Biochimica et Biophysica Acta, 311 (1973) 576–593

BBA 76372

RECOMBINATION OF HUMAN RED CELL MEMBRANE PROTEIN FRACTIONS WITH HOMOLOGOUS LIPIDS*

MOSHE BARZILAY[a], ELEONORA CONDREA[a], EITAN BEN-DAVID[b] and ANDRE DE VRIES[a]

[a] *Rogoff-Wellcome Medical Research Institute and* [b] *Department of Pathology, Tel-Aviv University Medical School, Beilinson Hospital, Petah Tikva (Israel)*

(Received February 1st, 1973)

SUMMARY

Human red cell membrane proteins were separated from membrane lipids by butanol–water partition, solubilized in sodium dodecyl sulfate and fractionated by gel filtration on a Sepharose 4B column. The eluates were pooled in two fractions: Fraction I containing the high molecular weight proteins, around 200000, and Fraction II comprising the proteins which migrate as 100000 and less.

Fractions I and II had different sialic acid content and pH-dependent solubility. Both protein fractions recombined with red cell lipids when mixtures in sodium dodecyl sulfate were dialyzed against aqueous buffers. The amount and composition of recombinate obtained at various pH values were different for the two fractions. The recombinates obtained at pH 3.6 from Fraction I and from Fraction II had different density and stability towards change in pH and ionic strength. The recombinates of both fractions exhibited a membrane-like structure on electron microscopy.

INTRODUCTION

The red cell membrane is a stable structure held together by protein–lipid, lipid–lipid and protein–protein interactions[1]. It has been shown that following dissociation of the membrane its proteins are able to recombine with lipids in a variety of experimental conditions, forming membrane-like aggregates[2–4].

In the present study human red cell membrane proteins have been separated by gel filtration into two fractions and their recombination with membrane lipids, achieved by dialysis from sodium dodecyl sulfate solutions, has been examined.

MATERIALS AND METHODS

Sodium lauryl sulfate was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. and used without further purification. Acrylamide, *N,N'*-methylenediacrylamide and *N,N,N',N'*-tetramethylethylenediamine were purchased from Fluka A.G., Buchs SG, Switzerland. Coomassie brilliant blue R and *N*-acetylneuraminic acid were obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A.

* Submitted by M. Barzilay in partial fulfilment of the requirements for a Ph.D. degree, Tel Aviv University.

All other chemicals and organic solvents were reagent grade and obtained from E. Merck A. G., Darmstadt, Germany.

Isolation of red blood cell membranes

Ghosts were prepared according to the method of Hoogeveen *et al.*[5] from human blood stored in acid–citrate–dextrose for 22–23 days performing a series of hemolyses at room temperature with decreasing osmolarity using 60, 30 and 20 mosM NaCl–phosphate buffer, pH 7.2, containing 1 mM EDTA. For preparation of large quantities of membranes, the cells were hemolysed with 30 vol. of 60 mosM buffer and centrifuged at 20000 × *g* in a Szent-Gyorgi and Blum continuous flow apparatus (Sorvall). The process was repeated with 30 and 20 mosM buffers. Care was taken to discard the red pellet formed under the lightly packed ghosts.

Extraction of membrane proteins

Membrane proteins were extracted by butanol–water partition according to the method of Maddy[6], as modified by Rega *et al.*[7]. By this procedure most of the lipids are retained in the butanol phase while the proteins are dissolved in the water phase. Since effective butanol extraction requires a salt-free membrane preparation to minimize the butanol–water interface, the ghosts were washed once with bidistilled water prior to the butanol partition. The water phase, containing the membrane protein, was dialysed against cold bidistilled water to remove traces of butanol.

Separation of membrane proteins

The membrane proteins were precipitated (100%) isoelectrically at pH 4.5. The protein precipitate was collected by centrifugation at 500 × *g* for 10 min, suspended in 3% sodium dodecyl sulfate solution containing 1 mM EDTA at pH 7, to a final protein concentration of 10 mg/ml[8], and incubated overnight at 37 °C for complete solubilization. The proteins (150–220 mg) were fractionated by gel filtration on a column (140 cm × 2 cm) of Sepharose 4B equilibrated with 1% sodium dodecyl sulfate and 1 mM EDTA (pH 7.0), at a flow rate of 4.5 ml/h. 3-ml fractions were collected and protein was estimated by absorption at 280 nm in a Unicam SP800 A spectrophotometer.

Preparation of protein solutions

The column eluates were pooled in two protein fractions, as described in Results (Fig. 1). The sodium dodecyl sulfate was removed from the fractions by dialysis against bidistilled water prior to concentration by ultrafiltration through an Amicon cell model 52 with Diaflo UM-2 ultrafilter. The protein fractions were adjusted to a protein concentration of 2 mg/ml and stored in 1% sodium dodecyl sulfate at −20 °C.

Preparation of lipid solutions

The butanol phase, obtained by butanol–water partition of the red blood cell ghosts, was evaporated to dryness under a N_2 stream. Lipids were reextracted in a mixture of chloroform–methanol (2:1, v/v), washed with NaCl solution according to Folch *et al.*[9] and stored in chloroform–methanol at −20 °C. For the recombination experiments the organic solvents were removed under N_2 and the lipids were suspended in buffer of the desired pH by shaking with glass beads, or they were dissolved in 1% sodium dodecyl sulfate. Lipid phosphorus was assayed and the solutions were adjusted to 2 mg phospholipid/ml.

Recombination experiments

Mixtures of protein and lipids were made up so as to contain in 2 ml of 1% sodium dodecyl sulfate, 2 mg protein and an amount of lipid phosphorus corresponding to 2 mg phospholipid. The mixtures, as well as single lipid and protein solutions of the same concentration, were dialyzed for 72 h at various pH values against 200 vol. of buffer with 3 changes of 24 h each. The dialyses were carried out at pH 3.6, 5.0 and 6.0 against 0.05 M sodium citrate–citric acid buffer, and at pH 7.5 against 0.05 M Tris–HCl buffer. Removal of labeled sodium dodecyl sulfate by dialysis has been found satisfactory by Rottem *et al.*[10] and Terry *et al.*[11]. Using the test of Mukerjee[12], as modified by Reynolds and Tanford[13], we failed to detect any sodium dodecyl sulfate in the last dialysis change. The insoluble material appearing in the dialysis bags was sedimented at $20000 \times g$ for 15 min and the supernatants were assayed for protein and lipid phosphorus. The amounts sedimented were calculated by subtraction and expressed as percentage of the total lipid and protein contents of the dialysis bag.

Effects of pH and ionic strength

The sediments obtained by dialysis of sodium dodecyl sulfate as described above were resuspended in 3-ml buffer solutions of various pH values with or without addition of NaCl to final concentrations of 1 and 5 M. The suspensions were thoroughly mixed and left overnight at 4 °C. The material was sedimented at $20000 \times g$ for 15 min and the supernatants assayed for protein and lipid phosphorus.

