THE PROTEINS OF THE ERYTHROCYTE MEMBRANE OBTAINED BY SOLUBILIZATION WITH AQUEOUS PYRIDINE SOLUTION+

Olga O. Blumenfeld*

Department of Biochemistry and Unit for Research in Aging
Albert Einstein College of Medicine, Yeshiva University
New York, N. Y. 10461

Received January 3, 1968

The nature of the proteins of the erythrocyte membrane and other membranes is under active investigation in several laboratories. Various procedures have been used for the isolation of the proteins; these include extraction of membranes with butanol in the presence or absence of solubilizing agents such as urea or sodium dodecyl sulfate (SDS) (Maddy, 1966; Eddy and Johns, 1964; Blumenfeld et al., 1967), fractional precipitation with ammonium sulfate of membranes in solution with deoxycholate or cholate, (Criddle et al., 1962; Richardson et al., 1963), and extraction of membranes with media of low ionic strength containing ATP (Marchesi and Palade, 1967). In most cases the protein obtained has relatively large molecular weight, and consists of aggregates which in some instances can be dissociated into smaller units by treatment with SDS and adjustment of the preparation to alkaline pH values.

In this communication we describe a new, mild method for the isolation of the membrane proteins of the human erythrocyte, and present evidence that at least two classes of proteins are present; one of the proteins contains all of the sialic acid of the membrane and the other has strong affinity for the lipid of the membrane. Some properties of the solubilized sialoprotein are described.

+ This work was supported by USPHS Grants #HD-00674 and #AM-05821 from the National Institutes of Health.

* National Institutes of Health Development Award #GM-5998.

Erythrocyte membranes were prepared from freshly drawn human blood, essentially by the method of Dodge et al.(1963), using 0.02M sodium phosphate buffer of pH 7.6 for lysis. After being washed six times with this buffer, the membranes contained less than 0.1% of hemoglobin and were intact in shape when viewed under the phase microscope. The membranes were dialyzed against several changes of cold distilled water to remove inorganic phosphate. This treatment did not affect their shape. The results of analysis of the chemical composition of the membrane are included in Table I. Their amino acid composition is included in Table II. These results agree well with those found by others (Maddy, 1966; Bakerman, 1967).

To one volume of an aqueous suspension of membranes (at 4°C) was added one-half volume of pyridine (at 4°C). The mixture was shaken gently to dissolve completely the membranes, and immediately the solution was dialyzed against cold distilled water. Dialysis was continued for about 18 hours with frequent changes of water to insure a thorough removal of pyridine. The new turbid solution was centrifuged in a Spinco Model L centrifuge at 30,000 RMP for 30 minutes. The clear supernatant solution was withdrawn and the pellet washed three times with cold distilled water and centrifuged as above.

Analysis of the supernatant revealed that it contained about 35-40% of the total protein of the membrane, all of the sialic acid, but none of the cholesterol and only a small amount of the organic phosphate. Analysis of the pellet showed it to contain most of the remaining protein, 83% of the total cholesterol of the membrane, but none of the sialic acid. These data are included in Table I. It should be noted that hexoses and N-acetyl hexosamines were present in both the soluble sialoprotein and the pellet. As seen in Table II, amino acid analysis of the sialoprotein and the pellet protein showed differences, the most pronounced of which were in the contents of aspartic acid, glutamic acid, and phenylalanine. The content of the acidic amino acids was higher in the sialoprotein and that of phenylalanine greater in the pellet protein.

TABLE I

Composition of erythrocyte membranes and distribution of various components of the membrane between the soluble sialoprotein and the insoluble pellet protein.

	Intact Membranes % dry wt.	% of total found in membranes: Supernatant (sialoprotein)	Pellet[1] (insoluble protein)	% Total Recovery
Total nitrogen (by Kjeldahl and amino acid analysis)	7.7	35-40	32	~ 70
Protein (assuming 16% N)	45	35-40	32	~ 70
Cholesterol	8.9	0.3	83	83
Sialic acid (thiobarbituric[2] assay method of Warren, 1959)	1.2	82	none	82
Phosphate (Organic)	1.1	5 or less	30	35[3]
Hexoses (anthrone method; glucose equivalents)	1.8	25	52	77
N-acetyl hexosamines (Elson-Morgan and amino acid analysis)	2.6	42	32	74

1. Because a suspension of the pellet in water presented a sampling problem, all analyses, except the sialic acid, were determined on the pellet redissolved in 33% pyridine. Total amino acid nitrogen was determined from an amino acid analysis; the sialic acid assay was performed on an aliquot of a water suspension of the pellet, since pyridine interfered with the assay. Since some losses of the pellet occured during the isolation and washing procedures, the values obtained are minimal.
2. Calculated from absorption at 549 mμ after a thorough extraction with isoamyl alcohol of the 532 mμ absorbing chromophore. The compound which gave rise to this chromophore was present in the intact membranes and the pellet, but was absent in the sialoprotein.
3. Low recovery most likely due to loss by dialysis of phospholipids soluble in aqueous pyridine.

The pellet could be redissolved in 33% pyridine and, when the solution was subjected to dialysis and centrifugation as outlined above for the intact membranes, the proteins again separated and none was found in the supernatant. This indicates that the original solubilization of the membranes caused the total extraction of the sialoprotein.

