BBA 75525

RECONSTITUTION OF MEMBRANES BY RECOMBINING PROTEINS AND LIPIDS DERIVED FROM ERYTHROCYTE STROMA

PETER ZAHLER AND EWALD R. WEIBEL

Theodor Kocher-Institute and Department of Anatomy, University of Berne (Switzerland); and Central Laboratory of the Swiss Red Cross, Blood Transfusion Service, Berne (Switzerland)

(Received May 19th, 1970)

SUMMARY

Reconstitution of membranes of the red blood cell has been obtained by recombining the membrane proteins with the membrane lipids. The native membranes have been solubilized in 90% 2-chloroethanol, and membrane proteins and lipids have been separated by gel filtration on Sephadex LH-20. The two components were combined in 2-chloroethanol and then dialyzed against aqueous buffer whereby precipitation of aggregated lipoprotein took place. Equilibrium density gradient centrifugation of original stroma and reconstituted stromal lipoproteins proved the binding of lipid to about 60% of the protein after recombination. Chemical analysis of the isolated zones of stroma and recombined material showed similarity in the composition of the proteins and the main lipid classes.

Electron microscopy of fixed and sectioned material revealed identical structure of original and recombined membranes; both were trilaminar, had an overall thickness of 70–80 Å and were found to have an identical granular substructure of the dense layers. The recombined membranes formed stacks or concentric shells which could be clearly differentiated from "myelin figures". The two subfractions of proteins and lipids did not form membrane-like structures.

INTRODUCTION

In previous studies on the solubilization of red cell membranes by 90% 2-chloroethanol at pH 2, it could be shown by electron microscopy that upon dialysis against aqueous buffers the reaggregated material revealed the typical trilaminar membrane structure. Part of the blood group antigens were recovered in this material but no catalytic functions were discernible[1,2].

In the present investigation we studied the question of the extent of recombination of the membrane proteins and lipids and the resemblance of the structure, density and chemical composition of the reconstituted membrane to the original red cell membrane.

As previously shown, the membrane components can be extensively dissociated with 2-chloroethanol. From Sephadex LH-20 molecular sieving experiments with this solvent, it is evident that membrane lipids are present as monomers or dimers and not as larger micelles[3,4]. According to the results of ultra-centrifugation studies, most of

the proteins are highly dissociated if not monomerized in 90 % 2-chloroethanol in contrast to other lipid solvents such as chloroform–methanol[4].

The membrane proteins retain or even increase their α-helical content in this solvent[5], and possibly related to this fact, they remain almost insoluble in aqueous buffers at neutral pH (refs. 3, 4). In contrast, *n*-butanol-extracted red cell membrane proteins, which have been shown to be water soluble[6–8], reveal circular dichroism spectra of largely coiled proteins[9].

After solubilization of red cell membranes in 2-chloroethanol, the protein was isolated quantitatively by chromatography on Sephadex LH-20 (ref. 4). The heterogeneity of the proteins was demonstrated by some 20 bands in disc electrophoresis[3]. These were shown to be completely free of lipids[3,4].

Lipid binding capacity of various lipid-free serum and membrane proteins has been described in the literature[10–13]. In the case of mitochondrial and chloroplast proteins, it could be shown that the extent of lipid binding is related to the amount of lipid present up to a certain point where saturation is reached[11,12]. It was also demonstrated that the various lipid classes are bound with different affinities and to various extents[13].

Since catalytic functions reappear after recombination of delipidated enzymes with lipids, it can be concluded that lipids act as structural co-factors of the lipoproteins[14–18].

In the case of membrane lipoproteins various recombinations after detergent solubilization have been described, and by use of electron microscopy the reconstitution of membranes has been demonstrated[19–21]. The extent of dissociation of lipids and proteins accomplished by the detergent is not known from these experiments. In the case of membrane reconstitution of pleuropneumonia-like organisms, the results indicate that in high concentrations of detergent the lipids may be completely split off from the proteins[19,21].

Up to now, no reconstitution of membrane structure has been described after solubilization experiments using organic solvents. The water-soluble membrane proteins obtained from red cell membranes by the *n*-butanol technique fail to bind any lipids[22], and the reconstitution of chloroplast lipoprotein aggregates from organic solvents has not been studied by electron microscopy[13].

MATERIALS AND METHODS

Isolation of red cell membranes

The general procedure of DODGE *et al.*[23], as applied by AZEN *et al.*[24] was used. The sediment of one 500-ml unit of human blood, collected fresh the previous day, was washed 3 times at 0–4° with 2 vol. of phosphate buffer (310 mosM, pH 8.0). Hemolysis was performed in 20 vol. of phosphate buffer 15 mosM at pH 8.0 at 0–4°. The sediment was washed with the same buffer 5–7 times; each time it was centrifuged for 30 min at 18000 rev./min in a Spinco Model L centrifuge (rotor 21). The volume was progressively reduced, ending with about 10 times the primary sediment volume. Each final preparation was analyzed for hemoglobin[25], protein[25], lipid[26] and phosphorus[27]; the results were in good agreement with the values of DODGE *et al.*[23], although a somewhat higher scattering of the protein/lipid ratios between various red cell membrane

preparations was found. The final sediment was suspended in 5 vol. of 0.30 M sucrose and stored frozen at —25° until used.

Solubilization[3,4]

The sample of stroma was thawed at 0–4°, washed 3 times with 10 mM Tris, pH 7.6, and suspended in the same buffer at a concentration of 10 mg protein per ml[25]. To 10 ml of the homogeneous nonaggregated suspension, 90 ml of freshly distilled 2-chloroethanol at an apparent pH of 2.0* were added in a few seconds with vigorous stirring at 0–4°. Instantaneous solubilization took place. Concentration of the resulting solution to a final volume of 15 ml in a rotatory evaporator at 1 mm Hg took 15–20 min (water bath temperature, 15°; evaporating temperature, 5–8°; condensor temperature, —30°).

