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THE CHARACTERISATION OF MEMBRANE PROTEINS BY CENTRIFUGATION AND GEL ELECTROPHORESIS
A COMPARISON OF PROTEINS PREPARED BY DIFFERENT METHODS

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SUMMARY

1. The value and importance of showing that proteins of similar characterstics may be extracted from membranes by different methods is argued.

2. It has previously been shown that proteins with similar characteristics can be isolated from erythrocyte ghosts by EDTA and dilute acetic acid.

3. It is now shown that such proteins may also be detected in a solution of proteins solubilised from the membrane by a butanol treatment in the presence of EDTA.

4. The butanol extract also contains the glycoproteins, which may be extracted separately by phenol, and a third category of proteins, the residual proteins.

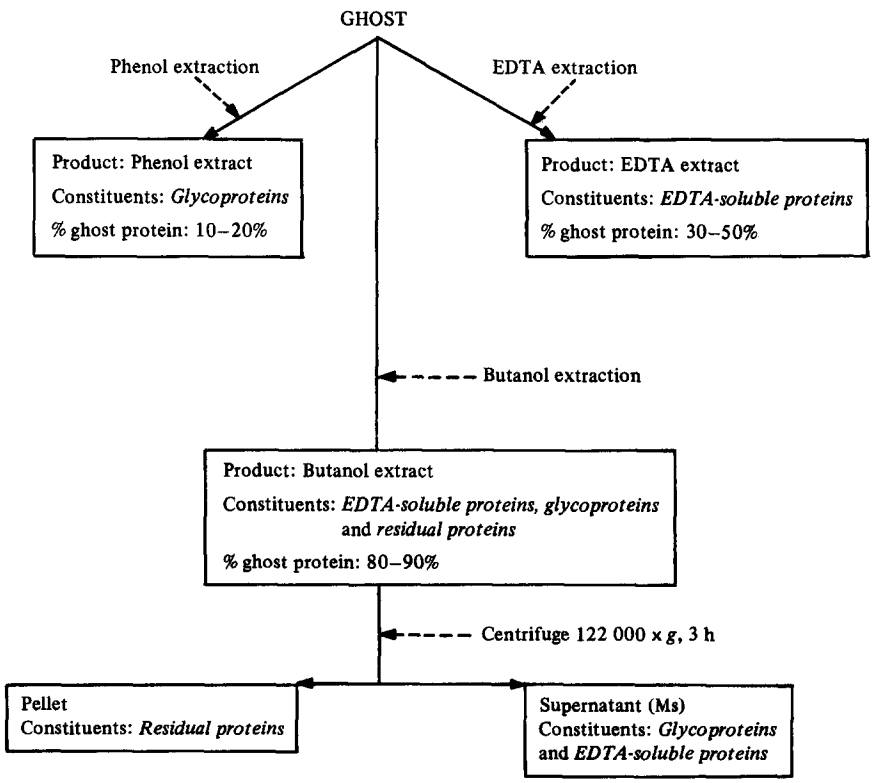
5. The presence of these three types of protein has been demonstrated by centrifugation and polyacrylamide gel electrophoresis.

INTRODUCTION

The confused state of the constitutional analysis of the proteins of the erythrocyte membrane is, at least in part, due to the almost embarrassing variety of results that have been obtained in different laboratories through the use of many solvent systems. Not only is the list of reagents used long, it is extremely varied. Although the end result of all the reagents, release of protein from the insoluble membrane, is the same, the variety of reagents that achieve this one end is so great it must be concluded that this result can be brought about by different mechanisms, *e.g.* some reagents releasing proteins by weakening electrostatic bonds, others by affecting hydrophobic interactions. Any one reagent is unlikely to abolish all interactions. It must reduce certain interactions to release the proteins, but other interactions between proteins will remain undiminished and possibly persist within the isolated protein. The state of the isolated protein can therefore depend on the conditions under which it was initially extracted as well as those under which it is being examined on any subsequent occasion. The probability of the characteristics of the proteins in any given extract being largely a consequence of the method of extraction is lower if similar characteristics can be demonstrated in proteins extracted by other

methods. We have shown[1] that proteins extracted from ox (*Bos taurus*) erythrocyte ghosts by both 0.26 M acetic acid and 0.5 mM ethylenediaminetetraacetic acid (EDTA) have similar characteristics: they have low sedimentation coefficients, are aggregated by salt, give similar band patterns in polyacrylamide gel electrophoresis and are free of carbohydrate and blood group substances. However, proteins with these properties cannot be found in the aqueous solution of membrane proteins prepared by a butanol treatment[2] even though 90 % of the proteins are solubilised. As the butanol method has some merit (see Discussion) we have investigated this discrepancy further.

The search for the acetic acid-soluble proteins (M_a proteins) in the butanol extract has already been briefly reported[3]. Proteins corresponding to the M_a proteins could only be detected in the butanol extract under strongly dissociating conditions (8 M urea + dithiothreitol) at low ionic strength. Under these conditions material of the sedimentation coefficient characteristic of the M_a proteins existed, and by polyacrylamide gel electrophoresis, at low pH in urea, a set of bands corresponding to that obtained from the acetic acid extract was seen within the gel in addition to slow moving protein. This result was analogous to that of Zwaal and van Deenen[4]



Scheme 1. An outline of the extraction procedures and the resulting products studied in this investigation.

except that we found thiol reagent to be essential, not only for the electrophoresis, but also for the appearance of slowly sedimenting material in the analytical centrifuge.

