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ACETIC ACID AS A SOLVENT FOR ERYTHROCYTE MEMBRANE PROTEINS

A. H. MADDY AND P. G. KELLY

Department of Zoology, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT (Great Britain)

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SUMMARY

The value of dilute acetic acid as a solvent for isolating erythrocyte membrane proteins is explored. Ox, human and sheep cells are used.

The acid liberates 30–40 % of the membrane protein, free of phospholipid and sialoprotein. The protein extracted fractionates into a complex set of bands on polyacrylamide gel electrophoresis using acetic acid as the electrophoretic solvent. The protein mixture is examined by analytical centrifugation under a range of conditions. It has a $s_{20,w}$ of 2 at low ionic strength but this value is markedly increased by the addition of salt.

The proteins extracted by acetic acid are very similar to those removed from the membrane by dilute EDTA solutions.

INTRODUCTION

A major difficulty in the constitutional analysis of membrane proteins is that they are locked in an insoluble complex with lipids and require to be released into solution before their analysis can proceed. Because of the present limited knowledge of the behaviour of proteins in non-aqueous media this usually involves liberation of the proteins in a lipid-free state into an aqueous medium. The removal of lipid and the conversion of the proteins into a water-soluble state are extremely drastic operations no matter how "mild" the conditions and at the very least will result in the rearrangement of any hydrophobic regions of proteins which interact with hydrophobic regions of lipid in the intact membrane. As the rearrangement that ensues will depend on the exact conditions pertaining during the separation of the protein and lipid, the state of any protein extract will depend not only on the conditions under which it is being examined but also on its history.

The range of methods for isolating proteins from membranes is now vast[1] but the variety of the results obtained by the different methods is almost as extensive and it follows from the preceding argument that little confidence can be placed on the results obtained by any one method. We have therefore been comparing various methods used on erythrocyte membranes to see whether proteins with similar properties may be extracted by different methods on the grounds that such results are less likely to be due to any one set of isolation conditions. We here report on proteins

which are extracted by dilute acetic acid (previously used to extract mitochondrial proteins[2]) and also show that these proteins (M_a proteins) are in many ways similar to those removed from ghosts by dilute solutions of EDTA. In a subsequent publication we shall show that these proteins may also be recognised in a solution containing all the ghosts proteins which was prepared by a butanol method. That proteins of similar properties may be isolated by three different methods provides some basis for believing that these properties are not a consequence of the extraction procedure. The proteins we describe are readily fractionated by gel electrophoresis and have lower sedimentation coefficients than have previously been observed for membrane proteins without the prior addition of some vigorous dissociating agent. Unless otherwise stated all findings relate to proteins extracted from ox (*Bos taurus*) cells.

METHODS

Extraction

Ghosts were prepared in phosphate buffer as previously described[3] except that the terminal water washes were omitted. Acetic acid: the ghost pellet was extracted for 20 min at 20° with 6 vol. of 0.26 M acetic acid (1.5 %), the insoluble residue centrifuged off and re-extracted twice. The final insoluble residue could, if desired, be completely solubilised by a butanol treatment[4]. EDTA: the ghost pellet was extracted for 48 h at 4° with 0.5 mM EDTA adjusted to pH 7.5 with NaOH[5]. The extraction was not affected by the presence of thiol reagent.

Neutralisation of the acetic acid extract

The pH of the solution was adjusted to 3.5 with 2.0 M NaOH, dialysed against 5 mM citrate–phosphate buffer (pH 3.5, 8 h, 4°), rapidly neutralised with NaOH and finally dialysed overnight against 5 mM phosphate buffer (pH 8.0).

Acrylamide gel electrophoresis

Gels of 7 % acrylamide + 0.1 % bisacrylamide were polymerised at 50° for 1 h using ammonium persulphate and *N,N,N′,N′*-tetramethylethylenediamine as catalysts. 0.26 M acetic acid was itself used as the electrophoresis solvent. The gels were prerun for 2 h, the protein applied (about 50 μg) and electrophoresed for 15 min at 3.5 mA per tube at 40 V/cm. The gels were stained in 0.05 % Coomasie Blue in 12.5 % trichloroacetic acid and stored in 7 % acetic acid.