The membrane solution remained colorless with only a trace of opalescence. The solubilization was less satisfactory if the starting material was aggregated or if the temperature was not strictly controlled during evaporation.

Isolation of membrane protein[3,4]

Membrane solution in 2-chloroethanol containing 6–7 mg protein per ml was placed on a column (4 cm diameter, 80 cm length) of Sephadex LH-20 equilibrated with 2-chloroethanol–water (9:1, by vol.) (applied volume: bed volume, 1:100), and eluted with the same solvent. The membrane proteins, identified by their 280-nm absorbance, eluted as a symmetrical peak in the void volume. Thin-layer chromatography on Kieselgel G with chloroform–methanol–water (65:25:4, by vol.)[28] was carried out with the last few fractions of the protein peak and the following fractions, applying 100 μl of each fraction. In some cases overlapping of the first phospholipid fractions with the more slowly eluted protein fractions occurred, but in most instances the separation was complete, often with 2–3 fractions separating the two components. The lipid-free protein fractions were pooled and concentrated by rotatory evaporation under the above conditions to 8 mg protein per ml. (See refs. 3 and 4 concerning the chemical analysis of the proteins and the evidence that no lipid phosphorus can be extracted from these protein eluates.) Subsequent recombination dialyses were always done immediately to limit exposure of the protein to 2-chloroethanol to 24 h at the most.

Isolation of lipids

Instead of collecting the lipid fractions obtained by Sephadex LH-20 chromatography, we used the technique of FOLCH *et al.*[26] for the isolation of stroma lipids. To the twice-washed chloroform phase, 2 vol. of 2-chloroethanol were added, followed by evaporation *in vacuo* of the solution at 10° to a concentration of about 0.15 mg of phosphorus per ml. The total lipid content was estimated gravimetrically. Thin-layer chromatography of lipids was performed by the method of JATZKEWITZ[28].

* It should be noted that 2-chloroethanol undergoes a slow decomposition resulting in HCl production. Also during distillation, HCl liberation takes place leading to an apparent pH of the distillate from Kp 127–129 of 1.9–2.1 (pH measurement after 1:1 dilution with water). Neutral 2-chloroethanol can be obtained by fractionated distillation *in vacuo* after neutralization with solid Tris base. We used the distilled solvent as long as the pH stayed above 1.8 and the absorbance at 240 nm (1 cm) was below 0.1.

Recombination (see Fig. 1)

The solutions of protein in 2-chloroethanol–water (9:1, by vol.) and of lipid in pure 2-chloroethanol were combined to give mixtures containing 1 mg of protein and 0.75 mg of total lipid per ml. This solution remained perfectly clear. It was then

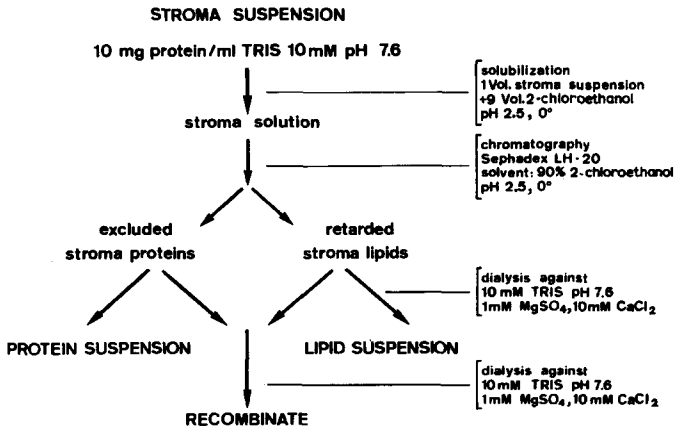


Fig. 1. Schematic outline of the solubilization, fractionation and recombination of red cell membrane.

transferred to a Visking dialysis bag, previously washed in distilled water for 24 h and dialysed for 6 h with constant stirring against 100 vol. of 10 mM Tris (pH 7.6), 10 mM $CaCl_2$ and 1 mM $MgCl_2$ buffer. With respect to other buffers used for recombination experiments see the section under RESULTS. The buffer solution was changed after 2 and 4 h. The resulting suspension was centrifuged for 1 h at 39000 rev./min in a Spinco SW-39 rotor; the sediment was taken up in 10 mM Tris (pH 7.6) and 0.25 M sucrose to a final concentration of 10 mg protein per ml. All operations were carried out at 0–4°. A survey of the overall procedure of recombination is given in Fig. 1.

Density gradient centrifugation

Linear sucrose gradients were formed by a Beckmann density gradient former in the density range of 1.09–1.26. The suspension of stroma or recombinate containing 3–15 mg dry weight per ml in 10 mM Tris (pH 7.6) and 0.25 M sucrose were mixed directly in the low density sucrose solution or overlayered on the gradient. Centrifugation was carried out at 24000 rev./min for 16 h at 4° (45000 rev./min for 14 h using the SW-50 rotor). Equilibrium conditions could be demonstrated by equal band widths in the same density regions of both the overlayered and admixed samples.

Chemical analyses

Depending on the analytical methods used, the gradients were treated in different ways. For profile analysis the gradients were collected in 1-ml portions from the top using Lang–Levy pipettes. The fractions were analyzed for protein[25], lipid phosphorus[27] and cholesterol[29]. The density profile was established by refractive index measurements. Lipids were extracted by the method of RENKONEN *et al.*[30].

For measurement of the overall chemical composition of the bands and for electron microscopy, the bands were collected, diluted with 10 mM Tris (pH 7.6) and

isolated by subsequent centrifugation for 30 min at 39000 rev./min in a Spinco 40 rotor. The sediments were analyzed for protein[25], total lipids[30], lipid phosphorus[27] and cholesterol[29]. Thin-layer chromatography was performed on the lipid extracts[28]. The lipid-free proteins were isolated using Sephadex LH-20 chromatography in 90 % 2-chloroethanol as described in a previous section and disc electrophoresis, by the method of NEVILLE[31], was made on these proteins.

