BBA 75462

ERYTHROCYTE MEMBRANE PROTEINS

THEIR STUDY USING AQUEOUS PYRIDINE SOLUTIONS

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(Received January 20th, 1970)

SUMMARY

1. A new method for the study of membrane proteins is described. It involves the use of aqueous pyridine which permits the separation of proteins of human erythrocyte membrane into two fractions: one water soluble and lipid free, the other a water-insoluble lipoprotein. Both fractions show multiple bands on gel electrophoresis and the band patterns of the two fractions and the intact membrane are similar. They suggest a close relationship of the proteins of the two fractions in spite of differences in lipid affinity. The sialoglycoprotein, containing the virus receptor activity, is present uniquely in the water-soluble fraction and its isolation is described.

2. The water-soluble proteins show a strong tendency for association. Such aggregation is promoted by certain salts. Aggregates of various sizes are still present in 33 % aqueous pyridine and they can be resolved and visualized by gel electrophoresis in 0.1 % sodium dodecyl sulfate as outlined by SHAPIRO *et al.*[22] into about twenty protein bands of a wide range of molecular weights. Certain proteins of the water-soluble fraction are not present as components of these aggregates. They include a sialoglycoprotein and three other proteins which can be visualized as distinct bands on polyacrylamide gels.

INTRODUCTION

Many laboratories are presently engaged in the investigation of membrane proteins from a variety of cells and subcellular organelles[1–13]. In these studies the nature of membrane components presents difficulties in solubility. Among the many approaches which have been suggested for solubilization of membrane proteins, detergents have been most widely used[1–7]. In general, conclusions derived from these studies suggest the presence of a heterogeneous population of proteins. Their affinity for each other and for the lipids of the membrane leads to problems in their further fractionation. These problems are yet to be solved in order to provide infor-

mation as to the number of proteins present, their relative quantities, chemical nature, functions and specific interactions.

In a search for other approaches to the study of membrane proteins we have used aqueous pyridine[14]. This solvent leads to solubilization of membrane components, and a facile separation of membrane proteins into two fractions, one water soluble and lipid free, the other a water-insoluble lipoprotein; each fraction shows multiple bands on polyacrylamide gel electrophoresis. Further studies of water-soluble proteins of the erythrocyte membrane are described in this communication. Their further fractionation leads to the isolation of a glycoprotein fraction probably identical with that previously obtained by phenol extraction[15–17].

MATERIALS AND METHODS

All chemicals used were reagent grade unless otherwise specified. Redistilled pyridine was used and ninhydrin was added to pyridine during distillation[18].

Preparation of erythrocyte membrane

Freshly drawn human blood was used and membranes were prepared by the method of DODGE *et al.*[19]. The cells were lysed in 8 mM sodium phosphate buffer (pH 7.6–7.8). Washes with this buffer were effected until the membranes appeared creamy yellow. They were then dialyzed for 24 h against several changes of distilled water to remove excess sodium phosphate buffer. They contained less than 0.1 % hemoglobin and their shape was intact when viewed under the phase microscope. Whenever possible, precautions were taken to prevent bacterial contamination. All buffers were sterilized and membranes were either solubilized rapidly in aqueous pyridine or stored in a screw-top tube, whose cap contained a filter paper pad soaked in toluene. Freezing was avoided as it led to a decreased resolution of membrane proteins on Sephadex chromatography as described below. Approx. 150 ml of membrane suspension containing about 0.5 mg nitrogen per ml (micro Kjeldahl nitrogen determination) were obtained per unit of blood.

Solubilization of membranes with pyridine and isolation of two protein fractions

To 1 vol. of membrane suspension in water at 4°, 0.5 vol. of ice cold redistilled pyridine containing 0.3 ml mercaptoethanol per 100 ml was added. The clear solution was immediately set to dialyze at 4° against a 10-fold volume of distilled water for 16 h to remove excess pyridine. Prolonged or exhaustive dialysis was avoided as this led to precipitation of all proteins. The turbid solution was centrifuged in a Spinco Model-L centrifuge for 45 min at 90000 × *g*, the clear supernatant set to dialyse against distilled water to remove residual pyridine (this led occasionally to precipitation of the proteins which are insoluble at pH values lower than 5–6; they could be brought back in solution by readjusting the pH to 7–8), and then reduced in volume by pressure dialysis (soluble protein fraction); the pellet was washed 3 times with distilled water (insoluble lipoprotein fraction).

Chromatography on Sephadex G-200 in 33 % aqueous pyridine 0.014 M mercaptoethanol

Sephadex G-200 (Pharmacia) was swollen in water and fines removed by decantation from water. The resin was suspended in 33 % aqueous pyridine containing

0.014 M mercaptoethanol. The columns, usually 2.2 cm × 70 cm, were packed and equilibrated with (2–3 vol.) 33 % pyridine–0.014 M mercaptoethanol. Pyridine and mercaptoethanol were added to the soluble fraction (final concn. 33 % pyridine, 0.014 M mercaptoethanol, protein concn. 5–10 mg/ml) and chromatographed at a rate of 10–15 ml/h. 2-ml fractions were collected and protein concentration monitored by a ninhydrin assay performed after alkaline hydrolysis[20]. Pyridine did not interfere with the assay. Successive fractions were pooled and various assays and electrophoresis on polyacrylamide gels performed after removal of pyridine by dialysis. For prolonged storage pooled fractions were kept in aqueous pyridine and aliquots dialyzed as needed. This prevented precipitation of protein and bacterial contamination.