The discovery of many similarities between the proteins extracted by acetic acid and those extracted by EDTA lead to a reappraisal of the butanol method to see whether it could be modified so as to produce, without recourse to strong dissociating agents, an extract containing proteins with the same properties as those extracted by EDTA. The requisite modification merely involved the introduction of EDTA into the butanol procedure. The resulting aqueous solution, which contains virtually all the membrane's protein, is subsequently referred to as the "butanol extract", and where it is compared with the solution prepared by the original butanol method[2] this latter will be termed the "original butanol extract". In both cases there is no butanol left in the protein solution. The effect of incorporating EDTA into the butanol extraction procedure is to yield a solution of membrane protein containing, in a free and recognisable state, (a) proteins with the characteristics of the EDTA-soluble proteins, (b) the glycoprotein(s) (which may be extracted from the ghost separately by phenol), and (c) a third major category of protein, residual proteins, which are not solubilised by EDTA, acetic acid or phenol (see Scheme 1). The presence of these various proteins in the butanol extract has been established by a combination of centrifugation data and polyacrylamide gel electrophoresis.

METHODS

Ghosts

Ox blood was collected into sterile acid–citrate–dextrose, the cells washed in 0.9 % NaCl and ghosts prepared as described previously[1] by a slight modification of the procedure of Dodge *et al.*[5]

Extractions

Acetic acid extraction: The haemoglobin-free ghosts were extracted at room temperature with 0.26 M acetic acid as previously described[1].

EDTA extraction: 4 vol. of 0.5 mM EDTA, adjusted to pH 7.5 with NaOH, were added to the ghosts and the suspension left to dialyse against this EDTA solution at 4 °C for 36 h. The insoluble material was removed by centrifugation for 1 h at 65000 × *g*.

Original butanol extraction: The procedure previously reported[2] was followed. The resulting protein solution is referred to as the "original-butanol extract".

Modified butanol extraction: The water employed in the foregoing method was replaced by 0.5 mM EDTA in 2.5 mM phosphate buffer adjusted to pH 8.0. The ghosts (8–10 mg/ml) were washed 3 times in this buffer, cooled on ice, 0.75 vol. of ice cold *n*-butanol (British Drug Houses, Analar grade) was added, and the mixture shaken. After 20 min on ice the two phases were separated by centrifugation (5 min at 22000 × *g*), the lower (aqueous) phase removed and dialysed overnight, to remove butanol, against the dilute EDTA buffer containing ice. The resulting protein solution was stored at 4 °C in the presence of 0.01 % NaN_3 as a bacteriostatic. This protein solution is known as the "butanol extract".

Butanol extraction of the insoluble residue left after EDTA extraction: The residue

after EDTA extraction was washed 3 times with the EDTA solution and extracted by the butanol method.

Glycoprotein-enriched fraction (M_8): This fraction constitutes the supernatant left after centrifugation of the butanol extract for 3 h in a Beckman 50 rotor at 122000 × *g*.

Phenol extraction: The protein solution or membrane suspension was extracted with an equal weight of phenol for 15 min at 68 °C, the mixture cooled, centrifuged to separate the phases, and the aqueous phase left to dialyse against the dilute glycine–Tris buffer used for electrophoresis until free of phenol.

Centrifugation

Analytical: The samples were examined at 20 °C in an M.S.E. analytical centrifuge with schlieren optics. Double-sector cells were usually used with protein in one sector and the appropriate buffer in the other.

Sucrose gradients: 5–20 % sucrose gradients in a glycine–Tris buffer (5.0 mM, pH 8.0) containing 0.5 mM EDTA were centrifuged in a Beckman S.W.25 rotor for 18 h at 60000 × *g*. 1 ml sample of the protein was applied to each gradient and after centrifugation the gradient displaced by 50 % sucrose, monitored continuously at 280 nm in an ISCO analyser, and fractions were collected in a Shandon–Jeffs fraction cutter.

Chemical analyses

The following were estimated: protein by a Lowry method in the presence of deoxycholate[6]; lipid phosphate by a Fiske–SubbaRow method after digesting with $KClO_4$ (ref. 7); sialic acid by the method of Aminoff[8]; hexosamine by the method of Gatt and Berman[9] using glucosamine as a standard; hexose by the method of Dubois *et al.*[10] with glucose as a standard.

Polyacrylamide gel electrophoresis

Glycine–tris(hydroxymethyl)aminomethane: Gels of 5 % acrylamide + 0.1 % bisacrylamide were polymerised with ammonium persulphate and *N,N,N*^{1},*N*^{1}-tetramethylene ethylene diamine as catalyst in a glycine–Tris buffer (5 mM glycine, 5 mM Tris, 0.5 mM EDTA; pH 8.0). Electrophoresis was carried out in this same buffer for 45 min at 40 V/cm, 2 mA per tube. The gels were prerun, before addition of protein, for 3 h at 20 V/cm. After electrophoresis the gels were fixed in 12.5 % trichloroacetic acid and stained in 0.02 % Coomassie Blue in 12.5 % trichloroacetic acid.

RESULTS

The butanol extract

The general features of the modified butanol extraction procedure are very similar to those previously reported for the original butanol extraction[2]. The most significant effect of the addition of EDTA is to facilitate the fractionation of the resulting protein solution. As in the original method, centrifugation of the emulsion produced by mixing butanol with an aqueous suspension of ghosts separates the aqueous phase, containing 80–90 % of the membrane's protein almost free of lipid,

and an organic phase, which in the EDTA modification usually contains a thick layer of fatty emulsion. The exact distribution of the protein is difficult to estimate for it is not possible to completely recover the lower aqueous phase, and a small proportion of protein remains at the interface and trapped in the fatty layer.