Electron microscopy

Suspensions of stroma and recombinate recovered from density gradients and of isolated protein and lipids were fixed for electron microscopy by addition of an equal volume of cold 2.5 % glutaraldehyde, buffered at pH 7.4 with veronal–acetate. After 90 min the suspension of fixed material was centrifuged for 10–15 min at about 1600 $\times g$ in a laboratory centrifuge. After washing 90 min in the above buffer, the pellet was postfixed for 90 min in cold 1 % OsO_4 buffered with veronal–acetate (pH 7.4)[32]. This was followed by blockstaining for 60 min with a 1 % solution of uranyl acetate (pH 4.8)[33].

Subsequently the pellets were dehydrated with graded ethanol and embedded in Epon 812, either by the standard method of LUFT[34] or by the rapid method of COULTER[35]. It was found that for our purposes the rapid method was far superior to the standard method, presumably due to a lower degree of lipid extraction. All electron micrographs presented here were derived from rapidly embedded material.

Dark grey to black sections were cut out on an LKB Ultrotome III using a diamond knife. To support the sections, copper grids were first fitted with a porous plastic film which was then overlaid with an extremely thin carbon film prepared on freshly cleaved mica[37]. Sections were briefly stained with lead citrate[38].

Electron micrographs were recorded in either a Philips EM 200 or EM 300 electron microscope at primary magnifications up to 246000 times, using an accelerating voltage of 80 kV with standard cathode, double condenser, an objective aperature of 40 μ, and the anticontamination device. Magnification was calibrated with a carbon-grating replica. For densitometer analysis and measurement, the negatives were printed on sheet film magnified 500000 or 1000000 times. Densitometer tracings were recorded using a slit 3 mm long and 0.1 mm wide, oriented parallel to the membrane profile; the slit width corresponded to 0.2 and 0.1 nm, respectively, on the preparation.

RESULTS

Recombination

During dialysis of the mixture of stroma-proteins and lipids in 2-chloroethanol against the aqueous buffer, the solution in the bag became opalescent after about 30 min, and the turbidity increased with time until about 4 h. With some exceptions, after 6 h the material precipitated. Table II shows the amount of protein recovered in the pellet after centrifugation of this suspension. No significant effect of the buffer composition could be detected, whereas the pH during dialysis had an influence on the extent of the formation of insoluble material. In addition to the buffers indicated in Table II, phosphate buffer, according to Sörensen[44], has been used in the pH region 5–8 and in concentrations from 10 to 310 mosM (isotonic). Between pH 5 and 7 no

systematic differences in the amount of insoluble protein formed during dialysis could be found (values between 91 and 96 %), whereas at pH 8 only about 80 % of the protein was insoluble. No effect of the buffer osmolarity could be detected. Addition of 0.25 M sucrose to the buffer solutions had no effect on the extent of formation of insoluble protein but led to less aggregated precipitates. In the region of pH 7 the highest yields of precipitate were obtained with Tris buffer containing 10 mM Ca^{2+} and 1 mM Mg^{2+}, and most of the recombination experiments for analytical purposes for electron microscopy have been performed with this buffer. When stroma was dialyzed under the same conditions as recombinates, we always found 5–10 % of the protein in the supernatant; this must be interpreted in terms of a partial spontaneous solubilization of the stroma during dialysis.

Density gradient centrifugation of stroma and chemical analyses

For several individual red cell membrane preparations, the density gradient centrifugation in most cases showed a narrow band and in some cases a double band at densities from 1.14 to 1.18 (Figs. 3(a) and 3(k)). Depending upon the lipid content of a given preparation which varied from 0.8 to 1.1 mg of total lipid per mg of red cell membrane protein, the centre of the band was located at densities from 1.14 to 1.16. The mean values from chemical analyses of the band of five individual red cell membrane preparations are shown in Table I. 90–98 % of the applied red cell membranes are recovered in the band demonstrating a rather homogeneous density distribution of the material. The determination of the various protein/lipid ratios showed close similarity between one individual stroma and the respective band after density gradient centrifugation, although the relatively wide variations of these ratios, especially of cholesterol, demonstrate the variability in composition of individual stroma preparations.

TABLE I

CHEMICAL ANALYSES OF RED CELL MEMBRANE AND RECOMBINATION PREPARATIONS AFTER DENSITY GRADIENT CENTRIFUGATION

	Red cell membrane *	*Recombinate* **
Density limits of band	1.14–1.18	1.12–1.22
Average band width (Δd ***)	0.02	0.06
Protein in band (% of total protein applied to the gradient)	90–98%	52–72%
Protein/lipid ratio (wt./wt.)		
Average	1.1	1.5
Limits	0.9–1.2	0.8–2.8
Protein/phosphatide ratio (wt./wt.)		
Average	1.7	2.5
Limits	1.5–2.1	1.6–3.1
Protein/cholesterol ratio (wt./wt.)		
Average	3.9	9.8
Limits	2.9–4.5	6.7–17

* Average values of five individual red cell membrane preparations.
** Average values of eight recombination experiments using three different red cell membrane preparations.
*** Δd indicates the difference of density on both sides of the lipoprotein band.