Polyacrylamide-gel electrophoresis

Two electrophoretic systems were used; 6 % polyacrylamide gels run in 0.005 M Tris–0.04 M glycine buffer (pH 8.5) at 4 mA per gel for 3 h, according to the procedure of MAIZEL[21] and 5 % gels in 0.1 % sodium dodecyl sulfate–0.1 M sodium phosphate buffer (pH 7.1), at 8 V/cm for 8 h, as described by SHAPIRO *et al.*[22]. For the former system the soluble protein fraction and pooled zones (pyridine free) obtained from chromatography on Sephadex G-200 were used. Electrophoresis in sodium dodecyl sulfate was performed on aliquots of these fractions and in addition on aliquots of intact membrane and the insoluble lipoprotein fraction. As protein standards, immunoglobulin and bovine serum albumin were simultaneously subjected to electrophoresis. In the latter system the solutions were subjected to the following treatment prior to electrophoresis as suggested by SHAPIRO *et al.*[22]. They were incubated at pH 7.1 in 0.02 M phosphate buffer containing 1 % sodium dodecyl sulfate and 1 % mercaptoethanol for 3–5 h and then dialyzed for 16 h against 0.01 M phosphate buffer (pH 7.1) containing 0.1 % mercaptoethanol and 0.1 % sodium dodecyl sulfate. After electrophoresis gels were fixed in 20 % sulfosalicylic acid or 50 % trichloroacetic acid and stained in 0.25 % Coomassie Blue. In several instances parallel gels were stained with the Schiff stain for carbohydrate[23] as follows: gels were fixed in 15 % acetic acid for 24 h with frequent changes of acid, reacted 2 h with 0.4 % HIO_4, fixed again in 15 % acetic acid and stained with the Schiff reagent. For elution of protein bands from gels, bands were located by comparison with parallel stained gels and cut from gels which were frozen immediately after electrophoresis. The gel particles, in a test tube, were ground with a few ml of water and the supernatant hydrolyzed for amino acid analysis.

Immunological studies

Glycoprotein with virus receptor and blood group activity (virus receptor substance) was prepared by warm phenol extraction followed by a chloroform–methanol extraction as described earlier[16].

Antisera were prepared in rabbits. Protein fractions (virus receptor substance) and a protein fraction (Zone XIII), analogous to Zone VIII, obtained from another experiment, were incorporated in Freund's complete adjuvant and used for immunizations[24].

Immunoelectrophoretic analyses were performed in agarose gels according to the method of GRABAR AND BURTIN[23] as previously described[16].

Assays

Assays were performed on aliquots of intact membranes, their soluble and insoluble protein fractions, and aliquots of pooled fractions from Sephadex chromatography. Since intact membranes and the insoluble lipoprotein fractions were in suspension, when sampling problems were encountered the suspensions were redissolved in 33 % aqueous pyridine, aliquots sampled, pyridine evaporated in a ventilating oven and the assay performed.

Protein concentration was determined by a micro-Kjeldahl nitrogen determination, assuming an average nitrogen content of 16 %. Occasionally, the nitrogen content was computed from amino acid composition.

Ninhydrin assay was performed by the method of ROSEN[25]; when a prior alkaline hydrolysis was performed, the procedure outlined by FRUCHTER AND CRESTFIELD[20] was used.

N-Acetylneuraminic acid was determined by the method of WARREN[26]. A Cary Model 14 recording spectrophotometer was used to determine the concentration of the chromophore at 549 mμ. Intact membranes and insoluble lipoprotein fractions showed a chromophore at 530 mμ, even after an extraction step with isoamyl alcohol.

Amino acid and hexosamine analyses were performed on a Spinco Model 120 C automatic amino analyzer[27]. Protein was hydrolyzed in 6 M HCl for 22 h at 105°, *in vacuo*. A correction factor was applied to estimate the amounts of hexosamines under these prolonged conditions of hydrolysis. Performic acid oxidations were carried out according to the procedure of MOORE[28], for the determination of cysteic acid.

Ultracentrifugation studies and intrinsic viscosity determinations

Sedimentation velocity experiments were performed at about 20° in a Spinco Model-E ultracentrifuge at 59 780 rev./min. Sedimentation coefficients were corrected to $s_{20,w}$. Conditions under which sedimentation was preformed are indicated in Table I. Various agents were added to a concentrated solution of protein in water to yield the final concentrations indicated. Final concentration of protein was 2–3 mg/ml. In one set of conditions the protein solution was dialyzed overnight against 0.1 M sodium phosphate buffer of the indicated pH values.

Viscosity measurements were carried out in Ostwald–Fenske (Series B) viscometers at 26.8°.