Density gradient analysis

The sediments obtained from the sodium dodecyl sulfate solutions were suspended in 2 ml of 0.05 M sodium citrate–citric acid buffer (pH 3.6) and layered over 28 ml of a linear sucrose gradient (13–50% (w/v) sucrose in the same buffer). The gradients were centrifuged at 34000 rev./min in an SW 35 rotor of a Spinco Model L ultracentrifuge for 18 h at 4 °C. 2-ml fractions were collected by puncturing the bottom of the centrifuge tubes. Densities were calculated from refractometer readings of fractions collected from a gradient which was over-layered with buffer only. Since sucrose interferes with the phosphorus determinations, the samples were dialyzed against bidistilled water prior to protein and lipid phosphorus assays.

Polyacrylamide gel electrophoresis

Sample preparations and application were performed according to Weber and Osborn[14] and polyacrylamide gel was prepared according to Maizel[15]. Gels contained 5% acrylamide, 0.13% *N,N'*-methylenediacrylamide, 0.1 M sodium phosphate buffer (pH 7.0), 0.1% sodium dodecyl sulfate, 0.05% *N,N,N',N'*-tetramethylethylenediamine and 0.1% ammonium persulfate. Samples of up to 100 μg protein were loaded on the gels and run at 8 mA per tube untill the Bromophenol blue marker approached the bottom (approx. 3.5 h). The gels were fixed overnight in a mixture of methanol–water–acetic acid (5:5:1, v/v/v), stained for 1 h in the same mixture containing 0.25% Coomassie brilliant blue, and destained by washing with a methanol–water–acetic acid mixture (2:35:3, v/v/v).

Electron microscopy

Samples of recombinate and of isolated protein were taken from the sucrose

gradient and prepared for electron microscopy essentially as described by Zahler and Weibel[2], except for replacing the veronal–acetate buffer (pH 7.4) by 0.05 M sodium citrate–citric acid buffer at pH 3.6. The sections were examined and photographed in a Phillips EM 300 electron microscope.

Chemical determinations

Protein was measured by the method of Lowry *et al.*[16] with bovine serum albumin (Armour Pharmaceutical Company) as standard. Sialic acid was determined by the thiobarbituric acid method of Warren[17] with *N*-acetylneuraminic acid as standard. Lipids were extracted according to Marinetti *et al.*[18], lipid phosphorus was determined by the method of Bartlett[19] and phospholipid was estimated by multiplying the lipid phosphorus content by 25. Sodium dodecyl sulfate was assayed by the methylene blue method of Mukerjee[12], as modified by Reynolds and Tanford[13].

RESULTS

Fractionation of membrane proteins

Gel filtration of the delipidated membrane proteins dissolved in sodium dodecyl sulfate through a Sepharose 4B column achieved separation of the proteins in two main peaks (Fig. 1). Gel electrophoresis of individial samples showed fractionation of the proteins according to molecular weight. We have repeatedly observed that the protein band corresponding to the Component IV of Steck *et al.*[20] starts to elute from the column before the band corresponding to the Component III in the same nomenclature. This apparent discrepancy confirms the reported abnormal migration of the sialoglycoprotein present in the Component III of Steck *et al.*[20] on sodium dodecyl sulfate–acrylamide gel electrophoresis[21,22].

The proteins were pooled in two separate fractions: Fraction I comprising the first elution peak and including mainly high molecular weight proteins (around 200000[21,23,24]), and Fraction II containing the middle and low molecular weight proteins (around 100000 and less[21,23,24]) eluted with the second peak. To sharpen the separation, a small number of intermediate fractions containing both high and low molecular weight proteins were discarded, so that the resulting two pools presented complementary gel patterns. The hemoglobin-containing fractions eluted at the tail of the column were also discarded.

Characterization of protein Fractions I and II

The protein Fractions I and II were characterized by a different sialic acid content, most of the ghost sialic acid being recovered in protein Fraction II (Table I). The electrophoretic patterns of the two fractions showed that the high molecular weight doublet known as spectrin[25], tektin[26] or the Bands I and II of Steck *et al.*[20] was present in the protein Fraction I. The electrophoretic pattern and the high sialic acid content or Fraction II indicate that it also contained, in addition to low molecular weight proteins, proteins which migrate with an apparent molecular weight around 100000, including the main sialoglycoprotein[21,22]. The presence of residual phospholipid in Fraction II (Table I) might be accounted for by saturation of the water phase with lipid-rich butanol during the procedure of membrane solubilization[7]. Such a lipid would elute at the end of the column, thus contaminating Fraction II.

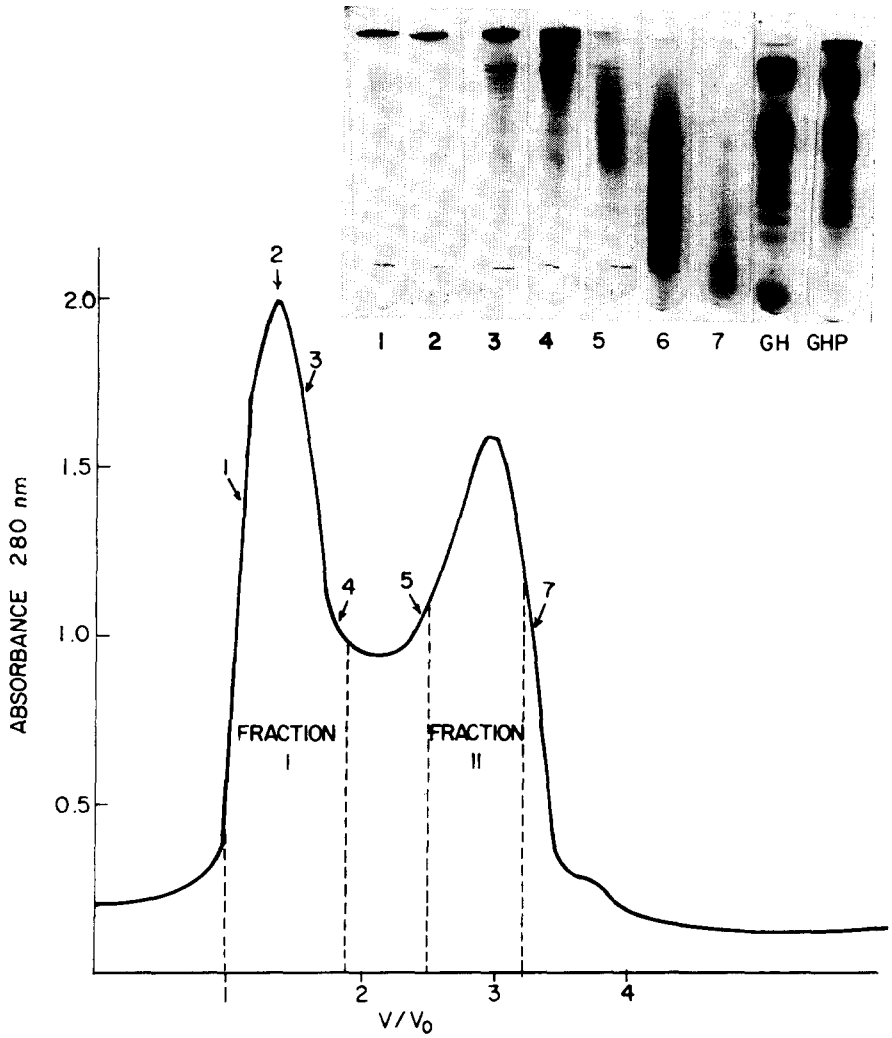


Fig. 1. Gel filtration chromatography of red cell membrane proteins in 1% sodium dodecyl sulfate on Sepharose 4B. For details see Materials and Methods. Arrows indicate samples analyzed by acrylamide gel electrophoresis. Of the total protein applied (190 mg), 43% was recovered in Fraction I and 21% in Fraction II. GH, ghosts; GHP, ghost protein.