The protein content of the various fractions of membrane protein is estimated by the Lowry method. The Lowry colour does not increase linearly with concentration and the extinction coefficients of the various fractions differ. The coefficient falls with increasing carbohydrate content but not in simple proportion. Consequently, calibration curves were constructed for each fraction separately (Fig. 1) and used

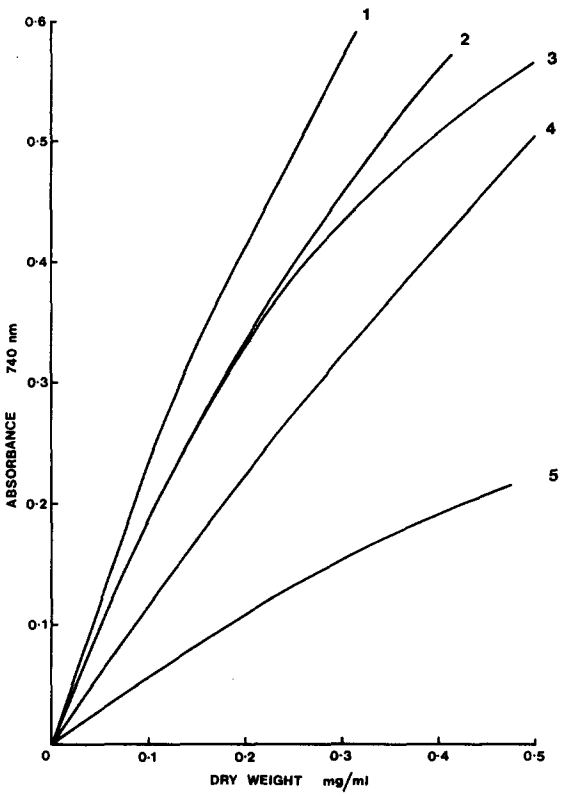


Fig. 1. Calibration of protein fractions for Lowry assay. The Lowry colour of each sample is plotted against the dry weight of a freeze-dried aliquot of that sample. (1) EDTA extract; (2) butanol extract of whole ghosts; (3) butanol extract of ghost residue after EDTA extraction; (4) glycoprotein enriched supernatant, M_8 of (3); (5) phenol extract.

subsequently to estimate dry weights. The change in Lowry sensitivity is not due to a simple interference with colour development by the carbohydrate, for when known mixtures of EDTA extract and M_8 are assayed the observed colour equals that calculated from the values obtained by the two components separately. Table I lists the carbohydrate compositions of the protein fractions. The EDTA extract is virtually free of carbohydrate[1]; one glycoprotein-rich fraction (M_8) has been recognised. The residual protein (see below) cannot be prepared free of carbohydrate,

but as it is made from the glycoprotein by centrifugation, contamination by the glycoprotein cannot be excluded, and therefore no reliable analytical data are available.

TABLE I

THE CARBOHYDRATE CONTENTS OF MEMBRANE PROTEIN FRACTIONS

Values are expressed as μg carbohydrate/mg dry weight of sample. The dry weights are estimated from the Lowry calibration curves shown in Fig. 1. A mean value followed by the range (in parentheses) is quoted for each sugar. The extent of the range is chiefly due to variation between individual animals[6]. The trace of sialic acid in the EDTA extract is believed to be due to a slight disruption of the ghost during its prolonged extraction[1].

	Sialic acid	*Hexosamine*	*Hexose*	% *carbohydrate*
1. Total butanol extract	23 (17–27)	40 (37–45)	41 (38–58)	11
Molar ratios	1 :	2.9 :	3.4	
2. Butanol extract of EDTA-extracted residue	50 (22–35)	51 (38–65)	61 (50–73)	14
Molar ratios	1 :	2.75 :	3.4	
3. Glycoprotein-enriched fraction (M_8) from 2.	49 (44–55)	104 (84–116)	122 (97–142)	27
Molar ratios	1 :	3.6 :	4.3	
4. Phenol extract	81 (70–95)	140 (104–188)	183 (158–210)	40
Molar ratios	1 :	2.9 :	3.8	
5. EDTA extract	Trace (see ref. 1)	0	0	0

To show the presence of EDTA-soluble proteins in the butanol extract

The EDTA extract, when examined at low ionic strength, shows a sharp but complex schlieren pattern as illustrated in Fig. 2a. The details of the pattern vary slightly from preparation to preparation. Addition of salt to the solution (0.1 M KCl) markedly increases the sedimentation coefficient and the band is considerably broadened[1]. The effect of protein concentration on the sedimentation of the major component of this extract at low ionic strength is shown in Fig. 3.

The schlieren pattern of the butanol extract is shown in Fig. 2b and may be seen to contain a component (a + b) which corresponds in sedimentation coefficient with the EDTA extract. The polydispersity of the solution and the low ionic strength prejudice precise comparison of the effect of protein concentration between this solution and the EDTA extract, but within the bounds of such limitations the effects of concentration on the slowly sedimenting protein in the butanol extract solution and the EDTA extract are similar (Fig. 3).

Addition of salt increases the sedimentation of the slower components in the butanol extract (Fig. 2c). Salt also increased the sedimentation of the EDTA extract[1]. Sedimentation in the absence of urea is not affected by reducing agents (dithiothreitol).