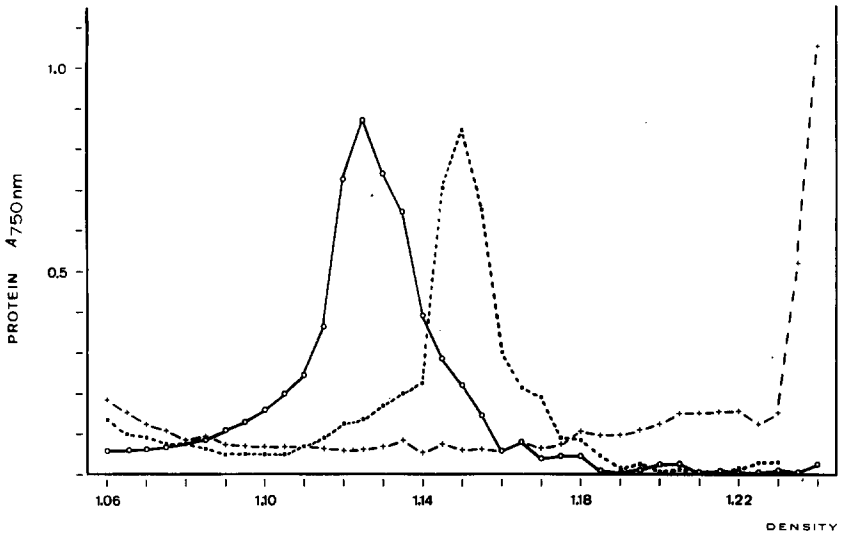


Fig. 2. Protein profiles (25) of red cell membrane (○—○), red cell membrane protein (+—+) and recombined red cell membrane lipoprotein (■---■) after equilibrium centrifugation in a sucrose gradient.

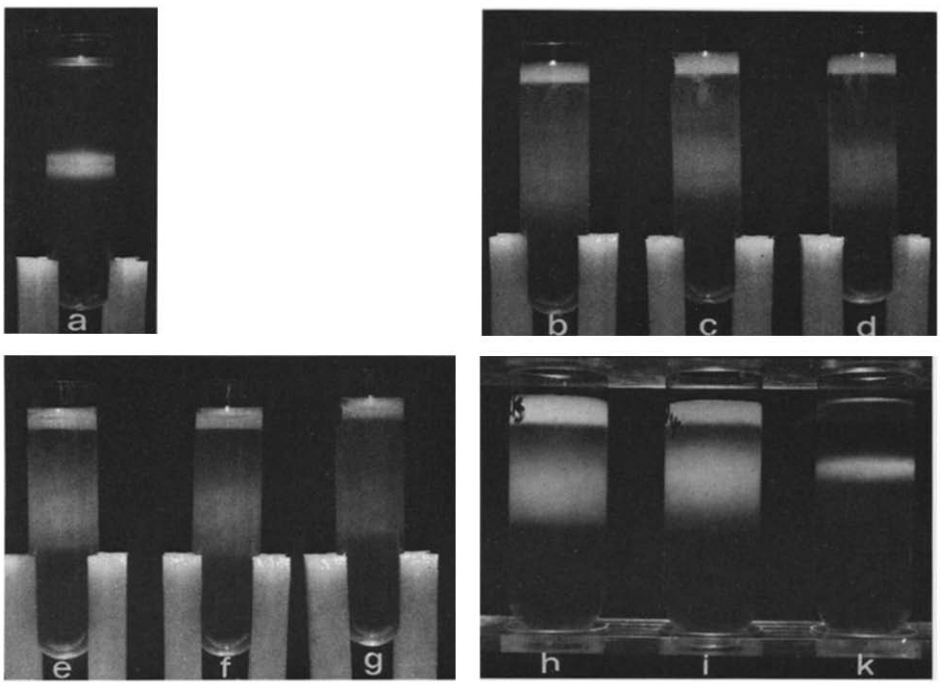


Fig. 3. Photographs of equilibrium centrifugation in sucrose gradient of (a) red cell membrane, 1 mg protein admixed to the low density sucrose. (b), (c), (d) recombination in Tris buffer (pH 7) containing Ca^{2+} and Mg^{2+} (see text) of three individual red cell membrane preparations. 1 mg protein per tube overlayered. (e), (f), (g) recombination in 10 mosM phosphate buffer pH 6 (e), 7 (f), 8 (g). 1 mg protein per tube admixed to low density sucrose. (h) recombination in Tris buffer containing Ca^{2+} and Mg^{2+} (see text), 16 mg protein overlayered. (i) recombination in phosphate buffer 10 mosM (pH 8). 16 mg protein admixed to low density sucrose. (k) red cell membrane used for Expt. (h) and (i); 5 mg protein overlayered.

Density gradient centrifugation of recombinate and chemical analyses

In the density gradient, recombined red cell membranes invariably showed a floating zone of free lipids indicating that not all lipids had recombined with the protein (Fig. 3). The lipoprotein band was found in density regions of 1.12–1.22, depending upon the stroma preparation and other uncontrollable factors during recombination. As seen in Figs. 3 and 4, the bands of the recombinates are much broader and in most cases in regions of higher density than those of stroma, indicating a more heterogeneous situation with respect to the chemical composition (Table I). Nevertheless, they clearly show that lipoprotein has been formed since as shown in Fig. 2, lipid-free protein sediments completely under the same centrifugation conditions.

The profile analysis of the density gradient in Fig. 4 further demonstrates the formation of lipoprotein by the fact that the amounts of the phosphatides and cholesterol parallel the protein estimation and by their coincidence with the maximum values of the protein peaks.

The chemical analysis of the lipoprotein bands reveals some similarity between

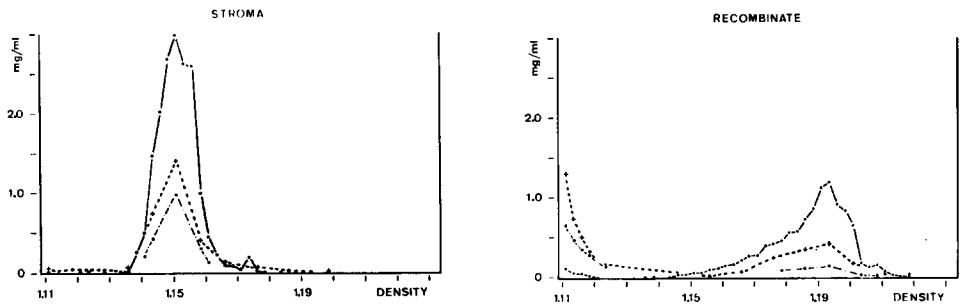


Fig. 4. Profiles of protein (○—○), lipid phosphorus (+---+) and cholesterol (□-·-□) of red cell membrane and recombinate after equilibrium centrifugation in sucrose gradient.