Chemical analyses

Sialic acid was assayed by the thiobarbituric acid method of AMINOFF[6]. Lipid phosphate by the Fiske-SubbaRow method[7]. Protein was assayed by the Lowry method modified by the addition of sodium deoxycholate[8]: the relationship between dry weight and Lowry absorbance was calibrated separately for each extract. The relationships are not linear.

Analytical ultracentrifugation

An M.S.E. analytical centrifuge was used with double sector cells and schlieren optics under conditions described in RESULTS. Unless otherwise stated, $s_{20,w}$ values at 10 mg protein per ml solution are quoted.

SITS (4-acetamido,4'-isothiocyanostilbene-2,2'-disulphonic acid) labelling[9]

Intact cells: Ox erythrocytes were washed 4 times in isotonic saline and then reacted for 20 min at 20° with 50 μM SITS in buffered isotonic saline (9 vol. 0.9 % NaCl: 1 vol. 0.2 M phosphate buffer, pH 7.4). Free, unreacted, SITS was removed by 2 washes with the buffered saline and 4 washes of isotonic NaCl. (No haemoglobin leached out of the cells during these operations.) The still intact cells were haemolysed in 5 vol. of 0.01 M phosphate buffer at pH 6.0, washed twice in this buffer, then repeatedly with 0.01 M phosphate buffer at pH 8.0 until free, or almost free of haemoglobin. The washed ghosts were extracted with 0.26 M acetic acid and the residue solubilised by butanol extraction[4]. The two solutions were finally inspected for fluorescence.

Ghosts: The above experiment was repeated with the difference that ghosts were first prepared and then reacted with SITS under the conditions used to label the intact cells. The ghosts were washed free of excess reagent with 2 washes of buffered saline and 4 washes of 0.01 M buffer at pH 8.0 and finally extracted with acetic acid. They were solubilized as above and the fractions again inspected for fluorescence.

RESULTS

Extraction

The yield of protein in the acetic acid supernatants is shown in Table I. The extracts were free of sialic acid and lipid phosphorous.

Electrophoresis

The complex pattern of bands produced by the ox extract is shown in Fig. 1a. The same pattern was exhibited by extracts taken directly from the ghost, *i.e.* after only 20 min exposure to the acid as after several weeks in the acid at 4°. The low pH did not therefore appear to degrade the proteins unless the splitting of some extremely labile bonds occurred during the initial extraction. Other very mild treatments caused the pattern to be obscured by the appearance of slow moving material trailing from the gel origin, presumably aggregated protein. This effect was produced by neutralisation and reacidification, treatment with KCl at 0.1 M but not 0.01 M, *i.e.* the salt was added and dialysed away before electrophoresis, and passage through a P300 column using acetic acid as the solvent. (All the protein appeared as one peak at the void volume). The pattern was not altered by thiol reagents.

TABLE I

Extraction of proteins from ox erythrocyte ghosts by dilute acetic acid. Results expressed as % of protein in the unextracted ghosts which passes into the acetic acid.

	Protein extracted (%)		
Animal No.:	*TAG 967*	*HZ 135A*	*U93B*
1st supernatant	23	25	26
2nd supernatant	5	11	5
3rd supernatant	2	2	2
Total extracted protein	30	38	33

Protein may be extracted in similar amounts from other species, human, sheep and guinea pig and each has a characteristic pattern (Figs. 2 and 3). Human protein differed from other species investigated as the pattern changed after 2–3 days in the acetic acid (Fig. 4). The change was not prevented by dithiothreitol. The complexity of the human extract hampers comparison of the acetic acid extract with the EDTA extract (see below).

