected by gel electrophoresis patterns.

REFERENCES


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