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PROPERTIES OF AN ERYTHROCYTE MEMBRANE LIPOPROTEIN FRACTION

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SUMMARY

Membrane vesicles and fragments were prepared from ghosts of red blood cells by extracting the water soluble proteins (50% of the total). On incubation in 8 M urea, about half of the remaining protein and of the lipid could be extracted. The remaining lipid (50% of the total in the ghost) and protein (25% of the total in the ghost) were present in small membrane fragments that could be sedimented by centrifugation at high speed. The protein of the pellet was solubilized by use of detergents. The major component (over 80% of the total) has a molecular weight of 95000 determined by gel electrophoresis. It contains most of the protein bound hexose of the ghost and lesser amounts of other sugars. It is intensively labelled by application of non-penetrating protein reagents to the intact cell, and is presumably located, at least in part, on the outer face of the membrane. The lipids associated with the urea pellet were not essentially different from those of the original ghost. It is suggested that a small fraction of the lipid is hydrophobically bonded to the pellet protein and that the remainder is held by lipid–lipid interactions.

INTRODUCTION

A large fraction, up to 50%, of the protein of the human erythrocyte membrane is bound by ionic forces and can, therefore, be solubilized by manipulations of pH, ionic strength, and chelating agents[1–4]. The remainder of the protein, associated with lipids in the form of vesicles and membrane fragments, is presumably bound by non-ionic forces. In the present study, the non-ionically bound protein has been fractionated by the use of urea. A number of proteins can be dissolved, but a vesicular fraction containing lipid and predominantly a single protein species is insoluble in urea. Several physical and chemical parameters of this protein and its associated lipid have been determined. In addition, the location of the protein relative to the permeability barrier has been investigated through the use of a non-penetrating reagent.

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MATERIALS AND METHODS

Chemical assays

Protein was determined by the method of LOWRY, *et al.*[5] using bovine albumin (Pentex) as a standard. Hexose and hexosamine were measured by the method of WINZLER[6]. Sialic acid was measured by the first method of AMINOFF[7]. Cholesterol was determined by a modification of the Liebermann–Burchard reaction[8].

Total phospholipid was determined as follows. Samples were ashed using the method of CHEN *et al.*[9] and the inorganic phosphate content determined by their modification of the Fiske–SubbaRow method. The number of mg of phospholipid in each sample was estimated by multiplying the mg of phosphorus by 25, the average ratio of phosphorus to phospholipid.

Lipid analyses

Lipid extractions were accomplished by a modification of the method of ROSENBERG AND GUIDOTTI[10] but the Soxhlet extraction used by these authors was replaced by an overnight incubation in ethyl ether at room temperature. Paper chromatography of phospholipids was carried out by the method of REED *et al.*[11] and the chromatograms were stained, using the tricomplex staining procedure[12].

Ultracentrifugation

Ultracentrifugation was performed in a Spinco Model E machine using an AN-D rotor and a photoelectric scanning system. The procedures used were those described in the Spinco manual[13].

Gel electrophoresis

Membrane protein fractions were characterized in an sodium lauryl sulfate acrylamide gel electrophoresis system. The procedure utilized was a modification of that of DUNKER AND RUECKERT[14]. The proteins or membrane fractions were dissolved in a solution containing 3 % sodium lauryl sulfate, 0.1 % mercaptoethanol, 5 M urea, 1 mM EDTA, and 0.1 M Tris–HCl at pH 7.6. The solution used for electrophoresis was similar except that the urea was omitted and the sodium lauryl sulfate concentration was 0.1 %. Staining was accomplished by the method of BERG[15] and the gels were scanned with a Gilford gel densitometer.

In sodium lauryl sulfate, the lipids and proteins are dissociated, the latter presumably into polypeptide monomers[15,16]. The molecular weights of the disaggregated membrane proteins can be estimated by calibrating the electrophoretic system with proteins of known molecular weight. Estimates made by this method are quite reproducible and agree with those derived from techniques such as equilibrium centrifugation. Few well characterized polypeptides of molecular weight greater than 100000 daltons are available for estimating molecular weights of larger membrane polypeptides. For this purpose bovine thyroglobulin (165000 daltons) and rabbit muscle phosphorylase A (95000 daltons) purchased from Sigma[17] were chosen. Other protein standards were also purchased commercially.