When sodium dodecyl sulfate was removed from the protein Fractions I and II solutions by dialysis against buffers of various pH values, the two fractions exhibited a different pH-dependent solubility (Fig. 2). While Fraction I was completely insoluble between pH 3.6 and 5.0, in agreement with the isoelectric precipitation area of the total ghost proteins[3], Fraction II was largely soluble at this pH range.

Recombination of protein fractions with lipids by dialysis from mixtures in sodium dodecyl sulfate

Attempts were made to recombine in aqueous buffers the protein fractions,

which had been freed of sodium dodecyl sulfate by dialysis, with lipids at pH values at which the protein was largely soluble. Regardless of the phospholipid–protein ratios used, no recombination was achieved, probably due to the negative charge of both the lipid and protein[3]. In subsequent experiments it was found that recombina-

TABLE I

SIALIC ACID AND PHOSPHOLIPID CONTENT OF THE RED CELL MEMBRANE PROTEIN FRACTIONS

The numbers in parentheses represent means ± S.E. of 5 individual separations.

Fraction I		*Fraction II*	
Sialic acid (μmoles/mg protein)	*Phospholipid (μg/mg protein)*	*Sialic acid (μmoles/mg protein)*	*Phospholipid (μg/mg protein)*
0.0208	18	0.2456	150
0.0201	2	0.2776	63
0.0165	20	0.1930	172
0.0162	0	0.2200	83
0.0126	26	0.2556	114
(0.0172 ± 0.0033)	(13 ± 12)	(0.2384 ± 0.0327)	(116 ± 49)

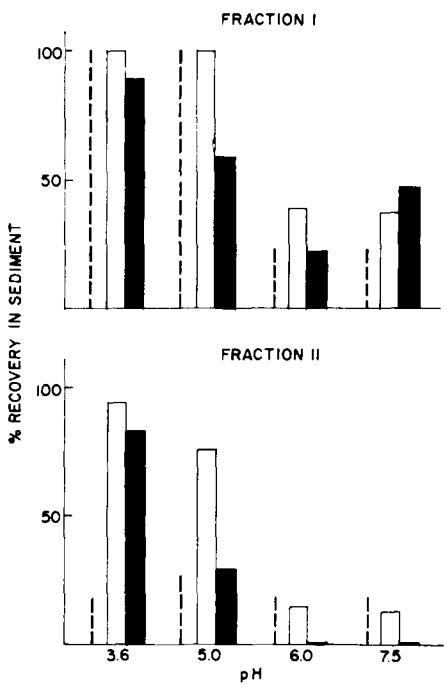


Fig. 2. Sedimentation of membrane protein fractions with lipids as function of pH. Solutions of protein and lipid in sodium dodecyl sulfate, alone or in 1:1 (w/w) mixtures, were dialyzed against buffers at various pH, as described in Materials and Methods. Open bars, protein in the sediment of mixtures; shaded bars, phospholipid in sediment of mixtures; interrupted line, protein alone. Lipid when alone was completely recovered in the supernatant at all pH values.

tion could be attained when protein–lipid mixtures in sodium dodecyl sulfate were dialysed.

Effect of pH. Protein and lipid solution in 1% sodium dodecyl sulfate were mixed in a 1:1 ratio (w/w) and the sodium dodecyl sulfate was removed by dialysis against buffers at various pH values (Fig. 2). Sample of lipid and protein alone were treated similarly. Insoluble material which accumulated at the end of the dialysis was sedimented by a brief centrifugation (see Materials and Methods). Lipid alone did not sediment at any of the pH values tested. The protein fractions alone behaved as described in the previous paragraph.

Removal of sodium dodecyl sulfate from the lipid–protein mixtures resulted in the formation of insoluble material whose amount and composition varied as a function of pH. Density gradient centrifugation and electron microscopical observation showed (see below) that the insoluble material represented protein–lipid recombinates. Although recombination of lipid and protein as soluble lipoprotein might have occurred, we chose to study the insoluble recombinates in view of their possible relevance to membrane reassembly.

As seen in Fig. 2, the two protein Fractions I and II showed a different capacity of binding lipid in insoluble form at varying pH values. While the sediments obtained with Fraction I contained lipid at all pH values studied, Fraction II was unable to bind lipid at pH 6.0 and 7.5. Furthermore, the amounts of protein Fraction I which sedimented in combination with lipids followed closely, at all pH values tested, the precipitation pattern of the protein alone. On the other hand, the ability of Fraction II to recombine with lipids did not correlate with its precipitation pattern when alone, as illustrated by the results obtained at pH 3.6.

The recombinates obtained at pH 3.6 from Fractions I and II were similar in both protein content and phospholipid/protein ratio (0.89). At pH 7.5, however, protein Fraction I showed the highest lipid binding capacity per weight (phospholipid/protein ratio 1.24) while protein Fraction II did not recombine at all. Aliquots of the sediments obtained by dialysis from protein alone and from protein–lipid mixtures were compared with undialysed protein fraction by acrylamide gel electrophoresis. The resulting gel patterns were essentially similar and did not reveal a selective enrichment in any of the protein bands in the sediments.

Effect of Mg^{2+}. Addition of 20 mM Mg^{2+} as $MgCl_2$ in the dialysis buffer caused an increase in the amount of insoluble material (Fig. 3). Mg^{2+}, by promoting the precipitation of protein Fraction II with lipid at pH 7.5, minimized the difference between the lipid-binding capacity of the two protein fractions.

The solubility as a function of pH of protein and lipid when dialyzed separately was changed by the presence of Mg^{2+}. The protein Fraction II became almost insoluble in the pH range 3.6 to 5.0, and thus the two protein fractions acquired a similar solubility profile (Fig. 3).

The lipid, when alone, flocculated and was recovered in the sediments at pH 3.6 and 7.5 (Fig. 3). Thus, the sedimentation of protein and lipid, when dialyzed from mixtures, did not differ much from that when dialyzed separately. Since Mg^{2+} was not an absolute requirement for protein–lipid recombination and indeed mitigated the difference in lipid-binding capacity of the two protein fractions, the sediments obtained in the presence of Mg^{2+} were not further studied.

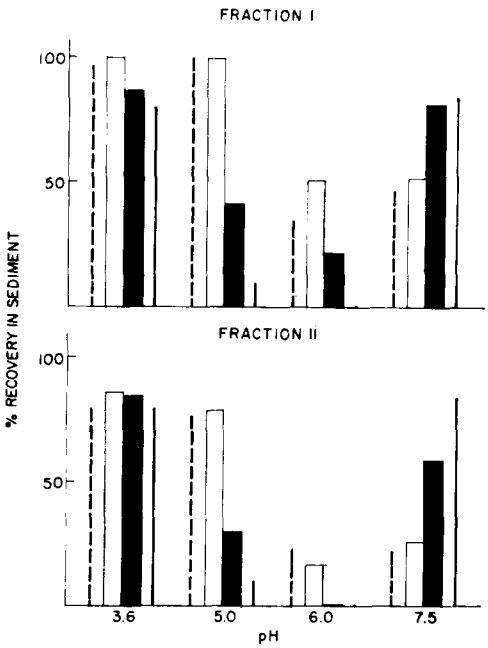


Fig. 3. Sedimentation of membrane protein fractions with lipids in presence of Mg^{2+}. Conditions as in Fig. 2, except for addition of 20 mM Mg^{2+} in the dialysis buffers. Open bars, protein in the sediment of mixtures; shaded bars, phospholipid in the sediment of mixtures; interrupted line, protein alone; solid line, phospholipid alone.