TABLE II

% PROTEIN RECOVERED IN THE SEDIMENT AFTER RECOMBINATION BY DIALYSIS AGAINST VARIOUS AQUEOUS SOLUTIONS

Solution **	*Concn.* (*mM*)	*pH*	% *Protein* *
Phosphate buffer (SÖRENSEN[44])	10	8	78
		5	96
Tris buffer	10	7.4	93
	100	7.4	94
Tris buffer	10		
$+CaCl_2$	10	7.4	97
$+MgCl_2$	1		
Acetate buffer[45]	10	4	72
HCl	10		27
Water			78

* Values established by protein determinations[25] in the supernatant after 60 min 100000 × *g* centrifugation.

** Same recombination conditions as described in MATERIALS AND METHODS.

stroma and recombined lipoprotein (Table I), although because of the more heterogeneous density distribution, the various protein/lipid ratios show much larger variations than in stroma. In most cases the recombinate showed a higher protein content

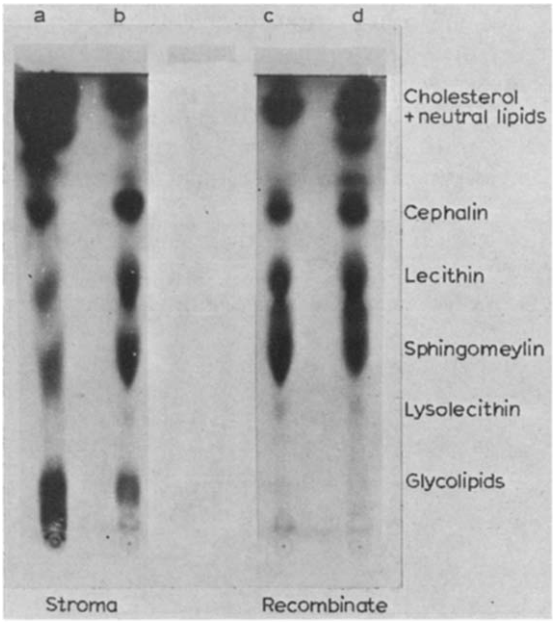


Fig. 5. Thin-layer chromatogram of the lipid extracts after density gradient centrifugation. a, red cell membrane floating free lipid; b, red cell membrane main lipoprotein band; c, recombinate floating lipid band; d, recombinate lipoprotein band (for technique see text).

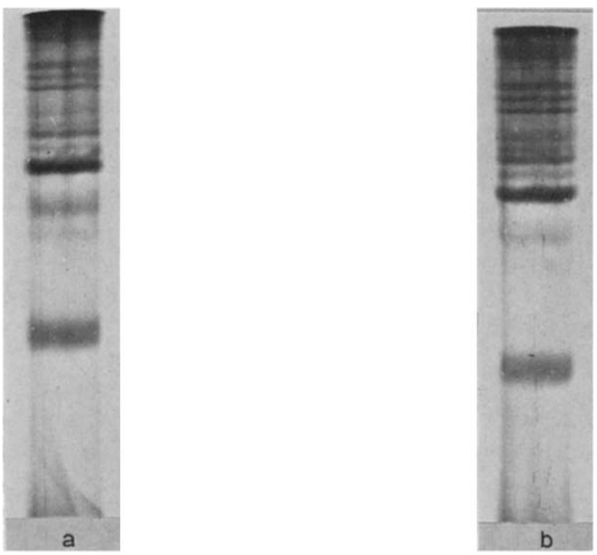


Fig. 6. Disc electrophoresis of the lipid-free proteins of (a) red cell membrane, (b) recombinated red cell membrane lipoprotein (for technique, see MATERIALS AND METHODS).

than the respective stroma, and in all cases, the cholesterol content in recombinates was less than half of the value found for red cell membranes.

Table II shows that 52–71 % of the total amount of protein overlayered on the gradient can be collected in the lipoprotein band. In contrast to our hypothesis that some of the membrane proteins with minor lipid-binding capacity or with higher susceptibility to the organic solvent would fail to recombine, the disc electrophoresis of the proteins from the band showed no evidence for disparities between the protein pattern of red cell membranes and recombinates (Fig. 6).

The qualitative thin-layer chromatogram of the lipids of the recombinates and red cell membranes (Figs. 5b and 5d) shows that, with the exception of the glycolipids, all major components of red cell membranes are also present in recombinates in similar proportions although considerable differences may be recognised in respect to the minor lipid components. From the patterns of the floating lipids (Figs. 5a and 5c), it may be concluded that the small amount of free lipid in red cell membranes differs distinctly in its composition from the protein-bound lipid. The chromatogram shows a much higher proportion of cholesterol glycerides and glycolipids in free lipid than in