Labelling of outer surface proteins

The outer surfaces of red cells were labelled with diazo [^{35}S]sulfanilic acid using a technique developed by BERG[15]. This reagent does not penetrate the cell and thus

only groups (amino and sulfhydryl) on the external aspect of the membrane are labelled[15]. A 50% red cell suspension was reacted with 1 mM diazo[^{35}S]sulfanilic acid in isotonic phosphate-buffered saline at pH 7.2 for 10 min at 20°. Excess reagent was washed away and ghosts prepared from the labelled cells. The ghosts were dissolved in a sodium lauryl sulfate-containing solvent. After electrophoresis on 6% acrylamide gels containing sodium lauryl sulfate, the gels were sectioned into uniform 2-mm slices and each was dissolved in 30% H_2O_2 and counted in a liquid scintillation counter.

Preparation of the lipoprotein fraction (PIV)

Ghosts (red cell membranes) were prepared as previously described[1]. The water-soluble membrane proteins designated PI and PII[1] were extracted from the ghosts by exposure to 1 mM EDTA at pH 9.5 for 1 h and to water at pH 11 for 15 min. A suspension (3–5 mg protein/ml) of the resulting membrane vesicles was mixed with 10 vol. of 8 M urea containing 1% mercaptoethanol and 1 mM EDTA and incubated at room temperature for 12 h. The mixture was then centrifuged at 150000 × *g* for 3 h and the pellet (designated PIV) recovered. The urea-soluble proteins are designated PIII.

RESULTS

Membrane vesicles from which water-soluble proteins have been extracted contain all of the membrane lipids and about 50% of the protein, distributed in several molecular weight species as determined by gel electrophoresis in the presence of sodium lauryl sulfate[1]. After the membrane vesicles are incubated in a solution containing 8 M urea and mercaptoethanol, and centrifuged as described in MATERIALS AND METHODS, about half of the protein and lipid remain in the supernatant, and the rest can be collected in a gelatinous pellet which contains about 25% of original ghost protein and 50% of total lipid. The pellet is composed of small (0.1–0.2 μm) vesicles and/or membrane fragments as seen by negative staining with uranyl in the electron microscope.

The distribution of proteins in the urea pellet can be compared with the distributions at various stages of fractionation in Fig. 1, using gel electrophoresis in sodium lauryl sulfate as described in MATERIALS AND METHODS. Treatments I, II, and III remove water-soluble membrane proteins (PI and PII), as described in a previous paper[1]. After treatment IV (urea), additional proteins (PIII) have been removed. One major component comprising 80% of the total, and one minor component remain in the urea pellet (PIV). The major peak corresponds with one member of the dense doublet (C and D of Fig. 1) seen in gels from intact ghosts. After urea treatment a small amount of protein fails to migrate and remains at the origin.

The components that are water-soluble include A and B (proteins II in ref. 1) and most of D, E, H, I, K and L which is residual hemoglobin (proteins I of ref. 1). Those that are primarily extracted by urea include G, J, and the remainder of D. The only two distinguishable peaks left in the urea pellet are the major component C, and the minor component F.

Component C behaves as a single peak under a variety of conditions. Electrophoresis was carried out at pH 11, in the presence of 8 M urea, at gel concentrations of

5, 6, and 7 %, and at sodium lauryl sulfate concentrations ranging from 0.1 to 1 %. These treatments and also heating at 65° had no effect on the homogeneity of component C.