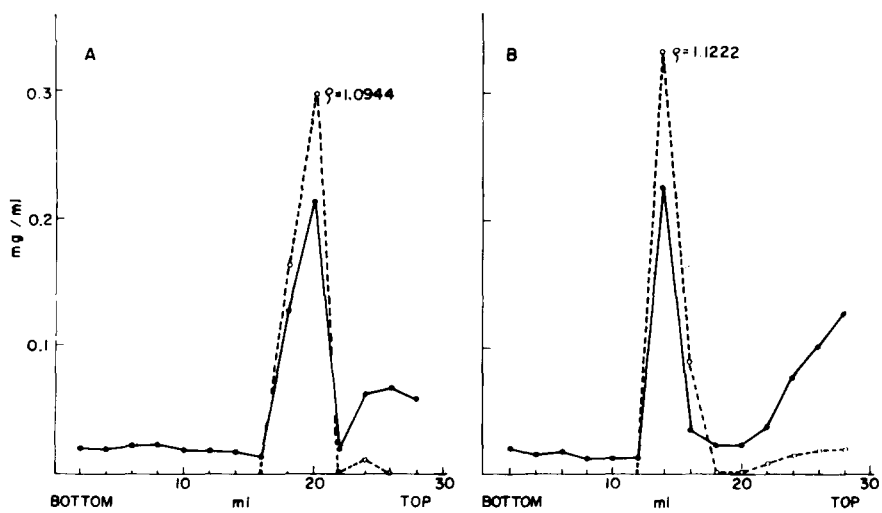


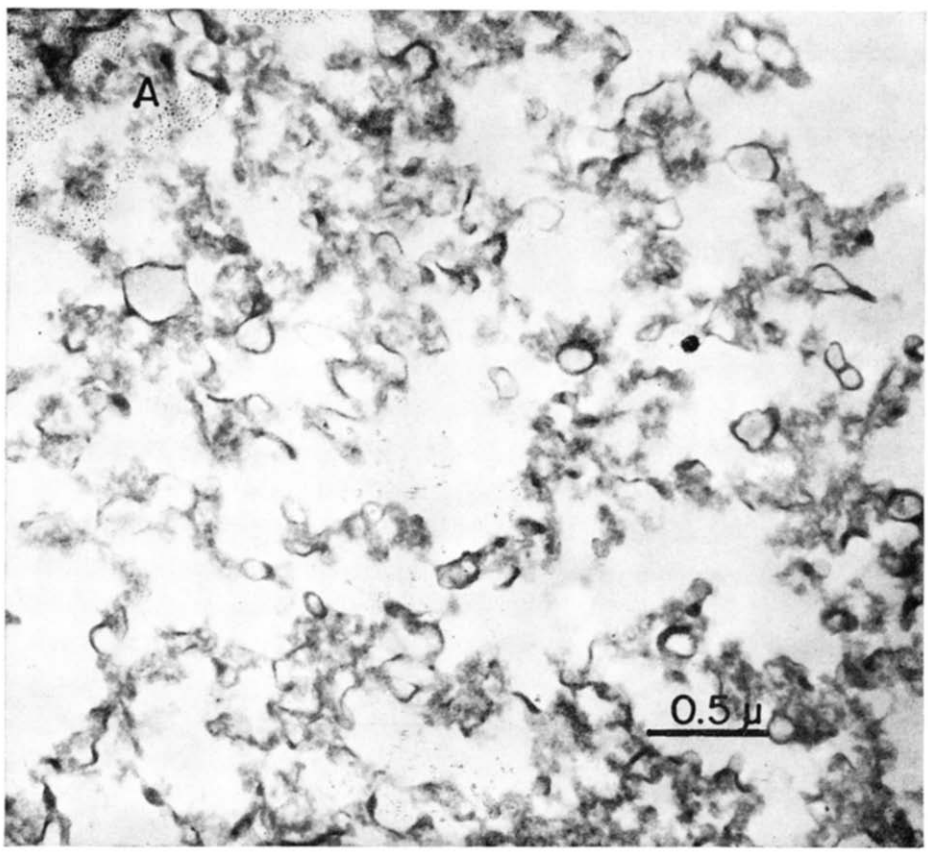
Fig. 4. Equilibrium centrifugation of pH 3.6 recombinates in a sucrose gradient. For details see text. A, recombinate of protein Fraction I with lipids; B, recombinate of protein Fraction II with lipids. ○---○, protein; ●—●, phospholipid.

Density gradient centrifugation of the pH 3.6 recombinates

We chose to study further those recombinates which were obtained by dialysis of mixtures of Fraction I or of Fraction II with lipid against pH 3.6 buffer in absence of Mg^{2+}. At this pH both protein fractions recombined almost completely with lipid. The fact that neither lipid alone nor protein Fraction II alone sedimented at this pH suggested that the massive coprecipitation from the lipid–protein mixtures was the result of lipid–protein recombination. In order to ascertain further that recombination of protein with lipids did occur, the sediments obtained at pH 3.6 were centrifuged at equilibrium in a sucrose gradient (Fig. 4). The precipitate of Fraction I with lipid showed a narrow band at density 1.0944, and a small amount of lipid-rich lipoprotein floating on top. The precipitate of Fraction II with lipid gave a slightly wider band whose center was at density 1.1222 and a larger amount of lipid-rich lipoprotein on top of the gradient (Fig. 4). Following separate density gradient centrifugation of protein Fractions I and II and of lipid, both proteins were recovered from the bottom of the gradient while the lipid from the top.