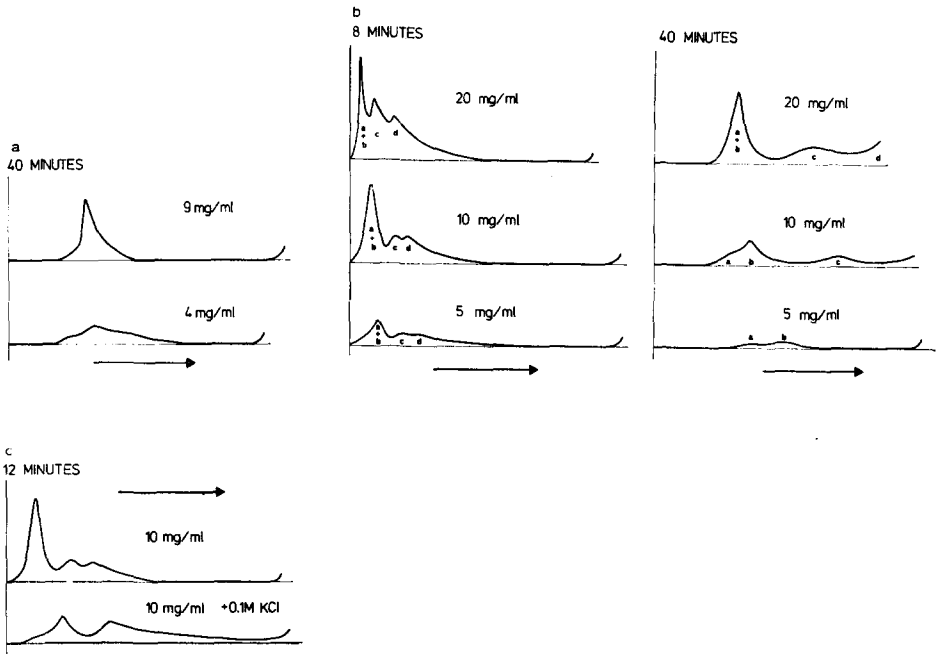


Fig. 2. a. Tracings of schlieren pattern obtained by centrifugation of EDTA extract. M.S.E. analytical centrifuge, 55000 rev./min, bar angle 15°, double-sector cells, solvent 2.5 mM phosphate buffer + 0.5 mM EDTA, pH 7.5. b. Centrifugation of butanol extract under conditions used in a. Four major components, a, b, c and d, are seen. c. The effect of the addition of 0.1 M KCl to the protein of b. Upper trace, 2.5 mM phosphate buffer + 0.5 mM EDTA, pH 7.5; lower trace, 0.1 M KCl added to phosphate-EDTA buffer.

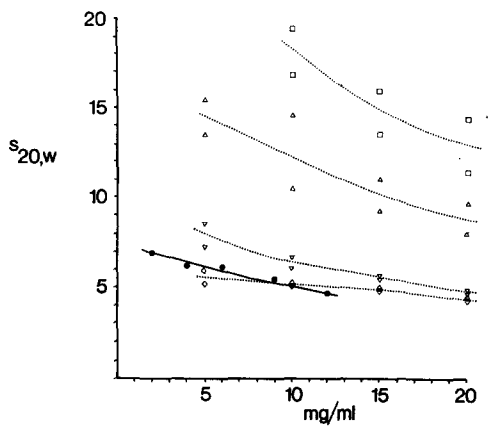


Fig. 3. The variation of the sedimentation coefficients of membrane proteins with protein concentration. Concentration is expressed as mg/ml of total protein in the mixture, not the concentration for each separate component. Proteins were concentrated by dialysis under negative pressure. ·····, components of butanol extract (Fig. 2b). ◇, a; ▽, b; △, c; □, d. ———, major deflection in EDTA extract.

Sedimentation coefficient alone would be insufficient evidence for identifying the slowly sedimenting material (Components a and b) in Fig. 2b as the protein of the EDTA extract, but the identification is confirmed by sucrose gradient centrifugation in conjunction with polyacrylamide gel electrophoresis. The distribution of butanol-extracted protein in a sucrose gradient after 18 h centrifugation is shown in Fig. 4a. The topmost fraction (i) is in the position occupied by the EDTA extract when centrifuged under these conditions (Fig. 4d). The analysis of the fractionated gradient (Fig. 4a) by electrophoresis in a glycine–Tris–EDTA buffer is seen in Fig. 5. When the unfractionated mixture (Gel A) is subjected to electrophoresis three features may be noted in the band pattern, a dense band at the origin, a broad, slow moving band which can be seen to partially obscure other slow moving bands, and a sequence of faster bands. This pattern can be compared with those obtained from the various peaks in the sucrose gradient. The material at the gel origin (Fig. 5,

