A MILD PROCEDURE FOR THE FRACTIONATION OF OX ERYTHROCYTE MEMBRANE PROTEINS

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1. Introduction

One of the major problems in assessing the role of proteins in membrane structure and function is that of preparing soluble, biologically active proteins from the insoluble membrane complex. A second difficulty is to relate the isolated protein to its former location in the membrane. In an attempt to overcome the first difficulty we solubilised the protein of ox erythrocyte ghosts by a butanol procedure [1] but until recently the resulting protein solution has been difficult to fractionate. We are now able to fractionate the mixture and begin to understand the aggregating interactions. To meet the second difficulty we have pursued our fractions, bearing in mind features that might enable us to relate the fractions to their original positions in the membrane. For this purpose we have used a fluorescent label, 4-acetamido, 4^1-isothiocyano stilbene-2, 2^1-disulphonic acid (SITS), that will react only with proteins outside the permeability barrier of the cell [2], and we have followed what may be regarded as an intrinsic label of the membrane – sialic acid – which is known to be outside the permeability barrier [3]. We now report the separation of the protein of ox erythrocyte ghosts into three fractions, one soluble in dilute acetic acid and possibly originating from the inner surface of the membrane, another bearing the bulk of the sialic acid and consequently containing material from the outer surface, and a third remarkable for its tendency to aggregate and insoluble when purified.

2. Experimental

2.1. *The acetic acid fraction, (M_A)*

Extraction of the haemoglobin free ghosts [4] with 0.26 M (1.5%) acetic acid (pH 2.7) solubilises about 30% of the protein without liberating any significant amount of lipid. This amount of protein is obtained by extracting the ghost pellet obtained from a 30,000 *g*, 30 min centrifugation with 6 volumes of acid for 20 min at room temperature (20°), repeating the extraction twice, and pooling the supernatants. More prolonged extraction does not release further protein. A complex pattern is obtained from this solution on polyacrylamide gel electrophoresis – up to 28 bands can be detected (fig. 1a). The electrophoresis is carried out in 0.26 M acetic acid, with gels polymerised in this acid at 50° using ammonium persulphate and *N,N,N′,N′*-tetramethylethylenediamine as catalysts. No additions such as urea, sulphydryl reagents or detergents are required. The separation takes 15 min at 3.5 mA per tube at 40 V. cm^{-1}. In an M.S.E. analytical ultracentrifuge the protein solution contains 2 partially resolved components of 0.7 S and 1.7 S but on addition of 0.1 M potassium chloride they aggregate to 4 S and 13 S and dissociate on removal of salt. The greater heterogeneity revealed by acrylamide gel electrophoresis is indicated by the broadness of the Schlieren peaks (fig. 2). (All sedimentation coefficients are quoted for a protein concentration of 10 mg/ml.) The M_A proteins are not labelled by SITS in the intact cell but are readily labelled when fragmented ghosts are treated with SITS. The extract is lacking in sialic acid and hexosamine but contains a small anthrone

positive component (table 1). The non-reactivity to SITS and the absence of sialic acid suggest, although it is not fully conclusive, that the M_A fraction is not on the outer surface of the cell.

Fig. 1. (a) Separation of acetic acid soluble proteins (M_A) on 7% acrylamide gels (0.1% *bis* acrylamide) in 0.26 M acetic acid. Gels stained Coomasie Blue.

(b) Electrophoresis of crude M_R protein (conditions as 1a, but shorter time) showing contaminating M_A bands + slow moving material at origin.

2.2. *The sialoprotein (M_S)*

The residue after acetic acid extraction is freed of acid by repeated washing in water, dispersed in water by one or two 10-sec bursts of ultrasound in an M.S.E. sonicator at an amplitude of 60 μm and the pH raised to between 8 and 9 with sodium hydroxide. The resulting clear dispersion is amenable to butanol extraction as described previously for ghosts [1]. Protein of ghosts not extracted with acetic acid prepared by this method consists, in the analytical centrifuge, of 2 major components of 10 and 20 S (not as quoted in [1] 5 S and 10 S) and a minor 5 S component; in protein from acetic acid extracted ghosts the 10 S component is unchanged, but the heavier fraction is diminished and appears as a broad band around 40 S (fig. 3). If the proteins of this latter preparation are centrifuged in water rather than the usual buffer the sedimentation coefficients have changed to 7 S and over 50 S respectively, a difference sufficient to permit separation in a preparative centrifuge. The supernatant after 3 hr at 100,000 *g* in a preparative centrifuge shows one homogeneous peak in the analytical centrifuge at 10 S (M_S). This supernatant contains the bulk of the sialic acid and hexosamine of the membrane (table 1) and about 25% of the membrane protein; in the acetic acid acrylamide gels the solution shows two components that move slowly towards the anode. The method of isolation prevents full recovery and also contaminates the pellet with M_S protein.