RESULTS

Properties of the two protein fractions obtained from the erythrocyte membrane by aqueous pyridine fractionation

The chemical composition of the intact erythrocyte membrane and the derived protein fractions was described previously[14]. About 40% of the protein of the membrane is in the soluble fraction and the insoluble fraction contains the remaining protein; their lipid composition is, however, strikingly different; the soluble protein fraction contains a small percentage of total cholesterol and phospholipid, whereas the insoluble lipoprotein fraction is greatly enriched in these components. The distribution of carbohydrates between the two fractions is of interest. Neutral hexoses are more abundant in the insoluble lipoproteins, whereas the sialic acid

is found exclusively in the soluble protein fraction. Thus, the glycoproteins containing sialic acid are present exclusively in the soluble fraction; whether the other carbohydrates are associated with other proteins or lipid components remains to be determined. No meaningful differences are noted in the amino acid contents of the two fractions.

In Fig. 1 are shown patterns obtained upon electrophoresis of intact erythrocyte membranes and its protein fractions on 5 % polyacrylamide gels, in 0.1 % sodium dodecyl sulfate at pH 7.1. It should be noted that in this system the mobilities of protein bands are related to their molecular weights, as shown by SHAPIRO *et al.*[22].

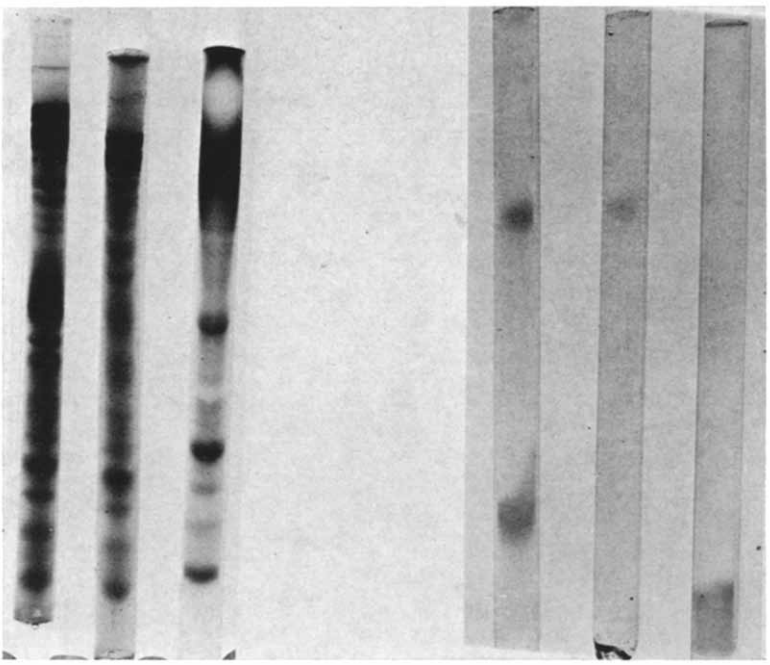

Fig. 1. Electrophoresis of intact erythrocyte membrane and its protein fractions on 5 % polyacrylamide gels in 0.1 % sodium dodecyl sulfate (pH 7.1). Left to right: stained with Coomassie Blue: intact membranes, water-soluble fraction, insoluble lipoprotein fraction; stained with Schiff stain: intact membrane, water-soluble fraction, insoluble lipoprotein fraction (mobilities of former two gels slightly slower).

Since the treatment before electrophoresis includes exposure to 1 % sodium dodecyl sulfate and 1 % mercaptoethanol for 3–5 h at 37° certain bands may consist of subunits resulting from cleavage of disulfide bonds, dissociation by sodium dodecyl sulfate, or by reassociation during electrophoresis[22]. The examination of the pattern obtained upon electrophoresis of the intact membrane shows many protein bands of a wide range of molecular weights (from about 200 000 or more, to 25 000 as determined with appropriate protein markers) (Fig. 1). The soluble protein fraction also shows many bands, many of which correspond to those found in the intact membrane. Bands are seen in the insoluble lipoprotein fraction which appear similar to those found in the intact membrane and/or the soluble protein fraction. When parallel gels are stained with Schiff reagent for carbohydrates, a glycoprotein band corre-

sponding to molecular weight of approx. 160000 is seen in intact membranes. A corresponding band is seen in the soluble fraction but not in the insoluble lipoprotein fraction. A Schiff-positive band which moves close to the front can be seen in intact membrane and the insoluble lipoprotein fractions. This band appears to have no corresponding protein band. It should be pointed out that similarities of patterns obtained in the intact membranes and the derived protein fractions indicate that treatment of membranes with pyridine does not lead to an alteration in the properties of proteins of the membrane responsible for electrophoretic mobility under the conditions studied.

TABLE I

PHYSICAL PROPERTIES OF PROTEINS OF THE SOLUBLE FRACTION

Intrinsic viscosity [η] in water, 0.44 and in 0.1 M sodium phosphate buffer (pH 7.0), 0.01.