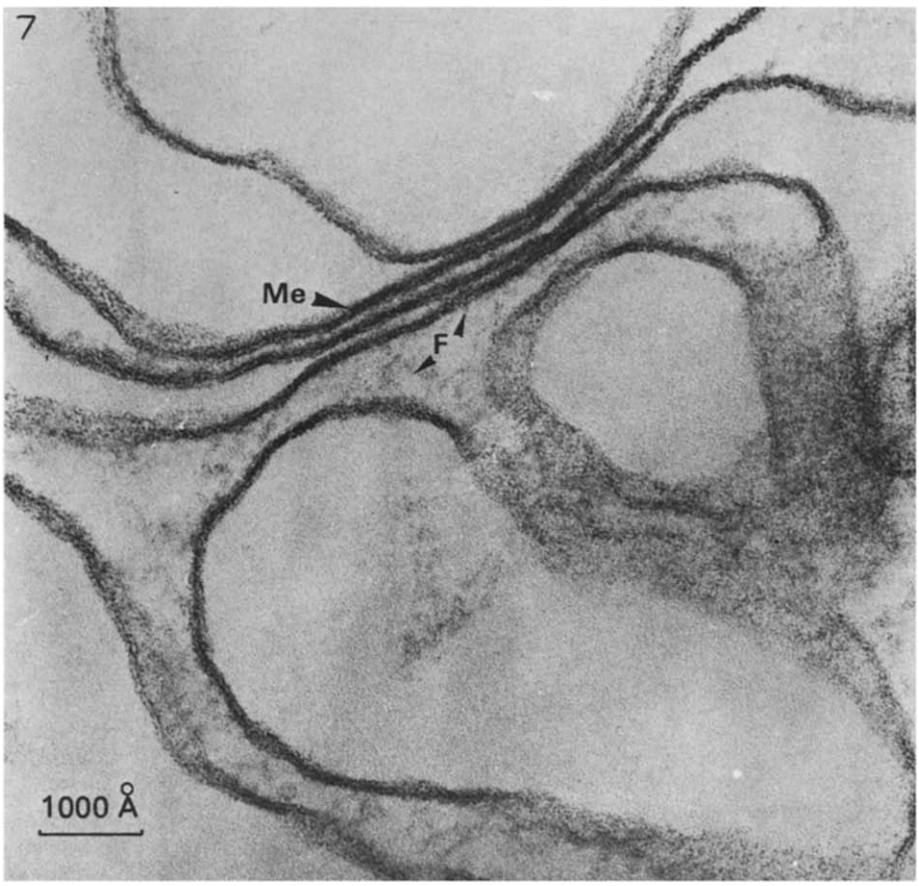


Fig. 7. Electron micrograph of stroma membranes (Me). Note filaments (F) attached to inner side of membrane (66). 135000×.

bound lipids. In the recombinate no important differences in the main lipid classes may be seen between protein-bound and free lipids.

The density gradient study and chemical analysis, as a whole, reveal that about

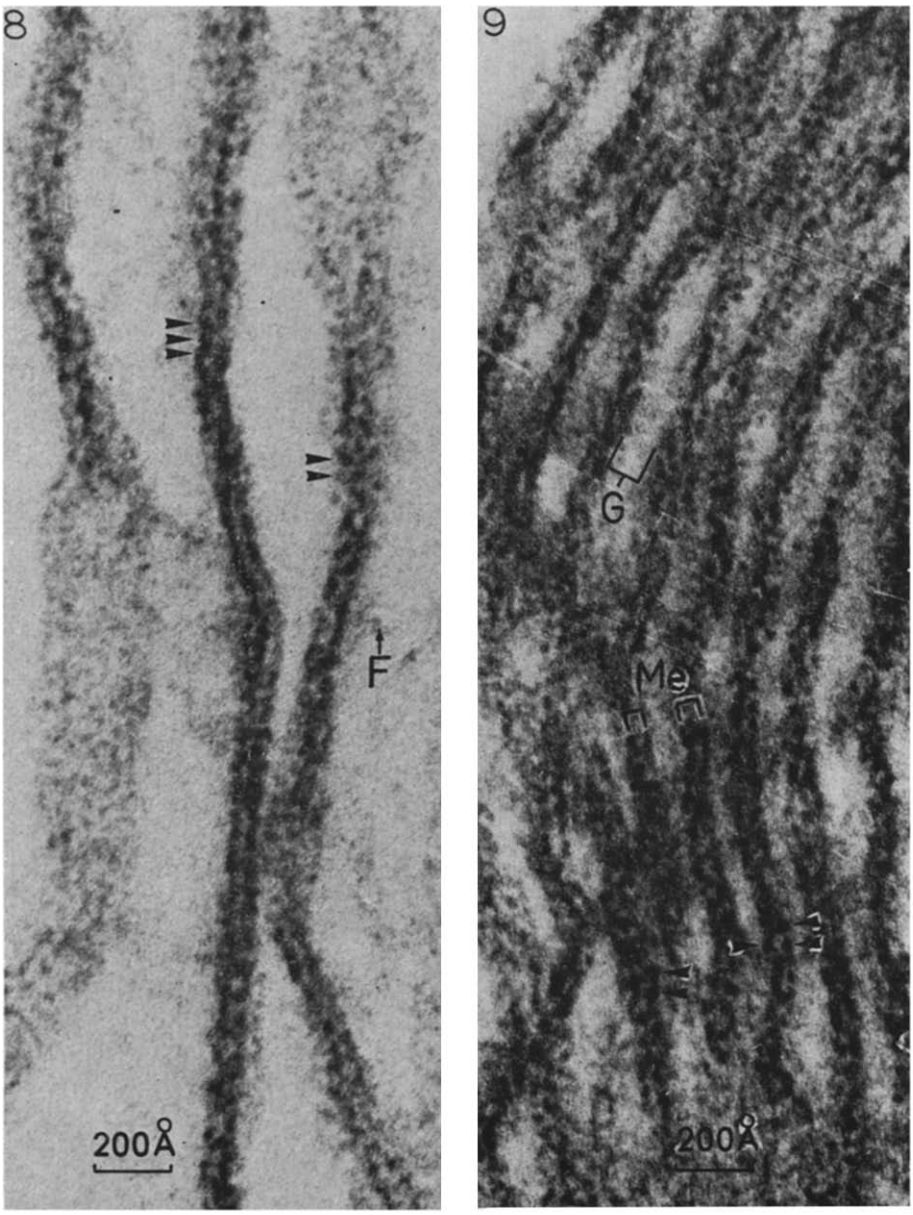


Fig. 8. Stroma membranes at higher power: note granular appearance of trilaminar structure (arrows). Filaments (F) adhere to one side of membrane. 500 000×.

Fig. 9. Stack of recombined membranes (Me) separated by irregular gaps (G). A granular substructure (arrows) is also evident. 500 000×.