2.3. *The residual protein (M_R)*

M_R – the pellet from the 3 hr centrifugation described above – is the most intractable of the fractions and also prevents the fractionation of a mixture of which it is a component. It will be contaminated with any pelleted M_S protein and possibly, as the result of incomplete extraction, with some acetic acid soluble proteins. The presence of traces of these M_A proteins can be demonstrated by dispersing the pellet in 8 M urea, acidifying to pH 2.7 with thioglycollic acid, dialysing into 0.26 M acetic acid and electrophoresing in 7% polyacrylamide gels in this solvent. The pellet is then seen to consist mostly of slow moving material together with small amounts of the bands of the M_A fraction (fig. 1b). The slow moving material (M_R) may be obtained free of the other bands by repelleting the crude mixture from

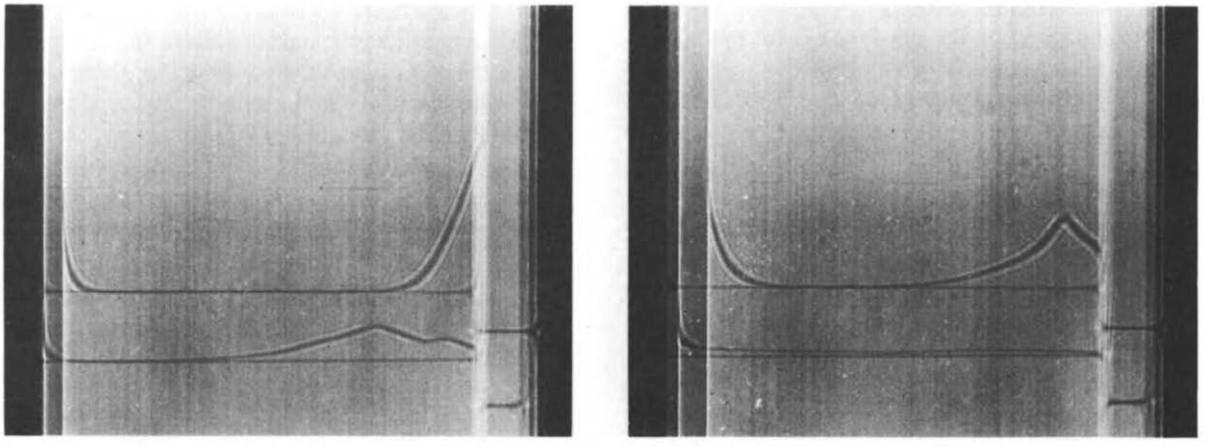


Fig. 2. Ultracentrifugation of acetic acid soluble proteins (M_A) in 0.26 M acetic acid – upper trace, and after the addition of 0.1 M potassium chloride – lower trace. a = 14 min, 54,750 rpm. b = 60 min, 54,750 rpm. M.S.E. Analytical centrifuge, double sector cells, bar angle 30°. Protein concentration = 10 mg/ml.

the acetic acid solution. There is a progressive increase in the size of the pelleted fraction as the M_A proteins are removed – in a solution of protein from ghosts untreated with acetic acid and with its full complement of M_A proteins there is the 20 S component – possibly an $M_A + M_R$ complex; in a solution of the acetic acid extracted residue this fraction is represented by a broad band of 30 S; and as the removal of traces of M_A (and M_S) proceeds, the residual protein increases in size until it is eventually insoluble. Although no 10 S component is detectable a significant amount of sialic acid remains with the M_R fraction.

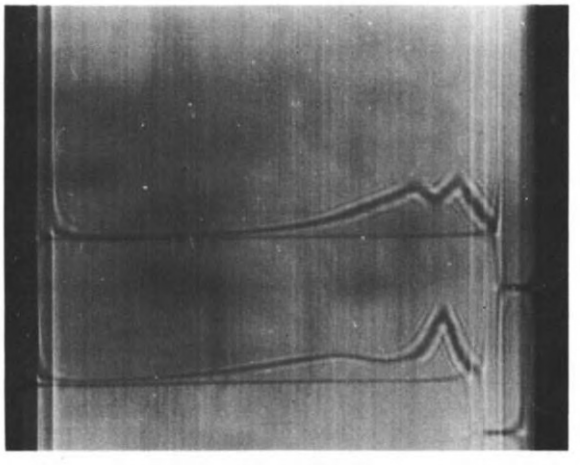


Fig. 3 Butanol solubilised protein of ghosts – upper trace, butanol solubilised protein of ghost residue after acetic acid extraction – lower trace. 8 min, 45,000 rpm, bar angle 25°. Protein concentration 10 mg/ml and 7.5 mg/ml respectively. Buffer–phosphate pH 7.5 ionic strength 0.02 + potassium chloride ionic strength 0.08.