Conditions	$s_{20,w}$				*Remarks*
	Peak I	*Peak II*	*Peak III*	*Peak IV*	
In water	3.5	5.7	—	—	—
In 0.08 M sodium phosphate buffer, pH 7.0	4.9	6.9	15.1	—	—
In 0.1 M sodium phosphate buffer (dialyzed overnight against buffer), pH 7.0	4.7	—	—	—	Precipitate
pH 9.0	4.7	—	—	—	Precipitate
pH 12.0	4.3	—	—	—	Precipitate
In 8 M urea–0.16 M mercaptoethanol, 48 h	10	49	—	—	—
98 h	8.5	30	—	—	—
2 weeks	8.5	30	—	—	—
33 % aqueous pyridine	1.8	2.4	3.6	6.5	
+ 0.1 M NaCl + 0.1 % acetic acid	—	—	—	—	Fast-moving peak >15 S
+ 0.1 M cysteine·HCl	—	—	—	—	Precipitate
+ 0.1 M cysteine	1.7	4.4	15.2	—	—
+ 0.05 M EDTA	2.6	4.9	8.9	—	—
+ 0.5 M KI	2.6	5.6	15.4	—	—

Studies of proteins of the soluble fraction

Some physical properties of water-soluble proteins. Examination of the proteins of this fraction in the analytical ultracentrifuge revealed that they easily aggregate and that aggregation is augmented by certain salt or urea solutions. In Table I are listed the sedimentation coefficients obtained under various conditions of sedimentation. It should be noted that, as expected, 33 % aqueous pyridine solutions lead to dissociation, but even in this solvent addition of salts promotes aggregation or precipitation of the proteins.

The relatively low value of intrinsic viscosity determined in 0.1 M sodium phosphate buffer (pH 7.0) suggests that aggregates found under these conditions are apparently globular and of low axial ratio.

Chromatography of the proteins of the water-soluble fraction on Sephadex G-200 in 33 % aqueous pyridine

The noted effect of pyridine solutions on dissociation of proteins of this fraction suggested the continued use of this solvent for their further fractionation. In Fig. 2 is shown the protein elution profile obtained upon chromatography of this fraction on Sephadex G-200 in 33 % aqueous pyridine. For comparison the peak positions of elution of dextran (mol. wt. 2 000 000) and trypsin (mol. wt. 24 000) when chromatographed under identical conditions are shown. It is apparent that under the conditions used about 8 % of total proteins are considerably retarded (Zones VI–IX). The contents of protein and sialic acid, of various zones are shown in Table II. No significant differences in amino acid contents are found in Zones I–V and their overall composition resembles that of the intact water-soluble fraction; only in Zones VI–VIII the contents of several amino acids and particularly of aspartic acid, serine, threonine and leucine, as well as the hexosamines are significantly different (Table III).

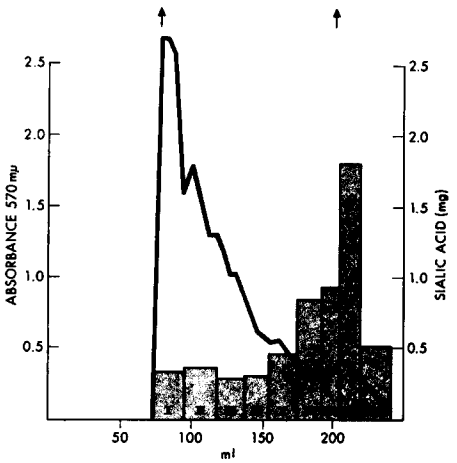

Fig. 2. Chromatography of proteins of water-soluble fractions on Sephadex G-200 in 33 % aqueous pyridine – 0.014 M mercaptoethanol. Column size 2.2 cm × 70 cm; rate 12 ml/h; at room temperature, 4 ml per fraction; —, ninhydrin after alkaline hydrolysis; shaded areas indicate amount of sialic acid in each zone; arrows indicate position of elution of dextran (left) and trypsin (right).

These zones are also enriched in sialic acid. This suggests that the sialic acid-bearing glycoproteins of the membrane are present in these zones and are separated from the bulk of the other proteins.

It can be concluded from the elution profile obtained that the size of proteins of the water-soluble fraction, in 33 % aqueous pyridine, varies from 200 000 or higher to 25 000, and that the sialic acid-containing glycoproteins are the only protein species which can as yet be distinguished by their amino acid and carbohydrate contents.

When the soluble protein fraction obtained from previously frozen membranes was chromatographed under identical conditions, the elution profile and analysis of pooled zones indicated a decreased degree of resolution in that most of the protein was present as one high-molecular-weight aggregate. This suggests that freezing of membranes may lead to protein aggregation which is irreversible under the conditions used.

TABLE II

COMPOSITION OF ZONES OBTAINED FROM CHROMATOGRAPHY OF WATER-SOLUBLE PROTEINS ON SEPHADEX G-200 COLUMN

Zone	*Protein* (%)	*Sialic acid* (%)	*mg sialic acid per mg protein*
I	32.5	5.5	0.005
II	26.5	6.2	0.006
III	19.8	4.7	0.007
IV	6.9	5.2	0.021
V	6.3	7.7	0.034
VI	2.6	14.6	0.159
VII	2.6	16.0	0.174
VIII	2.7	31.4	0.327
IX	<1	8.6	—
Intact membranes	—	—	0.025
Intact water-soluble fraction	—	—	0.050

TABLE III

AMINO ACID COMPOSITION OF GLYCOPROTEIN FRACTIONS

Values are expressed as residues per 1000 residues. n.d., not determined.