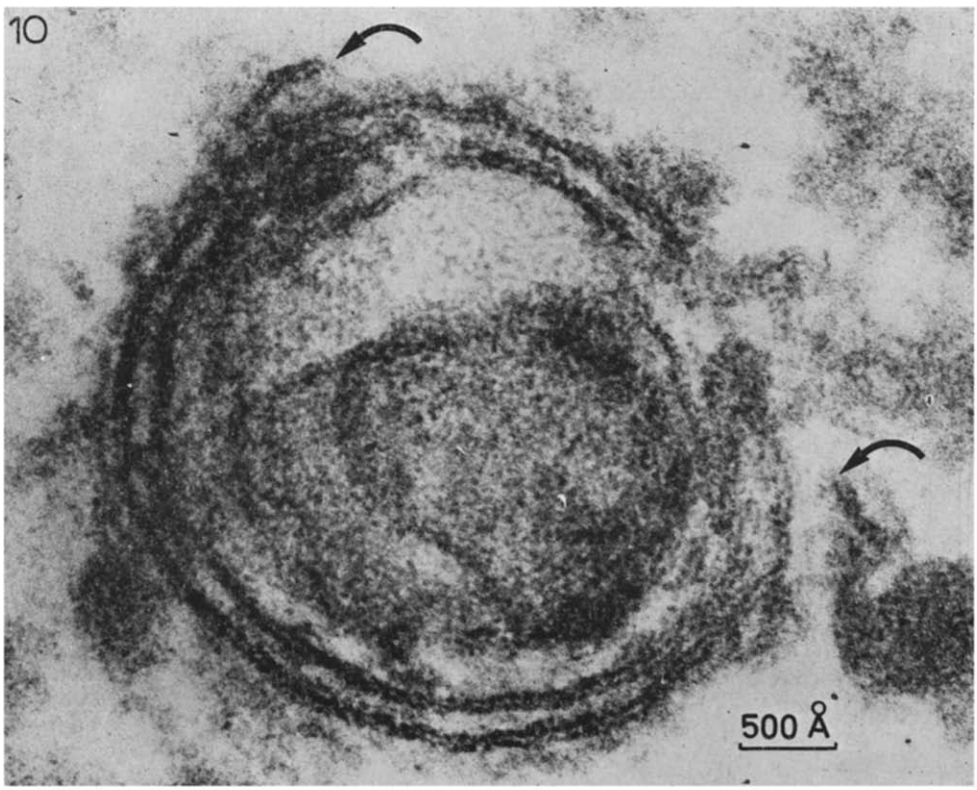
two thirds of the membrane proteins recombine with membrane lipids and form lipoprotein aggregates similar to stroma with respect to the proportions of the main lipid classes and the protein components.

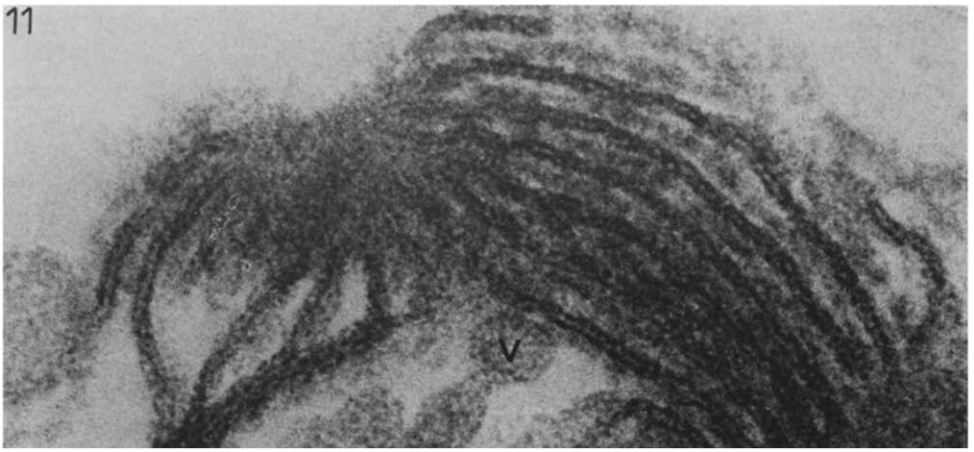
Electron microscopic findings

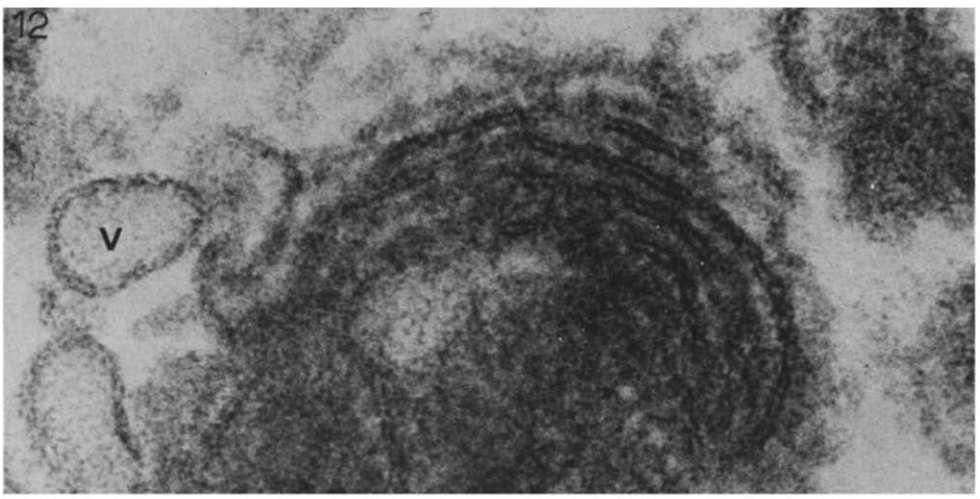
Figs. 7 and 8 show cross sections of a stroma preparation which exhibit the ordinary trilaminar structure of membranes. The overall thickness of these membranes is estimated at about 8 nm, when measured directly on the electron micrographs. In addition, this preparation shows a fine filamentous material of low electron density which is attached to one side of the membrane (Fig. 7). According to MARCHESI AND PALADE[39], this may represent remnants of internal filaments of the red cell which are always present in red cell ghosts.