Morphology of the recombinates

Electron microscopy of the pH 3.6 recombinates recovered from the density gradient bands showed the presence of predominantly membranous particles and of



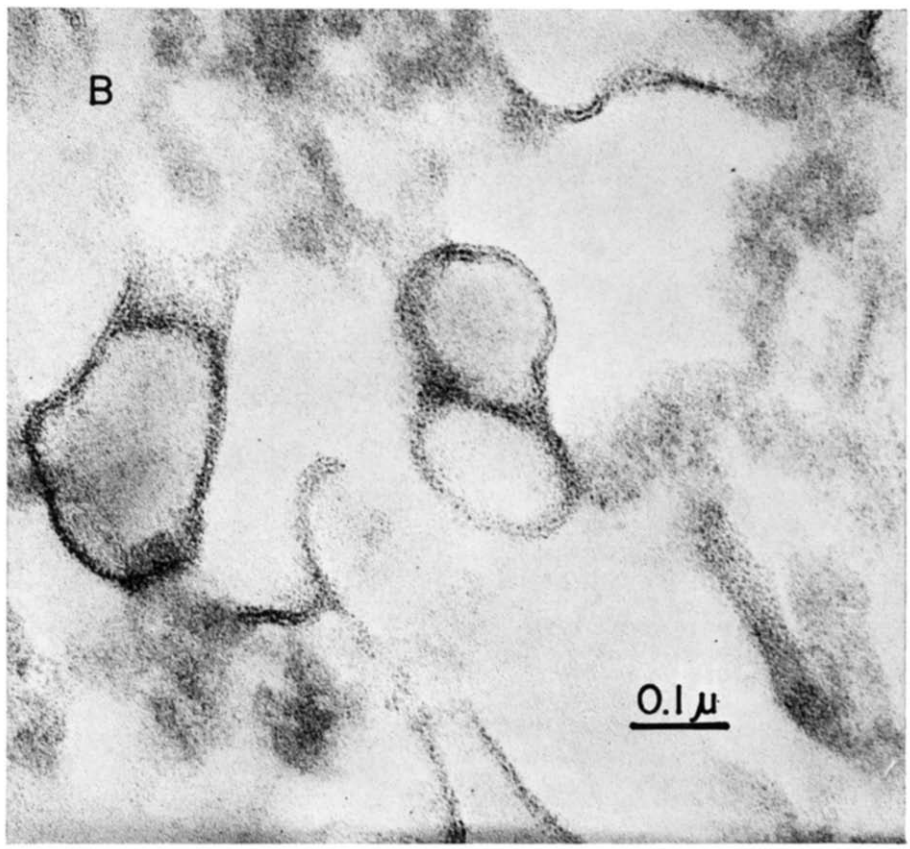


Fig. 5. Electron micrograph of the pH 3.6 recombinate obtained with protein Fraction I. Sample corresponding to the peak in Fig. 4A. A, ×32800; B, ×128000.

some amorphous material (Figs 5A and 6A). The membranous material which exhibited the typical trilaminar structure appeared both as closed vesicles and as open-ended threads. The latter were sometimes free, sometimes extending directly from a closed vesicle. While most of the membranous material consisted of a single unit membrane, close apposition of two or more unit membranes could be observed. There was no essential difference in the morphology of the recombinates obtained from protein Fractions I or II with lipids (Figs 5B and 6B). The distance separating the two lines of a unit membrane was 39.29 ± 3.04 Å in the recombinate of Fraction I and 40.63 ± 3.34 Å in the recombinate of Fraction II. In the intact ghosts the distance was 32.03 ± 4.00 Å. The values represent means $\pm$S.E. of 8 measurements.

When the pH 3.6 recombinates were examined directly without prior separation on sucrose gradient, free lipid appeared as typical myelin figures (Fig. 7) which, in agreement with the lipid assay of the gradient fractions (Fig. 4), were more abundant in the recombinate of protein Fraction II. Both protein Fractions I and II, in absence of lipids, appeared as amorphous material (Fig. 8).

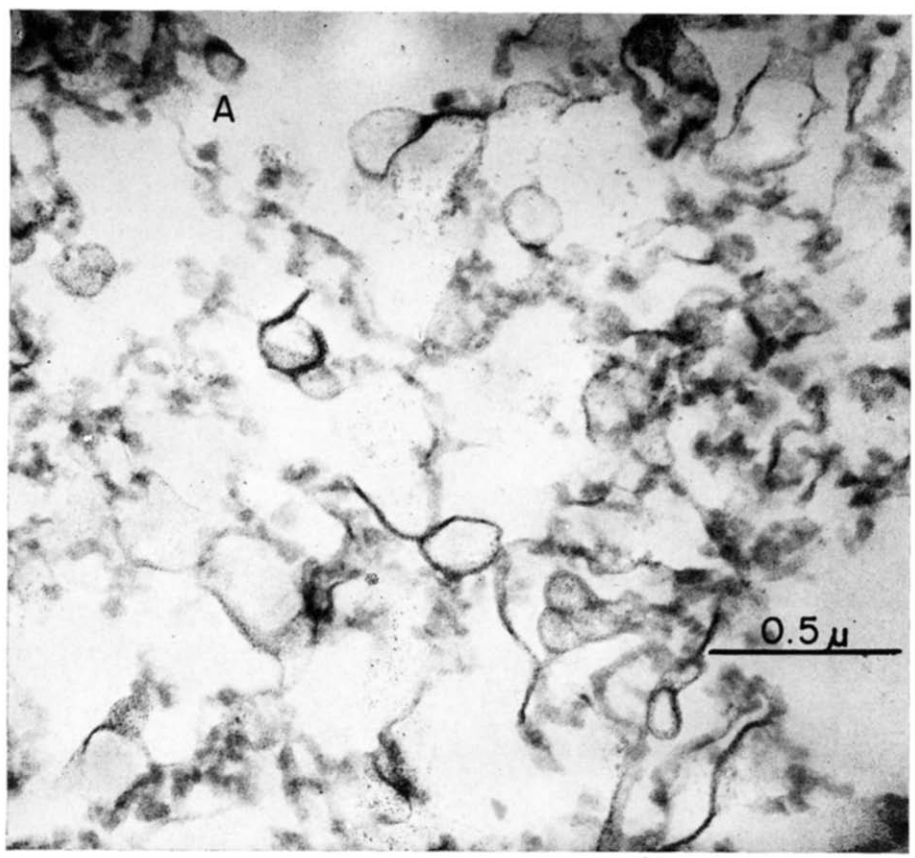
Effect of pH and ionic strength on the stability of pH 3.6 recombinates

The recombinate of the protein Fraction I with lipid obtained at pH 3.6 in absence of Mg^{2+} was markedly stable towards both a change in pH and an increase in ionic strength (Fig. 9). Although there was little initial recombination of Fraction I with lipid at pH 6.0 and 7.5, the recombinate obtained at pH 3.6 could be brought to these pH values without appreciable dissociation. At pH 7.5, however, some protein was lost from the recombinate resulting in an inversion of the phospholipid/protein ratios. This inversion was also apparent in all recombinates directly formed at this pH (Fig. 2). The pH 3.6 recombinate was stable when the ionic strength of the medium was increased by addition of 1 M and 5 M NaCl (Fig. 9).

The recombinate of protein Fraction II with lipid obtained at pH 3.6 was less stable than that obtained from Fraction I, both when transferred to pH 7.5 and when subjected to an increase in ionic strength, the latter resulting in an important loss of lipid (Fig. 9).