Amino acid	*Intact water-soluble protein fraction*	*Zone VI*	*Zone VII*	*Zone VIII*	*Band A Zone VIII*	*Zone XIII* Column V*	*Glycoproteins isolated by phenol extraction*		
							*Ref. 29***	*Ref. 37*	*Ref. 17*
Lys	54	44	36	40	51	42	36	24	45
His	23	39	30	32	43	33	38	30	39
Arg	52	47	45	43	53	45	40	41	42
Cysteic acid	11	n.d.	n.d.	n.d.	n.d.	n.d.	—	—	—
Asp	103	86	80	78	67	85	59	76	63
Thr	60	76	87	88	105	99	138	127	103
Ser	73	92	110	127	159	133	135	135	98
Glu	161	189	145	162	116	137	100	121	82
Pro	50	60	74	68	77	80	66	69	73
Gly	65	68	60	48	41	52	67	67	56
Ala	98	78	73	58	46	65	67	78	72
Val	70	67	70	92	95	75	77	81	75
Met	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	14	14	47
Ile	45	45	44	42	63	50	44	67	55
Leu	113	113	78	80	56	64	45	69	89
Tyr	17	23	22	23	11	22	36	13	28
Phe	28	31	22	21	17	19	35	28	33
GlcN	9	28	51	48	55	44	—	—	—
GalN	11	55	110	112	163	124	—	—	—

* Glycoprotein fraction (obtained from another experiment) used in immunological studies.
** Recalculated from data in this reference.

Polyacrylamide-gel electrophoresis of pooled zones obtained from chromatography on Sephadex G-200 in 33% aqueous pyridine

Electrophoretic patterns of consecutive pooled zones obtained from Sephadex columns are shown in Figs. 3, 4 and 5. In Fig. 3 electrophoresis was performed in 6% gels, in Tris–glycine buffer (pH 8.5), and no pretreatment was applied; in Fig. 4,

0.1 % sodium dodecyl sulfate was present in buffers and the samples were pretreated with mercaptoethanol and sodium dodecyl sulfate as outlined. As expected, the patterns obtained in these two systems differ as they most likely reflect different degrees of dissociation or aggregation of proteins during the two conditions of electrophoresis. Patterns in both gel systems reveal that Zones I–V contain protein aggregates of decreasing molecular weight sizes. Upon penetration into gels the aggregates show very similar band patterns. Thus proceeding from Zones I–V there is an increasing penetration into 6 % gels as seen by a decreasing amount of protein in spacer gels; in 5 % sodium dodecyl sulfate gels the band on top of gels and the cluster of bands immediately below decrease in intensity. (Note gels of Zones IV and V in Fig. 5, where protein bands are better visualized*.) In Zones VI–VIII a different band pattern becomes apparent showing two intensely staining Bands A and C, and Bands

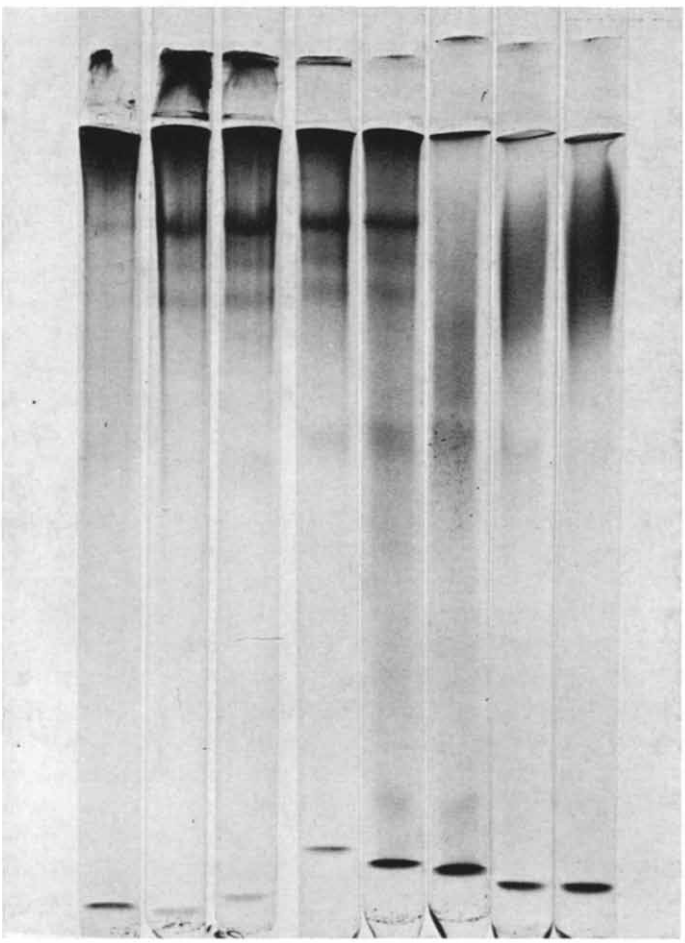

Fig. 3. Electrophoresis of Zones I–VIII obtained from Sephadex column on 6% polyacrylamide gels in Tris–glycine buffer (pH 8.5). Left to right: Zones I–VIII.

* These gels were fixed in 50% trichloroacetic acid.