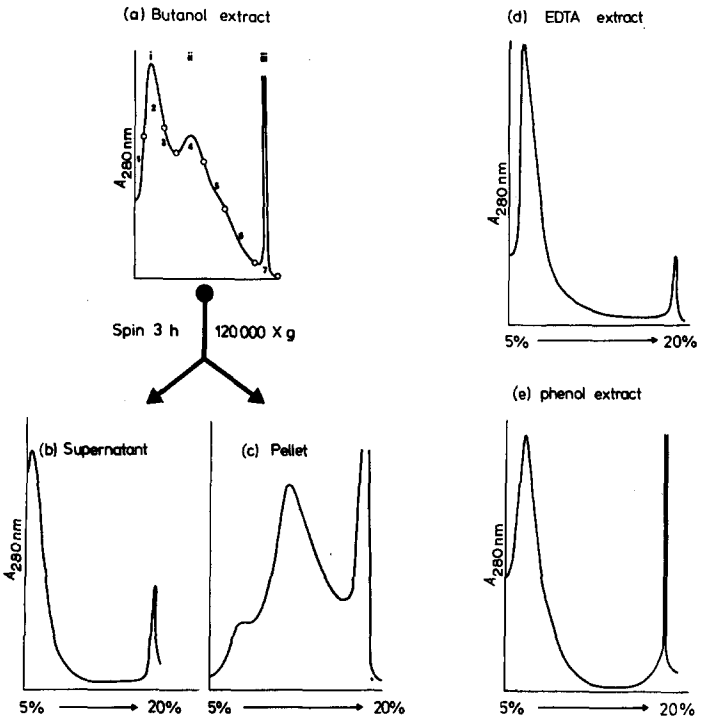


Fig. 4. The fractionation of membrane proteins by sucrose gradient centrifugation. Centrifugation was continued for 18 h at 60000 × *g* in a Beckman SW 25 rotor. (a) Total butanol extract; (b) supernatant (M_8); and (c) pellet from centrifugation of (a) for 3 h at 122000 × *g* in Beckman 50 rotor; (d) EDTA extract; (e) phenol extract. The numbers in (a) relate to fractions shown in Fig. 5.

Gel C7) is found in the pellet (Fig. 4a (iii)), the broad slowly moving band (Fig. 5, Gels C3–5) is in Peak (ii) (Fig. 4a), and the complex pattern of bands (Fig. 5, Gels C1–3) at the top of the gradient (Fig. 4a, (i)). Gels C1–3 are comparable with that of the EDTA extract (Fig. 5, Gel B).

However, the material in the slowly sedimenting complex (a + b) of the

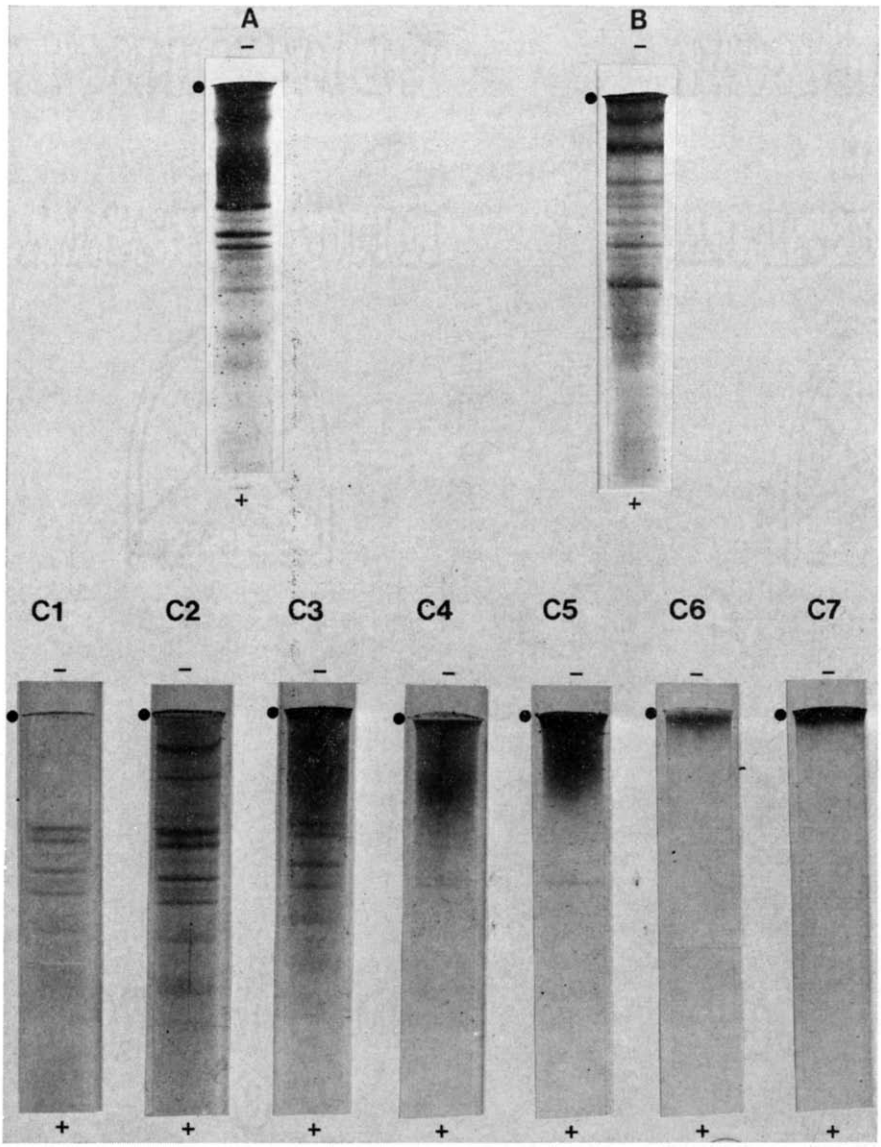


Fig. 5. Polyacrylamide gel electrophoresis of membrane proteins in a glycine–Tris buffer (details in text). (A) Butanol extract; (B) EDTA extract; (C) Fractions from sucrose gradient centrifugation of butanol extract. ●, gel origin; 1 = top of gradient → 7 = pellet.

butanol extract is not completely identical with the EDTA extract. Even after EDTA extraction of ghosts, slowly sedimenting material is present in the butanol extract prepared from the ghost residue, and sialic acid is present in the slowly sedimenting protein of butanol extracts. The EDTA extract contains little or no sialic acid. These observations suggest that the slowly sedimenting complex of the butanol extract of whole ghosts consists of a mixture of membrane proteins soluble in EDTA and membrane glycoprotein.