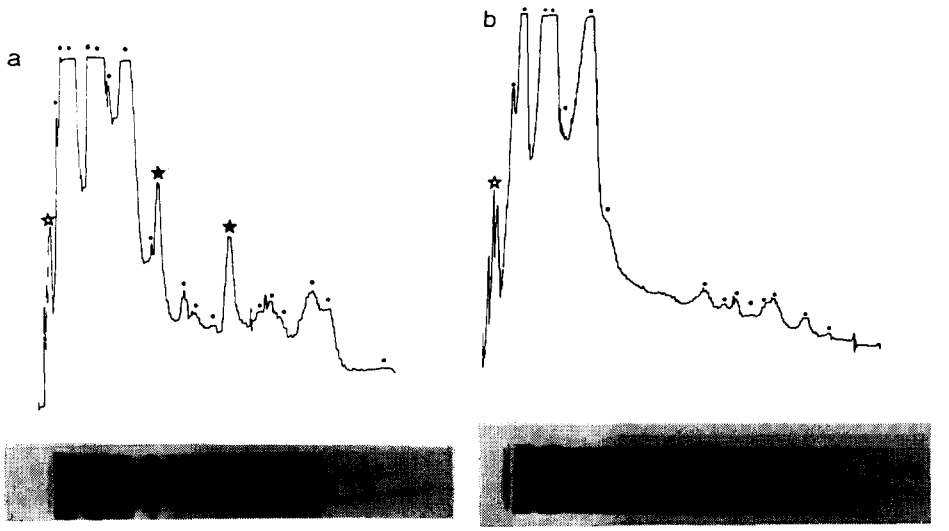

Fig. 1. Fractionation of proteins of ox erythrocyte membranes by polyacrylamide gel electrophoresis in acetic acid. Staining, Coomasie Blue. a, acetic acid extract; b, EDTA extract. The dots indicate positions in the gels where bands were seen by visual inspection. Open star, top of gel; filled star, band missing or diminished in b.

Ultracentrifugation

In 0.26 M acetic acid the extract sedimented as two partially resolved components with sedimentation coefficients of 1.0 S and 1.7 S at a protein concentration of 1 %, increasing to about 2.0 and 2.5 S at zero concentration. The slower component, which appeared as a shoulder on the trailing edge of the main peak, disappeared after passing the extract through P300 acrylamide gel, or after neutralisation (see below).

Addition of KCl to a final concentration of 0.1 M, resulted in the formation of a broad schlieren peak with a maximum at about 10 S, which sedimented to form a coherent gel. A small amount of material remained at 2–4 S. If the salt was removed by dialysis (against acetic acid) the original pattern was restored. In 0.01 M KCl–0.26 M acetic acid three components are seen with coefficients of 1.7, 3.0 and 7 S (protein concentration, 0.8 %) indicating that, even at this relatively low ionic strength, a salt dependent aggregation was taking place.

In 8 M urea–0.01 M HCl (pH 2.5) no dissociation was observed; after correction for viscosity and density, the sedimentation rates were similar to those seen in 0.01 M HCl alone and remained unaffected by the addition of 20 mM dithiothreitol. However, the urea was not totally without effect for it prevented the aggregation induced by salt. In the presence of urea it was necessary to replace acetic acid by HCl to achieve the low pH. In 0.01 M HCl the principal component was found to sediment faster and

to show a greater concentration dependence than in 0.26 M acetic acid. Its sedimentation coefficient was 2.0 S at 1 %, increasing to about 5 S at zero concentration. The slower boundary appeared to be more spread out and was difficult to distinguish in the schlieren pattern.

Location of the M_a proteins in the intact membrane

Even if the detailed composition of both the lipid and protein moieties of the membrane were known, the information would not itself reveal the structure of the membrane, which depends on the arrangement of the molecules relative to each other