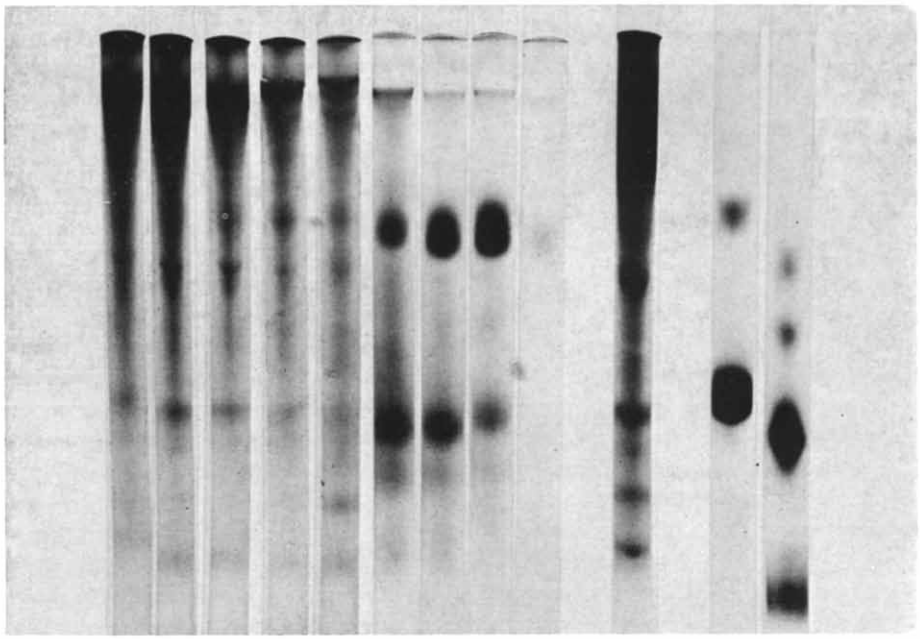

Fig. 4. Electrophoresis of Zones I–IX obtained from the Sephadex column on 5 % polyacrylamide gels in 0.1 % sodium dodecyl sulfate (pH 7.1). Left to right: Zones I–IX; intact water-soluble fraction; protein markers: bovine serum albumin, immunoglobulin.

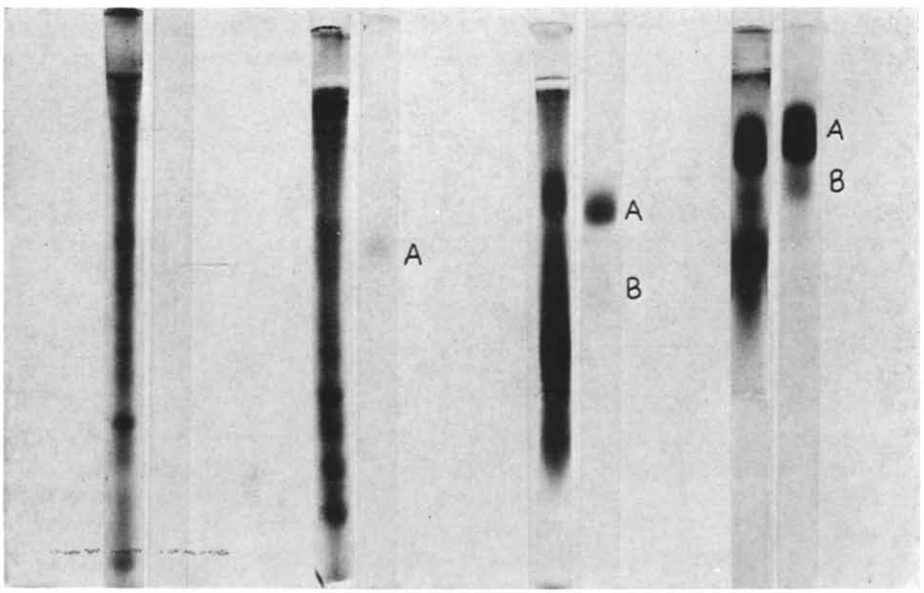

Fig. 5. Electrophoresis of Zones IV, V, VI and VIII on 5 % polyacrylamide gels in 0.1 % sodium dodecyl sulfate (pH 7.1). Parallel gels stained with Coomassie Blue and Schiff stain. Left to right: Zones IV, V, VI and VIII.

B, D, E of lesser intensity (Figs. 5 and 6). Parallel gels stained for carbohydrate show that only Bands A and B of Zones VI–VIII are glycoproteins; Zone V contains only a trace of glycoprotein and it is absent in the earlier zones. The amino acid composition of protein in Band A was determined after elution from gels. As shown in Table III its glycoprotein nature is confirmed by a high content of serine, threonine and hexosamines. Proteins in Bands C, D and E await further investigation.

An estimation of the molecular weight of proteins in Bands A and C, compared to appropriate standards yields a value of 160000 for the glycoprotein and 60000 for the protein in Band C. The glycoprotein of Band B has a molecular weight of about 80000 and may represent a smaller aggregate of the 160000 mol. wt. glycoprotein[30]. Its amount is much smaller than that of glycoprotein in Band A, as evidenced by the intensity of Schiff stain in Zones VI and VIII (see Fig. 5)*.