DISCUSSION

The protein–lipid associations in native red blood cell membranes are stabilized by both ionic and hydrophobic interactions[27]. When recombination of dissociated



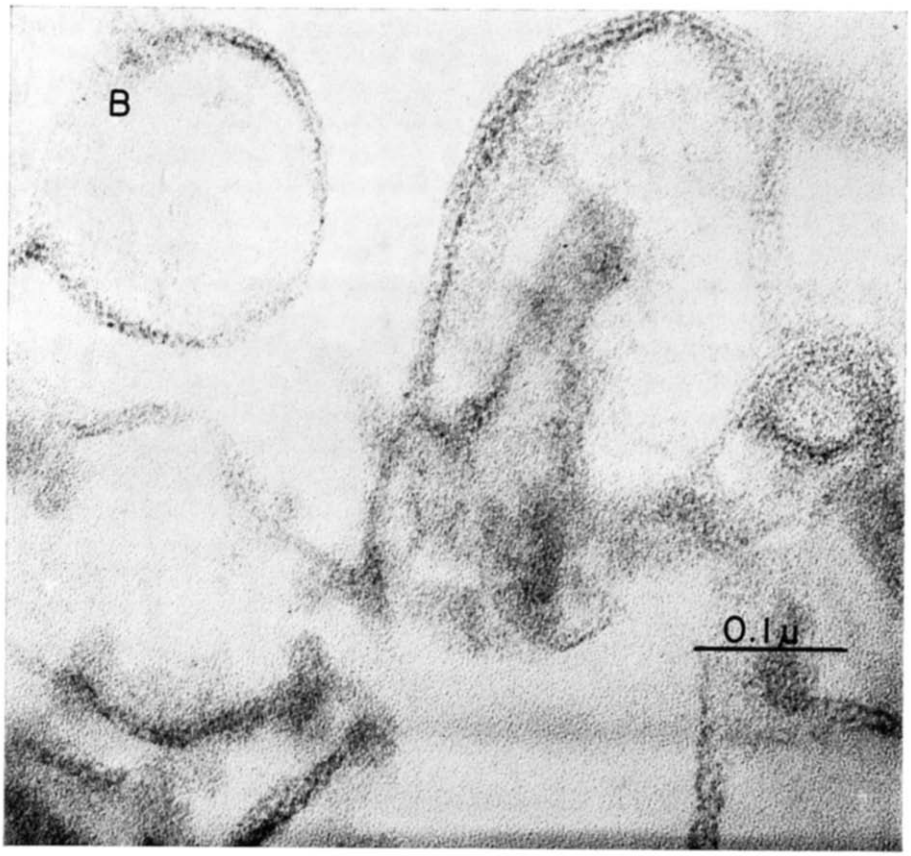


Fig. 6. Electron micrograph of the pH 3.6 recombinate obtained with protein Fraction II. Sample corresponding to the peak in Fig. 4B. A, ×51040; B, ×200640.

membrane components is carried out directly in aqueous media, it appears that electrostatic attraction between the charged proteins and lipids is required for their initial contact prior to the establishment of hydrophobic associations[3]. Since, because of thermodynamic requirements, phospholipids and proteins adopt conformations of minimal free energy with the hydrophobic regions internalized and the polar or ionic regions exposed in aqueous media, the importance of surface charge is obvious.

However, the presence of opposite charges on protein and lipid is not the single requisite for their association; both the red cell membrane protein spectrin and albumin fail to interact with positively charged liposomes at neutral pH[28]. Although in this case the condition of opposite charge is fulfilled, the conformation of the protein at this pH seems unfavorable to the interaction[28].

In the particular case of the proteins of the red cell membrane components, at pH values at which they are soluble they carry the same charge sign as the lipids and recombination does not occur. Indeed, Zwaal and Van Deenen[3], Zahler and Weibel[2] and we ourselves failed to obtain in aqueous media recombination between membrane lipids and proteins prepared by either butanol–water partition or 2-chloro-

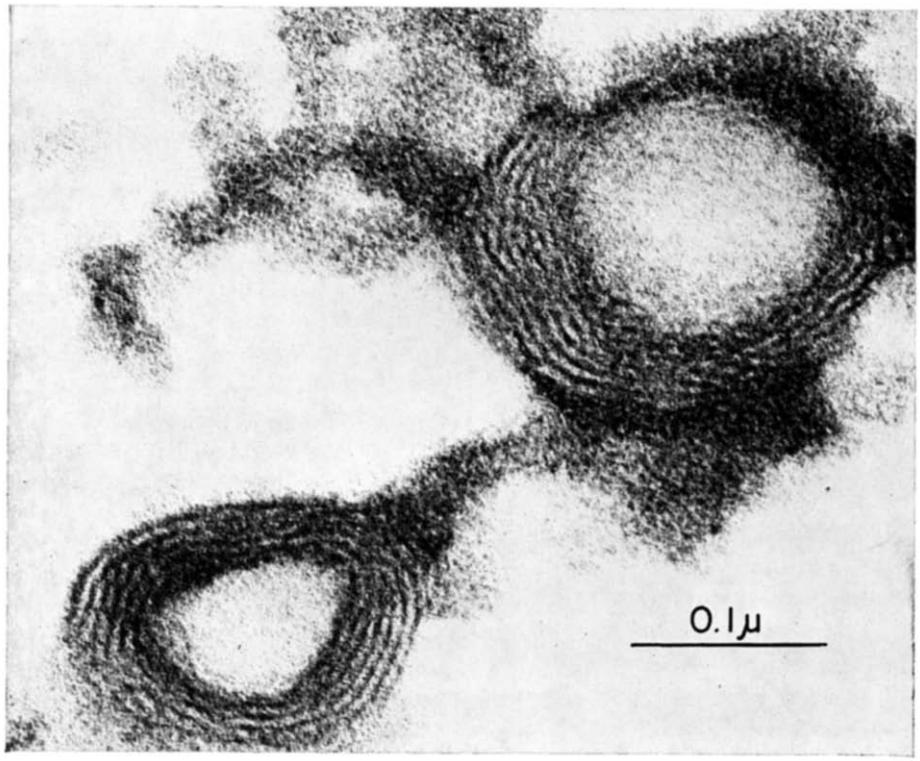


Fig. 7. Myelin forms indicating the presence of free lipid in the recombinate obtained with Fraction II not subjected to density gradient centrifugation. × 255360.

ethanol solubilization. Zwaal and Van Deenen[3] overcame this difficulty by recombining the lipid with oppositely charged protein from which sialic acids had been removed. Zahler and Weibel[2] showed recombination, without prior chemical modification of the protein, to occur when mixtures in 2-chloroethanol of the components were subjected to dialysis and the organic solvent was gradually replaced by aqueous buffers. In the view of these authors[2], the dissociating effect of 2-chloroethanol on both lipid and protein aggregates favored their contact and interaction.

The procedure used at present for recombining membrane lipids and proteins by dialysis from sodium dodecyl sulfate solutions resembles that of Zahler and Weibel[2] in that the components are brought together in a dissociated state. Sodium dodecyl sulfate is known to dissociate proteins and convert them into rod-like helices or pairs of helices complexed with sodium dodecyl sulfate molecules and carrying a uniformly negative charge[13,29]. By its denaturing effect, sodium dodecyl sulfate may bring hydrophobic residues to the surface of the protein and thus promote association with lipid[30]. Sodium dodecyl sulfate has been used for the dissociation and reassembly of mycoplasma membrane[10,11]. However, while reaggregation of mycoplasma components required the presence of Mg^{2+}, that of the red cell membrane components did not.