The glycoprotein

The membrane glycoprotein(s) is not extracted by either acetic acid or EDTA, but it is present in the butanol extract whose sialic acid content per unit protein is almost identical with that of the intact ghost. Various centrifugational procedures have shown that the glycoprotein is found amongst the slowly sedimenting proteins of the total extract. First, the sialic acid content of the fractions produced by sucrose density centrifugation (Fig. 4a) falls from 40 μg/mg in Peak (i) to 17 μg/mg in Peak (ii) and 5 μg/mg in Peak (iii). Secondly, the supernatant obtained by preparative centrifugation of the butanol extract at 122000 $\times$ *g* for 3 h is enriched in carbohydrate. The supernatant is referred to as the M1 fraction and analytical centrifugation shows it to consist almost exclusively of slowly sedimenting protein (occasionally a small amount of heavier material is included). From these observations, and the evidence presented in the previous section, it must be concluded that the a + b complex (Fig. 2b) of the butanol extract is a mixture of EDTA-soluble proteins and glycoproteins.

In an attempt to determine the proportions of the two categories in the complex, a method for the isolation of the glycoprotein was sought. Phenol extraction of the ghosts has been widely employed for this purpose[12,13], and is of some assistance in the present circumstance. The material extracted by phenol is considerably richer in carbohydrate than the other extracts (Table I) and, as predicted, is sedimented at the same rate as the EDTA proteins both in dilute buffer and after the addition of salt. However, its sedimentation differs from the EDTA extract in 8 M urea, for while the sedimentation coefficient of the latter falls to around 2 S on adding 8 M urea + dithiothreitol, the material in the phenol extract is unaffected. Through this effect the relative proportions of EDTA-soluble proteins in Complex a + b may be approximately estimated. The schlieren pattern of the M_8 in 8 M urea + dithiothreitol reveals a large component at 2 S, representing the EDTA extract, and another at 6 S, representing the glycoprotein (Fig. 6). If the refractive increments of the two components are the same, and ignoring Johnston–Ogston effects[11], the ratio of EDTA-soluble protein to glycoprotein is 2:1. In Fig. 6 it is also seen that the deflection assigned to the EDTA-soluble protein is markedly diminished, although not absent, in the preparation made from the ghost residue left after EDTA extraction. A second approach to the problem of the relative amount of glycoprotein

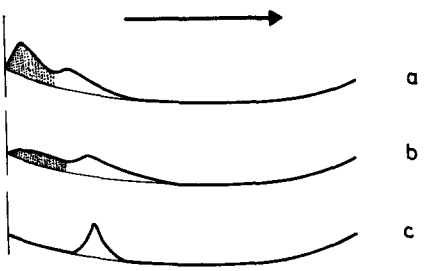


Fig. 6. Tracings of schlieren patterns obtained by centrifugation of glycoprotein-rich fractions of membrane proteins in 8 M urea + 10 mM dithiothreitol, pH 7.5 (M_8). a, Supernatant from butanol extract after centrifugation at 122000 $\times$ *g* for 3 h; b, supernatant comparable to a derived from insoluble ghost residue left after EDTA extraction. Note diminution of EDTA-soluble proteins; c, phenol extract. The shaded component represents the EDTA soluble proteins.

can be made by comparing the peak area of a phenol extract of known sialic acid concentration with that of the a + b complex in a butanol extract of known sialic acid content. On this basis the ratio of EDTA-soluble protein to glycoprotein is 3:1. This calculation assumes that all the sialic acid of the total extract is in the a + b complex.

The remaining protein

Two categories of proteins from the complex mixture that constitute the protein complement of the ghosts have so far been considered, (a) the readily extractable proteins, which by EDTA extraction constitute about 30–50 % of the total and by acetic acid extraction 30–40 % of the total, and (b) the glycoprotein(s). As carbohydrates amount to about 10 % of the dry weight of the lipid-free membrane protein and the glycoprotein contains around 40 % carbohydrate, the glycoprotein may be concluded to account for approximately 10–20 % of the membrane's protein. Hence, these two categories account for only about half of the membrane protein.

The residual protein is represented by the third component seen in the butanol extract however that butanol extract is examined. During centrifugation in dilute aqueous buffers (and in 8 M urea) it forms the faster sedimenting components (Fig. 2b, Components c and d) and it appears as a diffuse slow moving band during gel electrophoresis. Figs 4 and 5 relate the faster component (ii) on the sucrose gradient to the slow moving band in electrophoresis in glycine–Tris buffer (Fig. 5, Gels C3–5). The residual protein has also been demonstrated as a slow moving band by electrophoresis in acidic urea[3]. Similarly, it forms the slow moving material during gel electrophoresis of both ghosts and butanol extract by a Takayama gel system[14]. The other, faster bands visible on such gels are much fainter after EDTA extraction of the ghosts, and are virtually absent in the pellet obtained by centrifugation of the butanol extract as in Fig. 4c. It follows that these faster bands represent the proteins of the EDTA extract.