The similarity of electrophoretic gel patterns of Zones I–V in both gel systems suggests that proteins of these zones form aggregates in pyridine which can be further resolved by polyacrylamide-gel electrophoresis. This is mostly apparent when electrophoresis is performed in sodium dodecyl sulfate and the proteins are pretreated with this reagent. Under these conditions dissociation into proteins of a wide range of molecular weights occurs. The sialic acid-bearing glycoproteins are considerably retarded on Sephadex G-200 suggesting a molecular weight of about 25000 in 33% aqueous pyridine; their molecular weight upon electrophoresis in

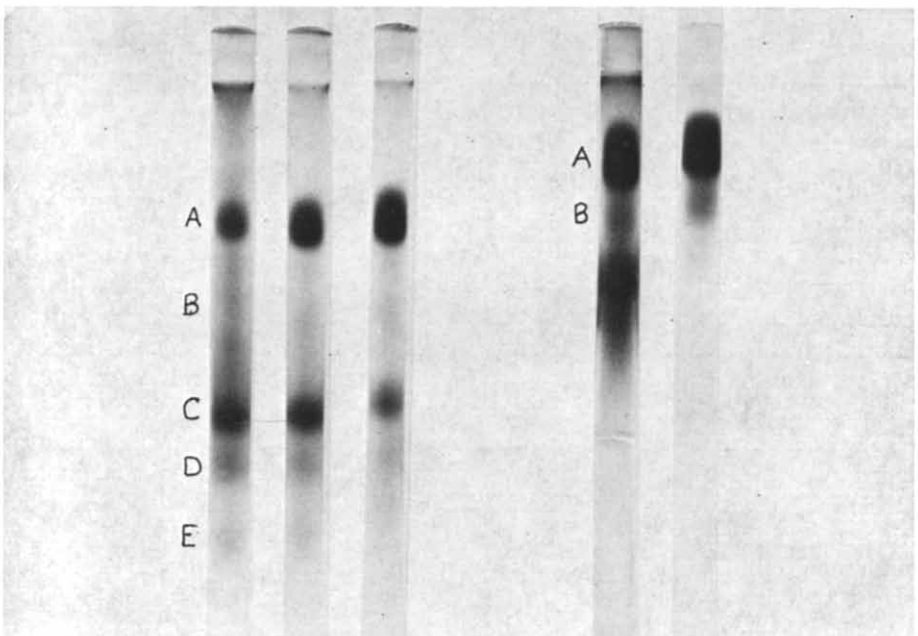

Fig. 6. Proteins which are retarded upon chromatography of soluble proteins on Sephadex G-200 in 33% aqueous pyridine visualized by electrophoresis of Zones VI–VIII on 5% polyacrylamide gels in 0.1% sodium dodecyl sulfate (pH 7.1). Left to right: Zones VI, VII, VIII; zone VIII with parallel gel stained with Schiff reagent (shorter time of electrophoresis; Bands C, D, E not resolved and present in band below Band B).

* It is likely that because of its low concentration this glycoprotein cannot be visualized either in Zone V or in the intact membrane or the soluble protein fraction (see Fig. 1).

sodium dodecyl sulfate gels was estimated to be 80 000–160 000. It appears that in the case of these proteins conditions used in electrophoresis cause aggregation.

Properties of the isolated glycoprotein fractions

The sialic acid-bearing glycoproteins emerge in Zones VI–VIII as evidenced by their contents of sialic acid, serine, threonine and the hexosamines, and by protein bands, seen on polyacrylamide gels, which stain with the Schiff reagent. The sialic acid and amino acid composition of these fractions strongly suggest that the glycoprotein they contain is related to those containing the MN antigens and virus receptor substance previously isolated from the erythrocyte membrane by phenol extraction[15–17]. This is shown in Table III where the amino acid analyses of glycoproteins isolated by phenol extraction are included for comparison. The close relationship of these glycoproteins to those isolated by phenol extraction was further confirmed by immunological studies. By double diffusion in agarose gels, as well as by cross diffusion and counterelectrophoresis using both antisera to virus receptor substance and to the glycoprotein-containing column fraction (Zone XIII) (see Table III for amino acid content) identity was demonstrable between glycoprotein of Zone VIII on the one hand and the virus receptor substance on the other. In contrast, the earlier column fractions (Zones I and II) which failed to stain by the Schiff reaction and had a low sialic acid content, also failed to react with antisera to either the virus receptor substance or Zone XIII. Further results of these immunochemical investigations are to be reported in detail in a subsequent communication.

DISCUSSION

In this communication another approach to the investigation of membrane proteins is presented. Aqueous pyridine was selected for this study for the following reasons: it is a weakly basic solvent, of both lipophilic and hydrophilic properties, which is completely miscible with water. As shown earlier, it leads to the solubilization of membrane components and permits a separation of membrane proteins into two fractions, which differ in their contents of lipid, and exhibit differences in water solubility. The fact that about 40 % of membrane proteins become water soluble and lipid free facilitates their further characterization.

Aqueous pyridine solutions have previously been used to dissociate aggregating proteins such as insulin[31] and tobacco mosaic virus protein[32] into subunits. No deletorious effects of aqueous pyridine treatment on these proteins have been noted. In this study it can be assumed that pyridine disrupts the membrane lipoprotein structure and causes protein dissociation without alteration of the proteins present. The fact that intact membranes and the derived protein fractions yield similar polyacrylamide-gel patterns reinforces this assumption.