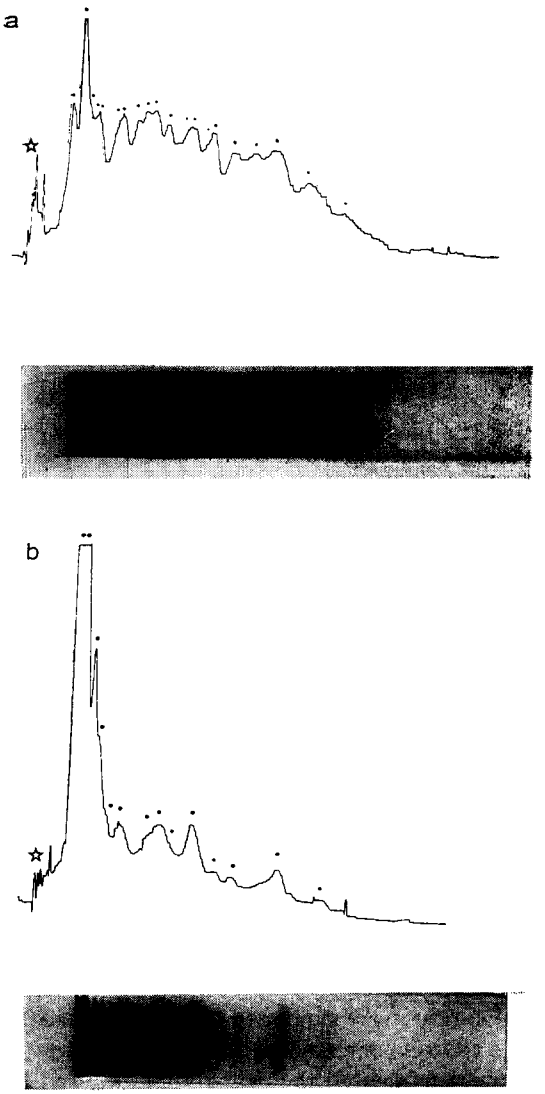

Fig. 2. Fractionation of proteins of human erythrocyte membranes by polyacrylamide gel electrophoresis in acetic acid. Staining, Coomasie Blue. a, acetic acid extract; b, EDTA extract.

It is therefore desirable to relate any isolated component to its original position in the membrane. The simplest question that may be posed in this respect is whether a given protein or set of proteins is inside or outside the permeability barrier of the cell. Three lines of evidence suggest that the M_a proteins are not on the outside of the cell. (a) They lack sialic acid, a marker known to be outside the permeability barrier[10]. (b) The solution is free of blood group antigens associated with the protein[11]. (These antigens are not destroyed by the exposure to acetic acid required to solubilise the proteins.) (c) The M_a proteins are not labelled by the impermeable fluorescent label 4-acetamido, 4′-isothiocyanostilbene-2,2′-disulphonic acid (SITS) when the intact cells are treated but become labelled if the ghosts are reacted.

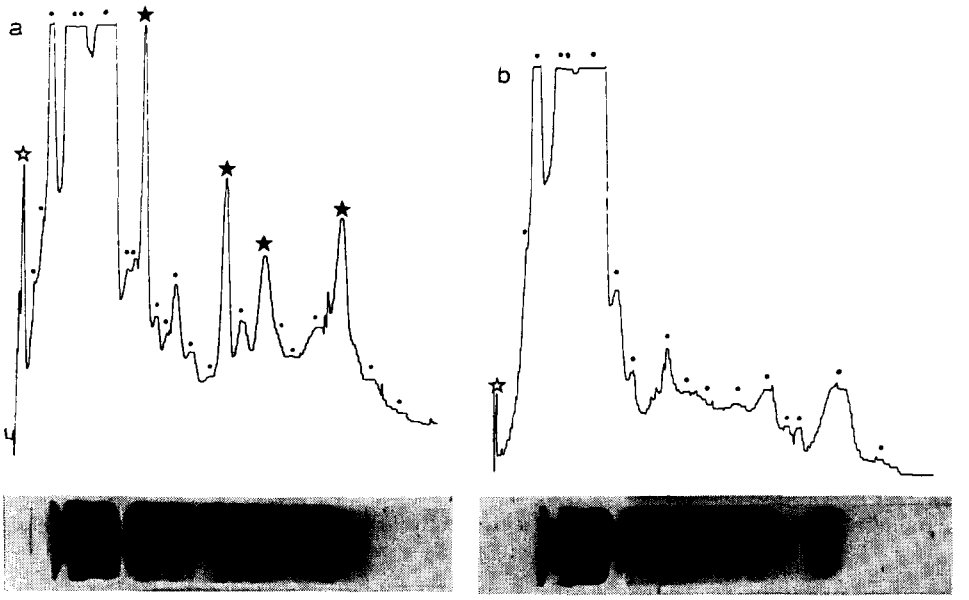

Fig. 3. Fractionation of proteins of sheep erythrocyte membranes by polyacrylamide gel electrophoresis in acetic acid. Staining, Coomasie Blue. a, acetic acid extract; b, EDTA extract.