Protein–lipid recombination experiments fall in one of two categories: (a) re-

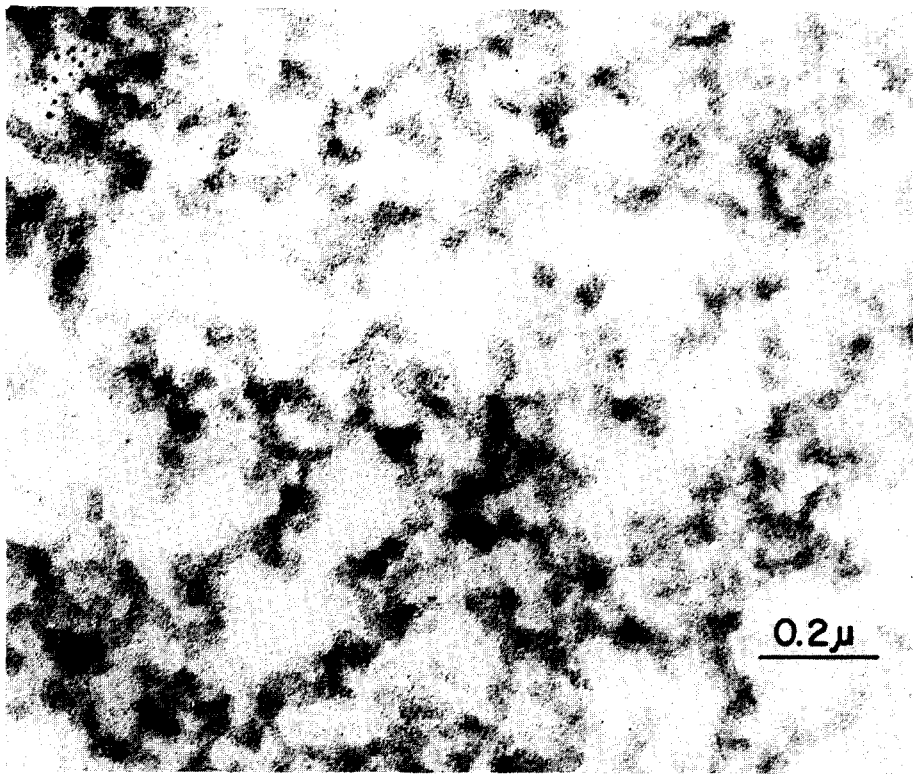


Fig. 8. Protein Fraction II. ×82080.

combination of aggregated protein and lamellar lipid in aqueous media, in which case opposite surface charges of the components are a necessary though not sufficient requisite; (b) recombination between depolymerized components which might have undergone shape changes and have become able to recombine regardless of charge sign.

Recently, Schubert *et al.*[4] reported on the preparation of a red blood cell membrane protein fraction by means of concentrated acetic acid. Since this fraction, redissolved at high pH, recombined with membrane lipids in aqueous buffer over a wide range of conditions, these authors concluded that opposite charges of proteins and lipids are not essential for the initial interaction. However, since concentrated acetic acid is a strong depolymerising agent and the exposure of the protein to high pH (9–10) at the time of lipid addition is known to induce conformational changes which favor lipid binding[31], the experiments of Schubert *et al.*[4] might fall into the second category mentioned above. Thus, their results would not be relevant to the essentiality of surface charge for recombination.

We have studied the recombination of lipid with each of two protein pools obtained from the red cell membrane. Electron microscopical examination of the recombinates formed at pH 3.6 from both Fractions I and II showed reassembly of a membrane-like structure to occur. Our data show that, using protein–lipid mixtures of a fixed ratio, the recombinates obtained from both protein Fractions I and II

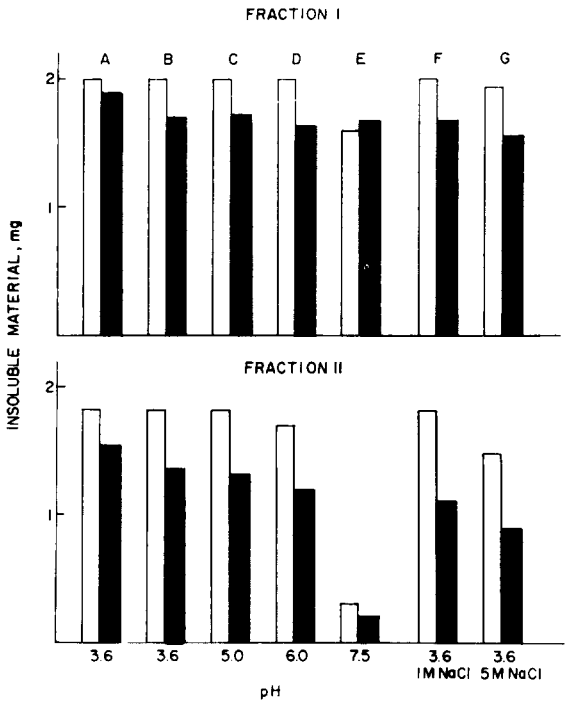


Fig. 9. Effects of pH and ionic strength on the stability of the recombinates obtained at pH 3.6. For details see text. A, recombinate obtained at pH 3.6; B, C, D, E, resuspension of Recombinate A at pH 3.6, 5.0, 6.0 and 7.5, respectively; F and G, resuspension of Recombinate A at pH 3.6 in presence of 1 and 5 M NaCl, respectively. Open bars, protein in sediment; shaded bars, phospholipid in sediment.

varied in amount and composition as a function of pH. A similar variation was found by Zwaal and Van Deenen[3] within the pH range 3.7–4.3. In contradistinction, in the experiments of Zahler and Weibel[2] and of Schubert *et al.*[4], variation in pH between 5 and 7 did not produce systematic differences in the amount of insoluble protein appearing in the recombinates. Thus, the pH dependence of the recombination process seems to vary with the method of protein preparation.

The two red cell membrane protein fractions studied by us were found to differ in the following properties: (a) pH-dependent solubility. While Fraction I behaved similarly to the total membrane protein, Fraction II was largely soluble within the pH range of 3.6–7.5. The total precipitation of unfractionated membrane proteins in the pH range 3.5–5.0 suggests that coprecipitation of Fraction II proteins occurred. The solubility of Fraction II proteins at pH 3.6–5.0 may result from their isoelectric point being more acidic, in agreement with their high sialic acid content.

(b) Yield and composition of recombinates as a function of pH. The overall lipid binding capacity of Fraction I was greater than that of Fraction II, especially in the pH 6.0–7.5 range. Comparison of the recombinates obtained at pH 3.6 revealed a difference in homogeneity and a difference in density, reflecting a different lipid/protein ratio.

(c) Stability of the pH 3.6 recombinates. Both recombinates showed hydro-

phobic association of protein with lipid, since they withstood changes in pH and ionic strength. However, while hydrophobic interactions firmly stabilized the recombinate of protein Fraction I, the recombinate of Fraction II was more labile, suggesting the participation of ionic interactions.

Zahler and Weibel[2] found total membrane protein to only partially recombine with homologous lipids. They considered the possibility of a difference in lipid-binding capacity of individual membrane proteins or of a partial recombination of each component with lipid, and provided evidence for the latter. Our results showed that at pH 7.6, at which the experiments of Zahler and Weibel[2] were carried out, there was a significant difference in the recombining capacity of Fractions I and II. This apparent discrepancy might be explained in various ways: different properties of the proteins due to different experimental procedures, presence of bivalent ions in the recombination media used by Zahler and Weibel[2] which, as we have shown, mask the differences between the two proteins fractions, or a possible co-recombination by protein–protein interactions between the unfractionated proteins.