Table 1
Carbohydrate content of protein fractions (expressed as *μmoles/mg protein) of individual* HZ127A. No fucose could be detected.

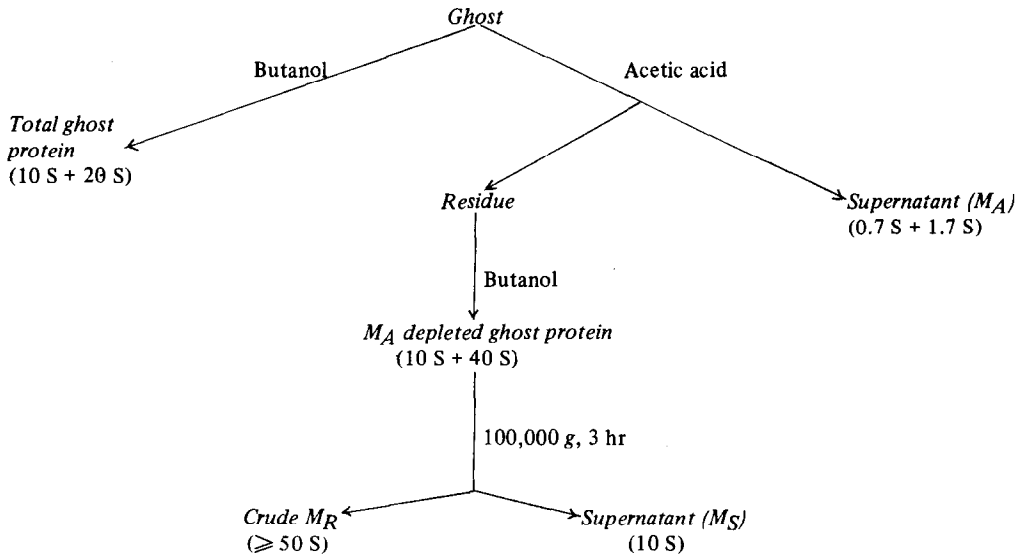
	Sialic acid [5]	Hexosamine [6]	Hexose [7]	% Carbohydrate by weight
Total ghost protein	0.07	0.12	0.10	6
Acetic acid extracted residue	0.09	0.15	0.13	7.5
M_S	0.16	0.28	0.20	13.5
M_R	0.02	0.09	0.01	2
M_A	0	0	0.17	3

4. Discussion

Several procedures have now been described for solubilising and fractionating the proteins of erythrocyte ghosts. Nevertheless the true number of molecular species of protein involved in the construction of the membrane is unknown, for it is not clear to what extent the heterogeneity that has been repeatedly observed is a consequence of the interactions of constituent proteins induced by the solubilisation procedures. Prior to the publication of a comparison of the results obtained by the various methods, we here report a new and relatively mild method of fractionation (table 2).

We have obtained one component originating from the cell surface (M_S), one highly heterogeneous fraction possibly from the inner membrane surface (M_A) and another intractable complex (M_R) of indeterminate origin, but we cannot yet deduce how these components combine with lipid to form a membrane. The magnitude of this task is apparent from the complexity of the interactions the proteins can undergo with each other in the absence of lipid. It is abundantly clear that the problem of the natural association of protein with lipid must be approached with great caution, and the importance of a concommitant study of the biological activity of the fractions as an indicator of their native state is paramount. This aspect is currently being considered.

Table 2
Fractionation of ghost protein.



Acknowledgements

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References

[1] A.H.Maddy, Biochim. Biophys. Acta 117 (1966) 193.
[2] A.H.Maddy, Biochim, Biophys. Acta 88 (1964) 390.
[3] E.H.Eylar, M.A.Madoff, O.V.Broady and H.L.Oncley, J. Biol. Chem. 237 (1962) 1992.
[4] J.T.Dodge, C.Mitchell and D.J.Hanahan, Arch. Biochem. Biophys. 100 (1963) 119.
[5] D.Aminoff, Biochem. J. 81 (1961) 391.
[6] C.J.M.Rondle and W.T.J.Morgan, Biochem. J. 69 (1958) 12.
[7] L.C.Mokrasch, J. Biol. Chem. 208 (1954) 55.