The molecular weight of the major peak of the PIV fraction (C) is 95000

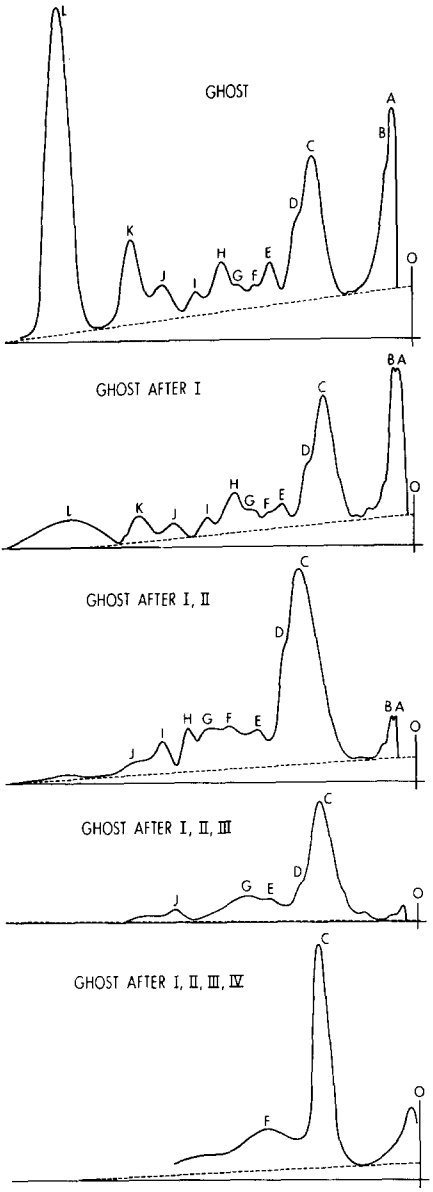

Fig. 1. Patterns of membrane proteins on 6% acrylamide gels containing sodium lauryl sulfate after various fractionations. Treatments were as follows: 1. Incubation with 150 mM KCl for 12 h. 2. Incubation at pH 9 in 1 mM EDTA for 1 h. 3. Incubation in double distilled water *plus* NaOH at pH 11 for 15 min. 4. Incubation in 8 M urea *plus* 1 % mercaptoethanol for 12 h. Gels were run at 8 mA per tube for 1 h.

daltons, while the minor peak (F) is 65000 daltons, as determined by sodium lauryl sulfate-gel electrophoresis. When PIV is freed of lipid, dissolved in 1–5% sodium lauryl sulfate and run in the ultracentrifuge, it sediments as a single component even though gel electrophoresis reveals at least two components (Fig. 1). This is undoubtedly due to the greater resolving power of gel electrophoresis as compared to ultracentifugation. With the latter technique, the minor component, F, may not be visible.

The lipid-free residue prepared from PIV contains 9% carbohydrate by weight as compared to 6% in the lipid-free ghost. The proportions of carbohydrates (hexosamine, hexose, and sialic acid) are also different in the urea pellet and ghost (Table I). The urea pellet is substantially richer in hexose than is the ghost, containing about 86% of the total[18].

TABLE I

LIPID AND CARBOHYDRATE COMPOSITION OF GHOSTS AND OF THE UREA PELLET

Units are μg of lipid or carbohydrate per mg of protein. The number of experiments are given in parentheses. The carbohydrate measurements were made on delipidated protein residue.

	Ghost	*Urea pellet*	*% of ghost carbohydrate in urea pellet*
Hexose	11.2 (4)	38.8 (4)	87
Hexosamine	20.0 (2)	28.5 (2)	36
Sialic acid	30.0 (2)	30.0 (2)	25
Cholesterol	150 (2)	250 (2)	
Phospholipid	390 (2)	750 (2)	

The urea pellet has a higher lipid-to-protein ratio than the intact ghost (Table I). The cholesterol-to-phospholipid ratio is 0:33 in the urea pellet and 0:38 in the ghost on a weight basis. When equal amounts of ghost and pellet phospholipid are applied to silica paper chromatograms, and the chromatograms developed, similar lipid spots appear in both cases. Thus the phospholipids of the urea pellet and of ghost are of the same types and are present in roughly the same proportions. Preliminary studies by gas chromatography indicate that there are no gross differences in the fatty acid composition of ghost lipids and urea pellet lipids.

The non-penetrating reagent, diazo [^{35}S] sulfanilic acid can be used to distinguish those proteins exposed to the outside medium from those that are not[15]. After exposure of the intact cells to the reagent, followed by preparation of membranes and fractionation of the proteins, virtually none of the diazo sulfanilic acid bound to ghosts appears in the water soluble components, about 50% is solubilized by urea, and the remainder is found in the urea pellet (PIV).

A more detailed analysis of the distribution of diazo sulfanilic acid by gel electrophoresis indicates a rather specific localization. Three peaks of labelling are found (Fig. 2), corresponding to three of the protein peaks seen in stained gels. The major peak has the same mobility as (C) the major component of PIV (urea insoluble protein), corresponding to a polypeptide molecular weight of 95000. The other two peaks correspond to two smaller peaks (F and H), the former partially, and the latter largely extractable by urea (compare Figs. 1 and 2). Estimates of peak areas suggest that about 30% of the diazo sulfanilic acid in the ghost is in Peak C with the remainder

predominantly in Peaks F and H. In the urea pellet the proportion of label in Peak C is enriched to about 60 %. This result indicates that the PIV fraction is heterogeneous, and that Component C has not been fully separated from Components F and H. Minor components, not fully resolved by this procedure may also contribute to the distribution of diazo-sulfanilic acid.