DISCUSSION

The major advantage of the original butanol method was that it yielded a solution containing virtually all the membrane protein in a relatively undamaged condition. The relative integrity of the butanol-extracted proteins has been indicated by several observations. A number of enzymic and antigenic activities of the original membrane persist (refs 15–17; and M. J. Dunn and A. H. Maddy, unpublished work): the infrared spectrum of the isolated protein closely resembles that of the protein in the intact membrane[18], and is not converted to the β state as happens in some solvents, *e.g.* dimethylformamide[19], it retains its ability to interact with lipid although it has not been established that the lipid–protein complexes formed represent interactions that pre-existed in the intact membrane[20–23]. Butanol solubilisation was found, in a comparative study of a wide range of solvents, to cause the minimal alteration in the conformation of membrane proteins as revealed by electron spin resonance labelling[24].

These advantages were counterbalanced by a major defect, the resistance of the solution to fractionation, so that certain proteins, which were readily fractionated when extracted by other means, were quite intractable after butanol treatment.

The modification of the butanol method by the addition of EDTA has overcome this problem of aggregation to a certain extent. Using the modified method it is now possible to recognise within the butanol extract proteins isolated by other methods (EDTA, acetic acid and phenol). A further category of protein, not isolated by these methods, is present although in an aggregated and intractable form. The EDTA-soluble proteins may originate from the inner surface of the membrane[1]. Part, at least, of the glycoproteins lie on the outer surface[25]. It is tempting to speculate that some, if not all, of the residual protein fraction is made up of protein that lies buried in the hydrophobic regions of the membrane, and that its aggregative nature is a consequence of its hydrophobicity.

Our observations are also of general relevance to other methods of protein isolation. It has previously been argued that the molecular status of protein fractions isolated from membranes is, largely as a consequence of protein aggregation, always in doubt, especially when the fraction has been isolated by just one method. However, if protein solutions prepared by different methods have similar characteristics it is less likely that these have been imposed upon the proteins by the extraction procedure. It has already been shown that essentially the same proteins are isolated from the membrane by acetic acid and EDTA[1], and we have here demonstrated the presence of proteins with the properties of EDTA-soluble proteins in an aqueous solution of membrane protein prepared by quite a different method, *i.e.* butanol extraction. Our results also suggest that EDTA extracts a large proportion of a particular set of membrane proteins, which consitute about 30–50 % of the total membrane's protein, rather than a random 30–50 % sample of all the membrane's proteins. The conclusion that the EDTA-extractable proteins represent a particular category of membrane proteins is held by most workers[1,26–29], but one group[30] reports that EDTA extraction of human ghosts is not selective, and on prolonged exposure (96 h) up to 90 % of the membrane protein passes into solution. In our experience with ox cells such lengthy treatment does not greatly increase the amount of protein extracted and the extraction remains selective. These observations apply to extraction with both 5.0 mM and 0.5 mM EDTA. The possibility that the residual protein contains some aggregated EDTA-soluble proteins cannot be excluded. It should be made clear that we have not yet conclusively established that any of the fractions we isolate represent distinct molecular species as opposed to stable multimolecular complexes. This ambiguity applies to all protein fractions, including those isolated from membranes by sodium dodecylsulphate[31], until their status has been verified unequivocally by classical techniques of protein chemistry (end-group analysis, peptide mapping, *etc.*). Considerable progress in the investigation of the role of proteins in membrane structure and function can be made in the present absence of this information, *e.g.* by various spectroscopic techniques, but ultimately this knowledge is essential for a full understanding of membrane structure.

Acrylamide gel electrophoresis in the presence of sodium dodecylsulphate has now achieved such a dominant position among techniques for the analysis of membrane proteins[29,31,32] that, although we are primarily concerned with the development of alternative methods, the relationships between these methods and the sodium dodecylsulphate method must be considered. It may be stated briefly here that the different fractions we have described correspond to specific components of the band pattern given by the ghosts in the dodecylsulphate gels. The pattern obtained by

sodium dodecylsulphate electrophoresis of the butanol extract is indistinguishable from the ghost pattern except for a small amount of protein at the gel origin. The EDTA extract in sodium dodecylsulphate consists predominantly of those proteins in the ghost pattern with an apparent molecular weight in the region of 200000 together with one component in the 40000 region, as has previously been reported by Fairbanks *et al.*[29]. In preparations from the ghost residue after EDTA extraction these bands are diminished. The pattern of ghost proteins from the ox is quite similar to that of the human with the major exception that in the former the periodic acid Schiff-positive bands are found very near the gel origin coincident with bands staining only faintly with Coomassie blue.

Sodium dodecylsulphate electrophoresis has been used in its own right as a monitor for proteolysis, which is recognised as a serious hazard in the study of ghost proteins, during the various extraction procedures. In common with Fairbanks *et al.*[29] potential proteolysis was kept to a minimum by removing leucocyte membranes as carefully as possible, and by working at low ionic strength in the presence of EDTA. As a possible result of these precautions and also perhaps due to a destruction of the proteases by butanol, proteolysis is not a major factor in the present investigation. As stated above, the pattern of proteins in the gels of the butanol extract is indistinguishable from that of the freshly prepared ghost, and it does not change after storage at 4 °C in the presence of 0.01 % NaN_3 for days or even a few weeks. However, after about 1 week under these conditions a small amount of faster moving protein appears in some preparations of the EDTA extract.