The two protein fractions on which we are reporting are nonhomogeneous pools, the proteins included in Fraction I differing from those in Fraction II not only in molecular weight (around 200000 in the former, 100000 and less in the latter[21,23,24]) but also in chemical composition, specifically sialic acid content. The high molecular weight proteins found in Fraction I are thought to be located as a fibrous network on the inner membrane surface[32], they form fibrils in the presence of divalent cations[32] and they have been implicated in membrane deformability[33] and structure maintenance[34]. They are referred to in the literature as spectrin or tektin[26,32].

The binding of spectrin to phospholipid vesicles has been studied by Juliano *et al.*[28] and by Sweet and Zull[35]. They found that binding of spectrin is favored by the conformation of the protein at pH 3.5 and by the presence of liposomes carrying opposite charges. However, when in high concentration, spectrin was found to bind to liposomes of the same charge sign at neutral pH, although in a much smaller amount. The hydrophobic nature of the spectrin–liposome interaction was evidenced by a marked increase in liposome permeability[35]. Our results compare well with those reported by Sweet and Zull[35]. Fraction I bound the largest amount of lipid per protein at pH 3.6 and was able to bind lipid at pH 7.5 in a high phospholipid/protein ratio, although with a much smaller yield of recombinate. The hydrophobic nature of the recombinate at pH 3.6 was demonstrated by its stability towards an increase in pH and ionic strength.

The type of interaction obtained in the recombination of membrane protein Fraction I with erythrocyte lipid is at variance with the accepted view on the nature of binding of these proteins in the native membrane. Since these proteins can be extracted by treatment of ghosts with alkaline buffers of low ionic strength in the presence of EDTA[5], they seem to be attached by ionic bonds and stabilized by interaction with Ca^{2+}. They are, according to the model proposed by Singer and Nicolson[36], "peripheral proteins". Apparently, the hydrophobic residues of these proteins, which in the native conformation are not exposed towards the membrane lipid core, have in our experiments been reoriented under the influence of sodium dodecyl sulfate.

Fraction II proteins included both the "fast-moving" ionically bound soluble proteins probably located at the inner surface[5], and those with an apparent molecular weight of 100000 which penetrate the membrane from side to side[22,37] and are firmly

bound to the membrane by hydrophobic interaction[38]. The participation of both hydrophobic and ionic forces in the recombinate obtained from Fraction II seems to reflect its nonhomogeneity. It cannot be stated at this stage whether the difference in lipid-binding capacity of Fractions I and II is due to a specific component of either one of them. A comparison of the lipid-binding capacity of individual red cell membrane protein components, obtained by selective elution from the red cell membrane, is in progress. Preliminary results (Barzilay, M., Condrea, E. and de Vries, A., unpublished) indicate that in the recombinates of these components with total lipid extract all phospholipid classes are present, in proportions not essentially different from those in the total lipid extract.

REFERENCES

1 Wallach, D. F. H. (1969) *Membrane Proteins*, pp. 3–26, Little, Brown and Co., Boston
2 Zahler, P. and Weibel, E. R. (1970) *Biochim. Biophys. Acta* 219, 320–338
3 Zwaal, R. F. A. and Van Deenen, L. L. M. (1970) *Chem. Phys. Lipids* 4, 311–322
4 Schubert, D., Poensgen, J. and Werner, G. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* 353, 1034–1042
5 Hoogeveen, J. Th., Juliano, R., Coleman, J. and Rothstein, A. (1970) *J. Membrane Biol.* 3, 156–172
6 Maddy, A. H. (1966) *Biochim. Biophys. Acta* 117, 193–200
7 Rega, A. F., Weed, R. J., Reed, C. F., Berg, G. G. and Rothstein, A. (1967) *Biochim. Biophys. Acta* 147, 297–312
8 Rosenberg, S. A. and Guidotti G. (1969) *J. Biol. Chem.* 244, 5118–5124
9 Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* 226, 497–509
10 Rottem, S., Stein, O. and Razin, S. (1968) *Arch. Biochem. Biophys.* 125, 46–56
11 Terry, Th. M., Engelman, D. M. and Morowitz, H. J. (1967) *Biochim. Biophys. Acta* 135, 391–405
12 Mukerjee, P. (1956) *Anal. Chem.* 28, 870–873
13 Reynolds, J. A. and Tanford, C. (1970) *Proc. Natl. Acad. Sci. U.S.* 66, 1002–1007
14 Weber, K. and Osborn, M. (1969) *J. Biol. Chem.* 244, 4406–4412
15 Maizel, J. V. (1966) *Science* 151, 988–990
16 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
17 Warren, L. (1959) *J. Biol. Chem.* 234, 1971–1975
18 Marinetti, G. V., Albrecht, M., Ford, T. and Stotz, E. (1959) *Biochim. Biophys. Acta* 36, 4–13
19 Bartlett, G. R. (1959) *J. Biol. Chem.* 234, 466–468
20 Steck, Th. L., Fairbanks, G. and Wallach, D. F. H. (1971) *Biochemistry* 10, 2617–2624
21 Tanner, M. J. A. and Boxer, D. H. (1972) *Biochem. J.* 129, 333–347
22 Bretscher, M. S. (1971) *Nat. New Biol.* 231, 229–232
23 Fairbanks, G., Steck, Th. L. and Wallach, D. F. H. (1971) *Biochemistry* 10, 2606–2616
24 Trayer, H. R., Nozaki, Y., Reynolds, J. A. and Tanford, C. (1971) *J. Biol. Chem.* 246, 4485–4488
25 Marchesi, S. L., Steers, E., Marchesi, V. T. and Tillack, T. W. (1970) *Biochemistry* 9, 50–57
26 Clarke, M. (1971) *Biochem. Biophys. Res. Commun.* 45, 1063–1070
27 Branton, D. (1969) *Annu. Rev. Plant Physiol.* 20, 209–233
28 Juliano, R. L., Kimelberg, H. K. and Papahadjopoulos, D. (1971) *Biochim. Biophys. Acta* 241, 894–905
29 Reynolds, J. A. and Tanford, C. (1970) *J. Biol. Chem.* 245, 5161–5165
30 Robertson, J. D. (1972) *Arch. Int. Med.* 129, 202–228
31 Richardson, S. H., Hultin, H. O. and Green, D. E. (1963) *Proc. Natl. Acad. Sci. U.S.* 50, 821–827
32 Marchesi, V. T., Steers, E., Tillack, W. and Marchesi, S. L. (1969) in *Red Cell Membrane*

(Jamieson, G. A. and Greenwalt, T. J., eds), pp. 117–130, J. B. Lippincott, Philadelphia and Toronto
33 Jacob, H., Amsden, T. and White, J. (1972) *Proc. Natl. Acad. Sci. U.S.* 69, 471–474
34 Juliano, R. L., Rothstein, A. and La Voy, L. (1971) *Life Sci.* 10, 1105–1113
35 Sweet, C. and Zull, J. E. (1970) *Biochem. Biophys. Res. Commun.* 41, 135–141
36 Singer, S. J. and Nicolson, G. L. (1972) *Science* 175, 720–731
37 Bretscher, M. S. (1971) *J. Mol. Biol.* 59, 351–357
38 Juliano, R. L. and Rothstein, A. (1971) *Biochim. Biophys. Acta* 249, 227–235