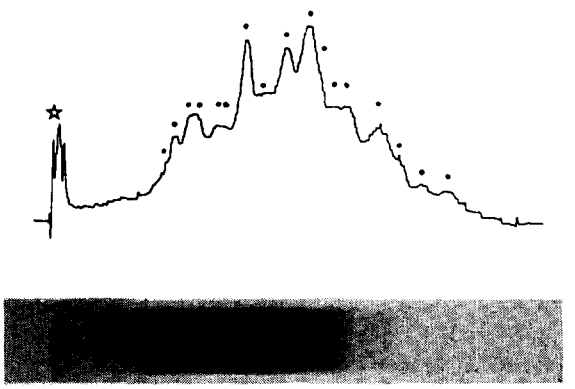

Fig. 4. The ageing of the acetic acid extract of human erythrocyte ghosts. Proteins stored 3 days at 4° in 0.26 M acetic acid before electrophoresis. Electrophoretic conditions as in Fig. 2.

When a ghost suspension, an acetic acid extract, and butanol-solubilised acetic acid residue of SITS-treated cells were examined under longwave ultraviolet light, stilbene fluorescence was seen in only the ghosts and the solubilised residue. Similar preparations from SITS-treated ghosts showed fluorescence in all three samples.

Ultraviolet spectroscopy confirmed that the fluorescence was due to the stilbene fluorophore. In fluorescent solutions an absorption band with a maximum at about 340 nm (the position of the *trans* stilbene isomer) was superimposed on the typical protein spectrum. The labelling was intentionally kept at a low level as the least perturbation of the normal membrane structure would be achieved by minimal substitution. This requirement unfortunately resulted in a fluorescence intensity too low for its distribution in a gel electropherogram to be investigated. The fluorescence was not removed from the labelled proteins by prolonged dialysis or passage through a P300 polyacrylamide gel exclusion column. No fluorescence was detected in experiments when the isothiocyanate was replaced by the non-reactive bisacetylated analogue. As fluorescent yield is markedly dependent on the molecular environment of the fluorophore the quantity of stilbene in any fraction could not be accurately determined from its emission, but some relative measure could be obtained. Measurements of the emission at 420 nm on a Locarte single side fluorimeter showed that the total fluorescence of the solubilised protein of treated ghosts had 3 times the emission of protein from treated cells.

Thus the acetic acid-extractable proteins after SITS treatment of the ghosts were reactive towards SITS. The most probable interpretation of this change is that the disruption of the cell's permeability barrier had exposed the previously unavailable M_a proteins, but some other membrane rearrangement could conceivably have been responsible. It is unlikely that the SITS substitution, which was at a very low level, affected the extraction procedure. The same amount of protein was extracted, and the electrophoresis pattern of the extract was unchanged, but there was insufficient SITS attached to the proteins to cause a detectable fluorescence within the acrylamide gel.

A comparison of the proteins extracted by acetic acid with those extracted by EDTA

During our investigation of acetic acid as a membrane solvent the application of dilute EDTA for this purpose was reported[5], and consequently we compared the proteins extracted by EDTA using the procedure established by MARCHESI *et al.*[5] with those extracted by acetic acid. EDTA extracted about 30 % of the total ghost protein. The proteins in the two extracts were similar with respect to electrophoretic properties, sedimentation coefficients under varying conditions, and location within the membrane.

The electrophoretic patterns obtained in acetic acid gels for the two extracts for 3 mammalian species ox, human and sheep are shown in Figs. 1–3. In all three cases the fast moving bands are diminished or absent in the EDTA extracts, especially in the human samples.