TABLE II

Amino acid composition of intact membranes, sialoprotein and the pellet protein

Residues per 1000 Residues

	Membranes (av.10 preparations)	Sialoprotein (av.4 preparations)	Pellet Protein (av.2 preparations)
Lysine	49	54	42
Histidine	23	23	17
Arginine	45	52	54
Aspartic Acid	94	103	76
Threonine	57	60	52
Serine	83	73	87
Glutamic Acid	129	161	119
Proline	58	50	49
Glycine	71	65	74
Alanine	73	98	82
Half Cystine	9	-	-
Valine	58	70	58
Methionine	11	-	-
Isoleucine	44	45	48
Leucine	125	113	129
Tyrosine	18	17	17
Phenylalanine	36	28	50
N-acetyl glucosamine	25	9	14
N-acetyl galactosamine	16	11	6

The solution of sialoprotein as isolated from the membranes contained about 1 mg of protein per ml. and had a yellow coloration. Upon concentration of this solution by pressure dialysis in the cold, a gel formed at the bottom of the dialysis tube. The protein, sialic acid and amino acid contents of the gel were identical to those of the solution from which it separated, suggesting that the sialoprotein is either a homogeneous protein or consists of very closely related proteins that have a strong tendency to aggregate. This is also suggested by the fact that the sialoprotein was completely retained on columns of DEAE cellulose; no protein peaks were eluted with buffers of ionic strength as high as 0.6 at pH 5.3.

The sialoprotein, upon ultracentrifugation in water or in 0.1M phosphate buffer pH 7.1 (1M phosphate buffer added to a solution of protein in water to yield 0.1M) showed two peaks with $S_{20,w}$ values of about 6 and 14S at protein

concentrations from 0.3-0.5%. However, the relative areas of the two peaks and the presence of the second peak were found to be dependent on the conditions and the media used in the ultracentrifugation. At higher protein concentration, in 0.1NKCl, or after dialysis against 0.1M phosphate buffer pH 7.1 gel formation became apparent during ultracentrifugation. By sedimentation equilibrium ultracentrifugation using the method of Yphantis (1964), the molecular weight of the smaller component present in a 0.03% solution in 0.1M phosphate buffer at pH 7.1 (after dialysis) was estimated to be about 500,000; the larger molecular weight components were present at the bottom of the cell. It is premature to conclude, however, that the 500,000 molecular weight components represent the smallest molecular weight species of the protein. Electrophoresis of the sialoprotein on 5% polyacrylamide gels at pH 8.5 with or without urea showed a single band near the origin with the bulk of the protein not able to penetrate the gel.

In this communication we have shown that two types of proteins can be prepared that account for the major protein content of the erythrocyte membrane. One protein is water soluble, contains nearly all of the sialic acid of the membrane and is virtually free of lipid; the other protein is insoluble in water, free of sialic acid and remains associated with the lipid of the membrane. Even though their amino acid contents are not extensively dissimilar, pronounced differences in their properties such as in solubility and association with lipid are remarkable. Such differences in behavior might in part be related to the high content of sialic acid in the sialoprotein and its absence from the lipoprotein (pellet).

The removal of the aqueous pyridine from the solution of lipoprotein (pellet) results in its reconstitution to an apparently structured insoluble aggregate whose properties and organization are worthy of further investigation.

The sialoprotein readily forms aggregates and gels. It also has an interesting yellow coloration. Marchesi (1967) has recently isolated a solu-

ble protein from the erythrocyte membrane which shows some properties of a fibrous protein; whether this protein is related to either the sialoprotein or lipoprotein described in the present report is yet to be determined.

Maddy (1966), using cold aqueous butanol isolated the erythrocyte membrane protein essentially free of lipid but which contained all the sialic acid of the membrane. The differences between this protein aggregate and the proteins isolated in this communication apparently lie in the different methods of isolation used.

The preparative methods used here, including the application of cold aqueous pyridine for solubilization of the membrane, are mild. In future investigations they promise to be of much utility because the component proteins are seemingly unaffected by the procedures of isolation.

REFERENCES

1. Bakerman, S. and Wasemiller, G. Biochemistry, 6, 1100, 1967.

2. Blumenfeld, O.O., Goldstein, M. and Seifter, S. Fed. Proc., 26, 831, 1967.

3. Criddle, R.S., Bock, R.M., Green, D.E. and Tisdale, H., Biochemistry, 1, 827, 1962.

4. Dodge, J.T., Mitchell, C. and Hanahan, D.J. Arch. Biochem. & Biophys., 100, 119, 1963.

5. Eddy, A.A. and Johns, P. (1964) In "Surface Activity and the Microbial Cell" Symposium, p. 24. Soc. Chem. Ind., London.

6. Maddy, A.H., Biochim. Biophys. Acta., 117, 193, 1966.

7. Marchesi, V.T. and Palade, G.E., Proc. Nat'l. Acad. Sci., 58, 991, 1967.

8. Richardson, S.H., Hulton, H.O. and Green, D.E. Proc. Nat'l. Acad. Sci., 50, 821, 1963.

9. Warren, L., J. Biol. Chem., 233, 1971, 1959.

10. Yphantis, D.A., Biochemistry, 3, 297, 1964.

Acknowledgment: The author wishes to thank Professor Sam Seifter and Paul M. Gallop for their invaluable advice and encouragement.

The technical assistance of Miss Stella Deloime and Mr. Norman Kinard is deeply appreciated.