Close to twenty protein bands with a wide range of molecular weights can be seen during electrophoresis of intact membranes on polyacrylamide gels in 0.1 % sodium dodecyl sulfate. Other investigators have similarly observed multiple protein bands during electrophoresis of erythrocyte and other membranes on polyacrylamide gels and several have concluded that a heterogeneous population of proteins exist in membranes[1–3,10,33,34]. In this study, electrophoresis in sodium dodecyl sulfate gels

aids in the visualization of the proteins in both the water-soluble (lipid-free) and more insoluble lipoprotein fractions. They show some similarities in band patterns even though their affinity for lipid appears to differ greatly. The reasons for the distribution of proteins between the two fractions and the nature of proteins present will require further study; however, the exclusive presence of the sialic acid glycoproteins in the soluble protein fraction leads to the conclusion that some of the proteins are uniquely distributed.

Such selective water solubility of membrane proteins has been reported by others. ROSENBERG AND GUIDOTTI[1] solubilized 50% of erythrocyte membrane proteins by extraction with EDTA and NaCl. Similarly, HOWE *et al.*[16], MARCHESI AND STEERS[9], BURGER *et al.*[35] and HARRIS[36] obtained a fraction of erythrocyte membrane proteins in a soluble form, and water solubility of the sialic acid-bearing glycoproteins led to their early partial characterization[17,29,37]. NEVILLE[11] reported solubilization of 70% of liver plasma membrane proteins in 0.05 M K_2CO_3. In a study using 33% aqueous pyridine as outlined here, SIMON *et al.*[10] solubilized 50% of liver plasma membrane proteins in a lipid-free form and showed that these proteins show polyacrylamide-gel band patterns similar to those solubilized with 0.05 M K_2CO_3. Both protein fractions show bands similar to those of intact membranes[10]. It is probable that in other membrane systems, as well, certain proteins are more water soluble and exhibit a lesser affinity for lipid.

The ease of aggregation of membrane proteins in aqueous solutions has been pointed out previously[8,12,30,33,38]. In this study the examination of proteins of the aqueous fraction in the ultracentrifuge revealed that aggregation was promoted by solutions of salts such as NaCl, sodium phosphate and agents such as urea. A similar effect of KCl on the aggregation of erythrocyte membrane proteins was noted by MAZIA AND RUBY[38]. In 33% aqueous pyridine solutions dissociation of protein aggregates was observed, but even here salts promoted aggregation. These observations are not usually found with most globular proteins and point out a somewhat unique property of membrane proteins. As a result of these observations, salt solutions were excluded from all operational procedures involving fractionation of membrane proteins. It was not determined here what effect divalent cations might have on these proteins. BURGER *et al.*[35], MARCHESI AND STEERS[9] and ROSENBERG AND GUIDOTTI[1] found that removal of these cations by EDTA leads to solubilization of certain membrane proteins.

Previous freezing of membranes or lyophilization of protein fractions resulted in increased aggregation as evidenced by loss of resolution during chromatography on Sephadex columns in aqueous pyridine or gel electrophoresis. A similar effect on the proteolipid protein of myelin was reported[39].

The observed effects of aqueous pyridine were used for further dissociation of the proteins of the water-soluble fraction. Their chromatography on Sephadex G-200 in aqueous pyridine led to the separation of several protein fractions which could be further studied by analysis of various components present and by polyacrylamide-gel electrophoresis. It appears that about 90% of the proteins still form aggregates in 33% aqueous pyridine solutions, some of which can be visualized on polyacrylamide gels as distinct bands of a wide range of molecular weights. Whether these bands represent multiple heterogeneous proteins, are subunits of a few proteins or are multiple units of a few proteins which aggregate into specific

arrays of various sizes awaits further investigation. Recent electron microscopic evidence of HARRIS[36] suggests that such arrays might indeed be present.

The remaining proteins of this fraction are not part of these aggregates as evidenced by their retardation on Sephadex and by different amino acid compositions and band patterns on gel electrophoresis. They are sialoglycoprotein and proteins in Band C, and perhaps D and E. Their tendency for self-aggregation is seen on electrophoresis in 6 % gels where they appear as a single band; however, they can be dissociated by electrophoresis in sodium dodecyl sulfate gels.

The close relationship of the glycoproteins in Bands A and most likely B to the glycoproteins exhibiting the MN antigenic activity and containing the influenza virus receptor sites is evidenced by similarities in amino acid and carbohydrate contents and the immunological studies. The high carbohydrate content of these glycoproteins may, in part, account for their exclusion from the bulk of the protein aggregate and their solubility in aqueous solutions following phenol extraction. Their tendency to aggregate, which has been noted here, has been pointed out by SPRINGER[30] who reported molecular weight sizes ranging from 31 000 to 12 000 000.

Other investigators have attempted to fractionate membrane proteins and have obtained protein fractions which show differences in water solubility, lipid, carbohydrate and amino acid contents[1]. It is clear from this investigation and others that membranes contain a heterogeneous mixture of proteins which exhibit unique structural properties which lead to specific aggregation. These properties need to be elucidated so that their further fractionation can be successfully achieved and the interrelationship and the specificity of their organization understood.

ACKNOWLEDGMENTS

This work was supported by grants form the National Institutes of Health, U.S. Public Health Service Grants No. GM-16389, GM-5998, AM-19435, AM-05821, HD-00674 and AI-03168.

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