The sedimentation behaviour of the two protein solutions under various conditions is compared schematically in Figs. 5 and 6. The EDTA extract at low ionic strength (2.5 mM phosphate buffer + 0.5 mM EDTA, pH 8.0) forms a double peak of 3.9 S and 4.5 S protein. Addition of 0.1 M KCl results in a broad band centred around 10 S and a small amount of material at 4 S (Fig. 5). In common with the acetic acid extract this change coes not take place in the presence of 8 M urea. In 0.26 M acetic

acid the EDTA extract shows only a single peak at 2.8 S (5 mg/ml) and when returned to the dilute EDTA buffer the sedimentation coefficient returns to 4.5 but the doublet nature of the material is no longer apparent. The acetic acid extract after neutralisation also looses its doublet appearance and the $s_{20,w}$ shifts from the original 2 to 4.5 S. On reverting to acetic acid the lower value is restored bu the doublet does not reappear.

The proteins extracted by the two methods appear to be derived from the same location in the membrane. The proteins extracted from ox membranes by acetic acid and those extracted from human cells by EDTA[5] are both unreactive in the intact cell towards SITS. Both extracts from ox cells are devoid of blood group activity[11] and the EDTA extract of guinea pig erythrocytes does not react with antibodies against these cells[12].

Although there are marked similarities between the two extracts, they are not identical. Perhaps the most significant difference is the inability of the neutralised acetic acid extract to form fibres of the "spectrin" type on the removal of EDTA and the addition of magnesium ions. The differences between the electrophoretic patterns