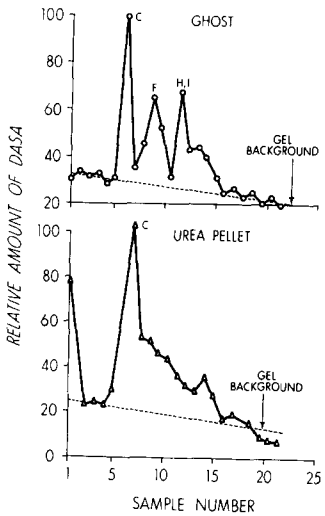

Fig. 2. Distribution of diazo[^{35}S]sulfanilic acid (DASA) labelled protein fractions in 6 % acrylamide gels containing sodium lauryl sulfate. Gels were run at 8 mA per tube for 1 h. The counts are normalized to give equal peak heights for Component C.

DISCUSSION

Several investigators[19,20,21], and notably LAUF AND POULIK[22] have used urea to fractionate erythrocyte membranes, finding that 8 M urea solubilizes 80 % of membrane protein and some membrane lipid, resulting in a residue with a higher lipid-to-protein ratio than the intact ghost. The present findings confirm the above results, and in addition, provide information concerning urea stability of specific membrane proteins, their location in the membrane, and their association with membrane lipids.

When the water soluble proteins are extracted from red cell ghosts, the residue containing all of the lipid and about half of the protein is in the form of vesicles of heterogeneous size[1]. Extraction of the vesicles with 8 M urea results in solubilization of half of their protein and lipid, leaving an insoluble residue of membrane fragments or vesicles. The extraction is relatively non-specific in terms of lipids but is highly specific for carbohydrate and protein. Hexose is enriched fourfold in the urea pellet (PIV) but it is not yet clear if the carbohydrate is an intrinsic part of a protein or if it represents tightly bound glycolipids[18] not removed by the procedure used for lipid extraction. In the case of protein, only two distinguishable components are retained, the major one of molecular weight 95000 designated as band C (Fig. 1), and a large portion of the minor one of molecular weight 65000 designated as band F.

Both protein components of PIV are labelled in the intact cell by the non-penetrating reagent, diazo [^{35}S]sulfanilic acid[15], indicating that they are at least partially exposed at the outer surface of the membrane. This result does not, however, rule out the possibility of a large portion of PIV being embedded in the interior of the membrane matrix.

PHILIPS AND MORRISON[23], using quite different techniques, have also reported the existence of a high molecular weight protein at the outer surface of the red cell membrane. These authors have used an enzyme (lactoperoxidase) to iodinate the exterior surface of red cells. They find only a single component of molecular weight 90000 to be labelled and conclude that only this component is on the exterior of the cell. In the present case at least three proteins are labelled by diazo [^{35}S]sulfanilic acid (peaks C, F, H.) These findings are not necessarily contradictory; there is little reason to believe that lactoperoxidase, a large enzyme, will have access to exactly the same set of surface sites as diazosulfanilic acid, a much smaller molecule. The labelling pattern seen in the present work qualitatively resembles that seen by BERG[15], in that one major and several minor peaks are seen. However, the molecular weight estimates of the major protein peaks of the ghost given by BERG are consistently higher than the present estimates and also those given by PHILLIPS AND MORRISON[23]. No explanation of this discrepancy can be offered at present.

The PIV fraction contains about 25% of membrane sialic acid. Since little sialic acid is recovered in the water soluble proteins[1], this result indicates that the bulk (75%) of ghost sialoprotein is extracted by urea treatment. A similar result was described by LAUF AND POULIK[22]. It is not yet known if the urea soluble polypeptides which are labelled by diazo-sulfanilic acid (peaks F and H) are also sialoproteins. It seems reasonable that the membrane sialoproteins should be labelled by diazo-sulfanilic acid, since $>$ 90% of red cell sialic acid is on the outer surface[24].

The association between PIV and membrane lipid is an intimate one. Extremes of pH (2–11), ionic strength (1 M salts), and strong denaturants (8 M urea) fail to separate PIV from membrane lipids. Presumably the association of PIV with lipid involves strong dispersion and "hydrophobic" forces. It is unlikely for several reasons that PIV is directly bound to all of the 50% of total membrane lipid with which it segregates. Firstly, COLEMAN *et al.*[25] have shown that phospholipase C treatment of erythrocyte ghosts results in the cleavage of 70% of membrane phosphate and that the hydrolysis products accumulate as lipid droplets which remain associated with the membrane. Thus a substantial portion of membrane lipid is accessible to the enzyme and is susceptible to rearrangement to form droplets; it is doubtful if such a process could occur if the lipid side chains were bound to membrane protein. Secondly, GLASER *et al.*[26] have coupled phospholipase C treatment with circular dichroism and nuclear magnetic resonance measurements. These authors show that enzyme treatment results in a large change in the physical state of 75% of the fatty acid groups of the membrane (they become more mobile), without appreciable change in the conformation of membrane protein. Thus most membrane lipid and membrane protein can change structure independently of each other. Thirdly, after removal of 50% of the membrane lipids by urea extraction, the remainder, associated with the PIV protein is not appreciably different in composition from the lipids of the intact membrane. If all of the lipids were bound directly to the protein, some degree of specific association might be expected.