Only a very limited understanding of the role of proteins in membrane structure and function is at present available. This communication has attempted to explore the diversity of proteins in membranes. Much effort is now expended in many laboratories on the chemical aspects of membrane proteins with the ultimate intention of elucidating their biological significance. Most of this work is carried out on isolated preparations of membranes not on intact, living membranes. It is always advisable to keep this distinction in mind. The problems of relating work on isolated preparations of membranes to the intact functional membranes of the living cell have been discussed in general terms elsewhere[33]. With specific reference to the ox ghosts used in the present study it can be pointed out that they are deficient in at least one component of the living membrane, *viz.* acetylcholinesterase[34]. Changes in membrane organisation during conversion of living membrane to ghost are not confined to the ox. Human ghosts which retain the ability to reseal effectively can only be prepared at a pH (6.0) which does not allow removal of haemoglobin[35], and further evidence for a rearrangement of the membrane is provided by the difference in reactivity of the membrane lipids towards acetic anhydride between the intact cell and isolated ghost[36]. One may feel that the chemical analysis of a multimolecular entity such as a ghost is sufficiently formidable a task without having to consider these further complications, but it is the existence of these, and even greater complexities, that distinguishes the biological from the chemical.

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REFERENCES

1 A. H. Maddy and P. G. Kelly, *Biochim. Biophys. Acta*, 241 (1971) 290.
2 A. H. Maddy, *Biochim. Biophys. Acta*, 117 (1966) 193.
3 A. H. Maddy and P. G. Kelly, *FEBS Lett.*, 8 (1970) 341.
4 R. F. A. Zwaal and L. L. M. van Deenen, *Biochim. Biophys. Acta*, 150 (1968) 323.
5 J. T. Dodge, C. Mitchell and D. J. Hanahan, *Arch. Biochem. Biophys.*, 100 (1963) 119.
6 A. H. Maddy and R. L. Spooner, *Vox Sang.*, 18 (1970) 34.
7 G. R. Bartlett, *J. Biol. Chem.*, 234 (1959) 466.
8 D. Aminoff, *Biochem. J.*, 81 (1961) 391.
9 R. Gatt and E. R. Berman, *Anal. Biochem.*, 15 (1966) 167.
10 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, 28 (1956) 350.
11 J. P. Johnston and A. G. Ogston, *Trans. Faraday Soc.*, 42 (1946) 789.
12 R. J. Winzler, in G. A. Jamieson and J. J. Greenwalt, *The Red Cell Membrane*, Lippincott, Philadelphia, 1969, p. 157.
13 G. F. Springer, Y. Nagai and H. Tegtmeyer, *Biochemistry*, 5 (1966) 3254.
14 T. K. Ray and G. V. Marinetti, *Biochim. Biophys. Acta*, 233 (1971) 787.
15 A. F. Rega, R. I. Weed, C. F. Reed, G. G. Berg and A. Rothstein, *Biochim. Biophys. Acta*, 147 (1967) 297.
16 N. B. Whittemore, N. C. Trabold, C. F. Reed and R. I. Weed, *Vox Sang.*, 17 (1969) 289.
17 A. H. Maddy and R. L. Spooner, *Immunology*, 21 (1971) 809.
18 A. H. Maddy and B. R. Malcolm, *Science*, 150 (1965) 1616.
19 D. Chapman, V. B. Kamat and R. J. Levene, *Science*, 160 (1968) 314.
20 A. H. Maddy, C. Huang and T. E. Thompson, *Fed. Proc.*, 25 (1966) 933.
21 R. F. A. Zwaal and L. L. M. van Deenen, *Chem. Phys. Lipids*, 4 (1970) 311.
22 R. J. Cherry, K. U. Berger and D. Chapman, *Biochem. Biophys. Res. Commun.*, 44 (1971) 644.
23 K. U. Berger, M. D. Barratt and V. B. Kamat, *Chem. Phys. Lipids*, 6 (1971) 351.
24 H. Schneider and I. C. P. Smith, *Biochim. Biophys. Acta*, 218 (1970) 73.
25 E. H. Eylar, M. A. Madoff, O. V. Broady and H. L. Oncley, *J. Biol. Chem.*, 237 (1962) 1992.
26 T. W. Tillack, S. L. Marchesi, V. T. Marchesi and L. Steers, *Biochim. Biophys. Acta*, 200 (1970) 125.
27 A. S. Rosenthal, F. M. Kregenouv and H. L. Moses, *Biochim. Biophys. Acta*, 254 (1970) 262.
28 A. J. Th. Hoogeveen, R. Juliano, J. Coleman and A. Rothstein, *J. Membrane Biol.*, 3 (1970) 156.
29 G. Fairbanks, T. L. Steck and D. F. H. Wallach, *Biochemistry*, 10 (1971) 2606.
30 J. A. Reynolds and H. Trayer, *J. Biol. Chem.*, 246 (1971) 7337.
31 A. H. Maddy, *Sub-Cell. Biochem.*, 1 (1972) 293.
32 J. Lenard, *Biochemistry*, 9 (1970) 1129.
33 A. H. Maddy, *Int. Rev. Cytol.*, 20 (1966) 1.
34 S. P. Burger, T. Fujii and D. J. Hanahan, *Biochemistry*, 7 (1968) 3682.
35 S. Lepke and H. Passow, *Biochim. Biophys. Acta*, 255 (1972) 696.
36 K. L. Carrway, D. Kobylka, J. Summers and C. A. Carraway, *Chem. Phys. Lipids*, 8 (1972) 65.