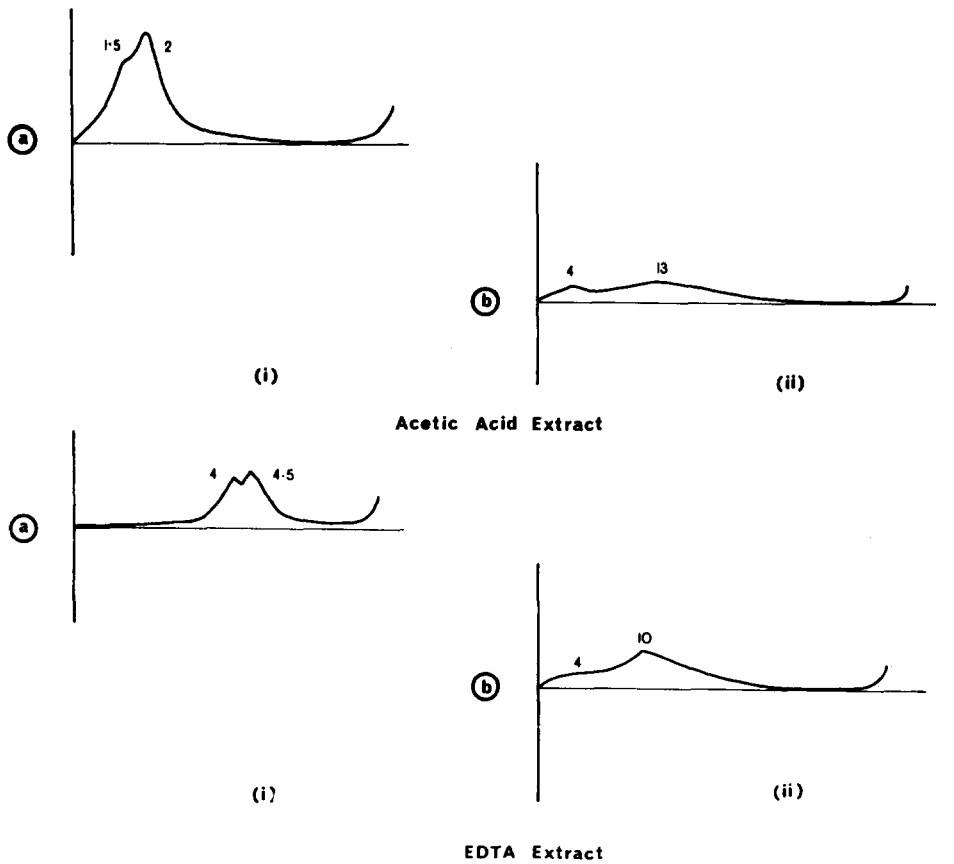

Fig. 5. Centrifugation of acetic acid and EDTA extracts of ox erythrocyte membranes (a) at low ionic strength. For the acetic acid extract the solvent was 0.26 M acetic acid; for the EDTA extract the solvent was 2.5 mM phosphate buffer, pH 8.0, *plus* 0.5 mM EDTA. (b) solvents used in (a) *plus* 0 1 M KCl. The figure represents tracings of the schlieren patterns and the $s_{20,w}$ values of the components are indicated. (i) after 100 min at 55000 rev./min; (ii) after 20 min at 55000 rev./min.

in acetic acid gels has already been noted and a further difference is seen in gels run in a glycine–Tris buffer containing EDTA. The EDTA extract resolves into several sharp bands but most of the protein in the neutralised acetic acid extract remains at the top

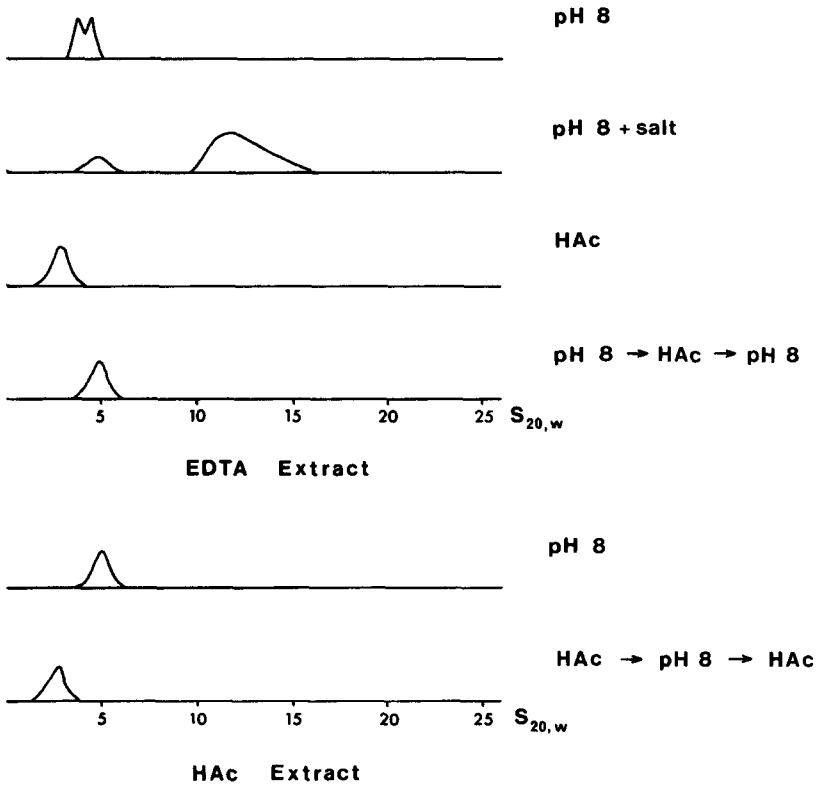

Fig. 6. Comparison of the sedimentation properties of the acetic acid and EDTA extracts of ox erythrocyte membranes. The schlieren peaks are shown schematically at the $s_{20,w}$ values observed under different conditions. pH 8, 2.5 M phosphate buffer + 0.5 mM EDTA; salt, 0.1 M KCl; HAc, 0.26 M acetic acid.

of the gel and the bands within the gel, although they correspond to those of the EDTA extract, are faint. The EDTA extract contains small amounts of sialic acid and lipid phosphate which do not occur in the acetic acid extract, but this is probably a consequence of the disruption of the ghost during the prolonged extraction with EDTA.

The apparent complexity of the EDTA extract is inconsistent with claims that EDTA extracts of several mammalian erythrocyte ghosts consist predominantly of one protein, "spectrin"[5,12,13]. This discrepancy is not due to the use of a different species of animal, for human ghost EDTA extracts are in our hands equally heterogeneous under all conditions. (The ghosts used in these experiments were kindly prepared by Dr. J. R. Harris.) We get the same pattern for the human EDTA extract in 8 M urea–glycine–Tris gels as the original authors, but the material excluded by G200 columns, *i.e.* purified "spectrin", gives not one broad band *plus* material at the gel

origin, as they describe, but four sharp bands together with material at the origin (Fig. 7).