From the above considerations it is suggested that only a fraction of the lipid is directly bound to PIV proteins and that the remainder is secondarily associated with the bound fraction. Thus most of the lipid may not be directly bonded to protein and yet may be ordered or patterned by a secondary interaction with PIV.

There exists an interesting asymmetry in the distribution of membrane proteins. PIV, a major fraction which is bonded by non-ionic forces, is at the outer surface of the cell. The water soluble membrane proteins, bonded by ionic forces, are located on the other hand, on the inner aspect of the membrane[1]. Thus the outer membrane proteins are tenaciously attached to lipid and seem ideally suited as a sort of armour plate for the cell. The inner proteins are ionically bonded and may respond to subtle changes in cellular milieu brought about by metabolic variation.

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REFERENCES

1 J. TH. HOOGEVEEN, R. JULIANO, J. COLEMAN AND A. ROTHSTEIN, *J. Membrane Biol.*, 3 (1970) 156.
2 R. JULIANO, *The Nature of the Association of a High Molecular Weight Water-soluble Protein with the Erythrocyte Membrane*, in preparation.
3 J. R. HARRIS, *J. Mol. Biol.*, 46 (1969) 329.
4 S. L. MARCHESI, E. STEERS, V. T. MARCHESI AND T. W. TILLACK, *Biochemistry*, 9 (1970) 50.
5 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
6 R. WINZLER, in D. GLICK, *Methods in Biochemical Analysis*, Vol. 2, Interscience, New York, 1955, pp. 279–311.
7 D. AMINOFF, *Biochem. J.*, 81 (1961) 384.
8 T. C. STEDTMAN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. III, Academic Press, New York, 1957, pp. 392–394.
9 P. S. CHEN, T. Y. TORIBARA AND H. WARNER, *Anal. Chem.*, 28 (1956) 1756.
10 S. A. ROSENBERG AND G. GUIDOTTI, *J. Biol. Chem.*, 243 (1962) 1985.
11 C. F. REED, S. N. SWISHER, G. V. MARINETTI AND E. G. EDEN, *J. Lab. Clin. Med.*, 56 (1960) 281.
12 G. J. M. HOOGEWINKEL, J. TH. HOOGEVEEN, M. J. LEXMOND AND H. G. BUNGENBERG DE JONG, *R. Acad. Sci. Amsterdam*, B 62 (1959) 222.
13 C. H. CHERVENKA, *Manufacturer's Manual*, Spinco Div., Beckman Instruments, Palo Alto, Calif., 1969.
14 A. K. DUNKER AND R. R. RUECKERT, *J. Biol. Chem.*, 244 (1969) 5074.
15 H. C. BERG, *Biochim. Biophys. Acta*, 183 (1969) 65.
16 K. WEBER AND M. OSBORN, *J. Biol. Chem.*, 244 (1969) 4406.
17 I. M. KLOTZ AND D. W. DARNALL, *Science*, 166 (1969) 127.
18 J. LEONARD, *Biochemistry*, 9 (1970) 1129.
19 E. A. AZEN, S. ORR AND D. SMITHIES, *J. Lab. Clin. Med.*, 65 (1965) 440.
20 S. BAKERMAN AND G. WASEMILLER, *Biochemistry*, 6 (1967) 1100.
21 J. R. MCINTOSH, *Biochim. Biophys. Acta*, 103 (1968) 285.

22 P. K. LAUF AND M. D. POULIK, *Brit. J. Haematol.*, 15 (1968) 191.
23 D. R. PHILLIPS AND M. MORRISON, *Biochem. Biophys. Res. Commun.*, 40 (1970) 284.
24 G. V. F. SEAMAN AND G. UHLENBECK, *Arch. Biochem. Biophys.*, 100 (1963) 493.
25 R. COLEMAN, J. B. FINEAN, S. KNITTON AND A. R. LIMBRICK, *Biochim. Biophys. Acta*, 219 (1970) 81.
26 M. GLASER, H. SIMPKINS, S. J. SINGER, M. SHEETZ AND S. I. CHAN, *Proc. Natl. Acad. Sci. U.S.*, 65 (1970) 721.