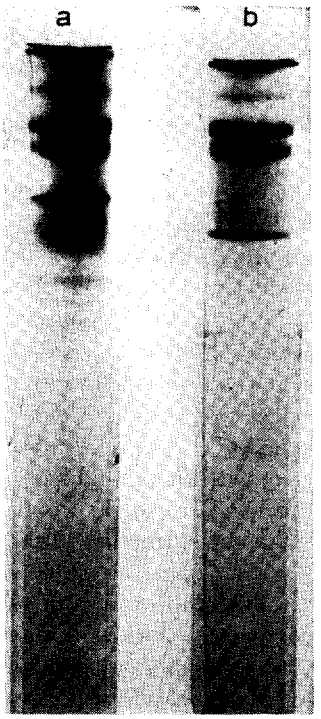

Fig. 7. Gel electrophoresis of human "spectrin" preparation using the electrophoretic conditions described by MARCHESI *et al.*[5] (5% polyacrylamide in Tris–glycine buffer (pH 8.4) + .8 M urea). a, total EDTA extract; b, purified "spectrin" from G 200 Sephadex column.

CONCLUSION

A number of laboratories have reported[12–15] that dilute EDTA solutions release protein from the erythrocyte membrane; we reported the effectiveness of dilute acetic acid as a solvent for a fraction of membrane protein[4]. It is now apparent that the two solvents extract similar proteins, which is not surprising if, as is probable, the release of protein by EDTA is due to the rupture of divalent ion bridges for acetic acid would have a similar effect by titrating the anionic elements of the bridges. Of the two systems, EDTA may be preferable as the conditions used are nearer physiological, but the acid extracts rather more protein and the presence of small amounts of sialic acid and lipid phosphate in the EDTA extract points to a general non-specific breakdown of the ghost during the lengthy extraction with the EDTA. As an electrophoresis solvent, acetic acid has the advantage, for all the protein enters the gel and the considerable ambiguity that exists in systems when material is left at the gel surface is avoided.

The large increase in sedimentation coefficient induced by salt in both extracts suggests a hydrophobic association of the proteins. Hydrophobic interactions have already been postulated as a basis for the interactions of proteins and lipids within

membranes and also between the isolated proteins[15–20]. Whatever the physiological significance of such interactions may be, they are of paramount importance in any attempt to fractionate the proteins. Most of the classical methods of protein fractionation implicitly assume that the interactions between the molecules are predominantly electrostatic, the concurrent existence of hydrophobic and electrostatic interactions alters and greatly complicates the problem of fractionation. Indeed, the difficulty of analysing membrane proteins can largely be attributed to this combination of forces.

The heterogeneity of the acetic acid extract indicated by electrophoresis, be this a consequence of true molecular variety or the formation of several different aggregates from a relatively small number of components, obstructs any straightforward analysis of molecular weight. In spite of the low sedimentation coefficient the protein is excluded by a P300 acrylamide column in acetic acid. By sodium dodecyl sulphate electrophoresis, a method to be regarded with considerable reservation for the estimation of the molecular weights of membrane proteins, the pattern obtained from the acetic acid and EDTA extracts is similar to that of the whole ghost, although there is rather more of the slow moving bands.

An itemised comparison of the proteins we isolate here with those obtained by the numerous other methods available would be of limited value. The central problem of the study of membrane proteins is why different methods of extraction usually result in different patterns of protein fractions. When so many disparate results are being obtained it is not unwelcome that similar results can be obtained by different procedures. Both acetic acid and EDTA leave the greater part of the membrane's protein in the insoluble residue, and from the point of view of membrane structure this could well be the more interesting moiety. A subsequent publication will report a butanol procedure which yields an aqueous extract containing all the membrane proteins, and which permits the study of the interactions between those described in the present communication with those the acetic acid and EDTA are unable to extract.

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