In the recombinate preparations, membrane-like structures of striking similarity with stroma membranes are observed (Figs. 9–13). They are likewise trilaminar and have an overall thickness of 7–8 nm. The membranes constitute the bulk of this preparation, with some amorphous material and a few myelin figures present (Figs. 13 and 14), and form vesicles of varying size and shape. Most frequently these vesicles appear as concentric shells which lead to an apparent stacking of the membranes in form of whorls (Figs. 10–13). Occasionally, small bits of membrane with "free edges" are observed (Fig. 10).

The stacks of recombined membranes are easily distinguished from "myelin







Figs. 10–12. Recombinate membranes showing typical configuration of concentric and parallel stacking. Note vesicular formations (V) and short profiles of sheets with apparent free edges (arrows). 250 000×.

figures", which are presumably composed of multilayers of phospholipids. Whereas the latter exhibit a regular spacing of about 4.5 nm, the stacked membranes are clearly separated by an irregular gap of 10–20 nm which contains some amorphous material of moderate electron density (Figs. 9–13).

It should be noted that the recombined preparation was structurally less homogeneous than the original stroma preparation. The whorls of concentric membranes were of very different sizes, and some contained large myelin structures, whereas others were associated with amorphous material. It can be expected, therefore, that the structural units of the recombinate will have varying densities.

Examination of the two subfractions of stroma (*cf.* Fig. 1) reveals that the lipid-free protein preparation consists of a homogeneous flocculent material of moderate electron density (Fig. 15) with no substructures discernible. The protein-free lipid

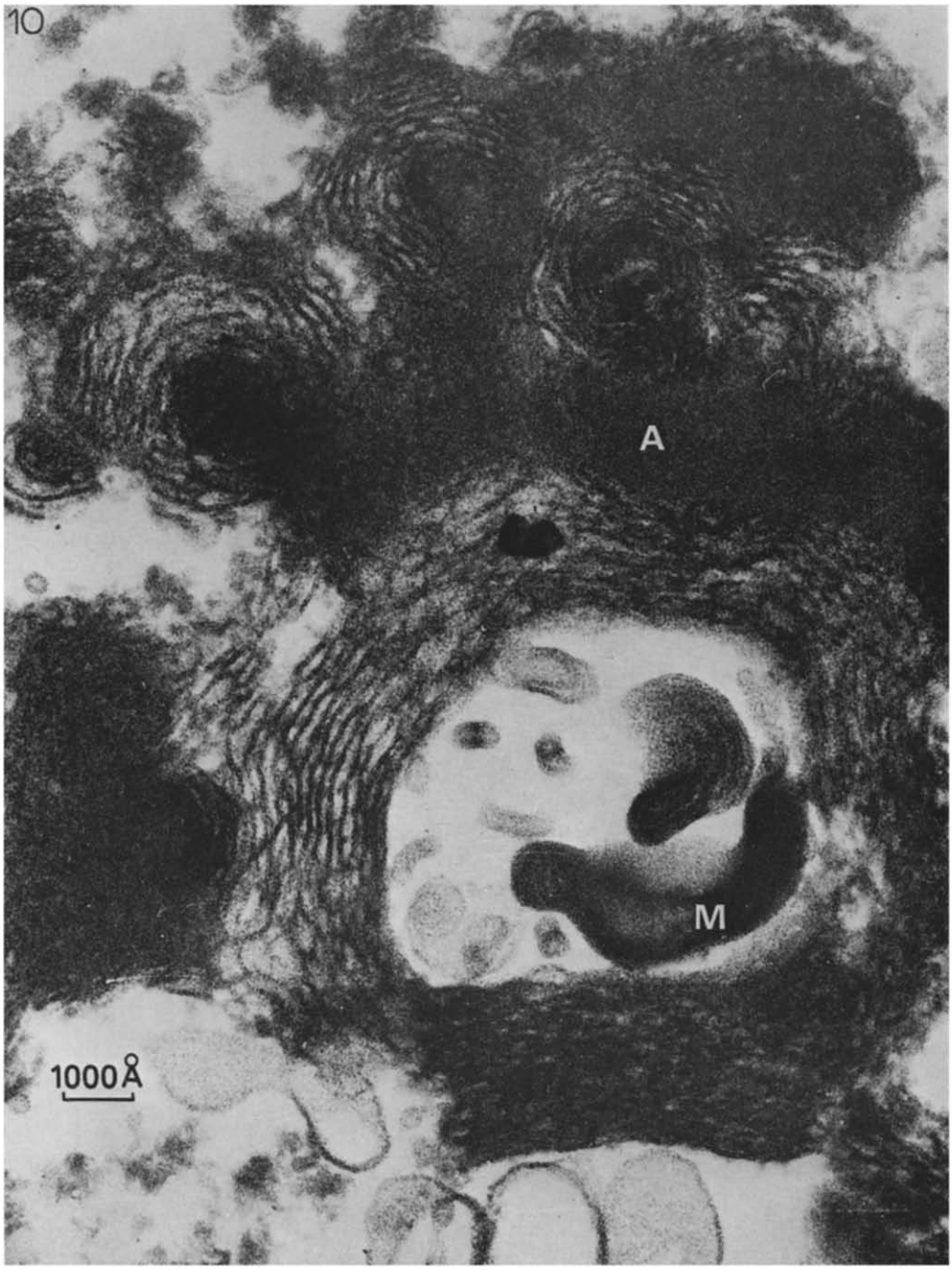


Fig. 13. Large "whorl" of recombined membranes containing a larger myelin figure of phospholipids (M) and regions with dense amorphous appearance (A) which may possibly be due to oblique sectioning. 100 000 ×.

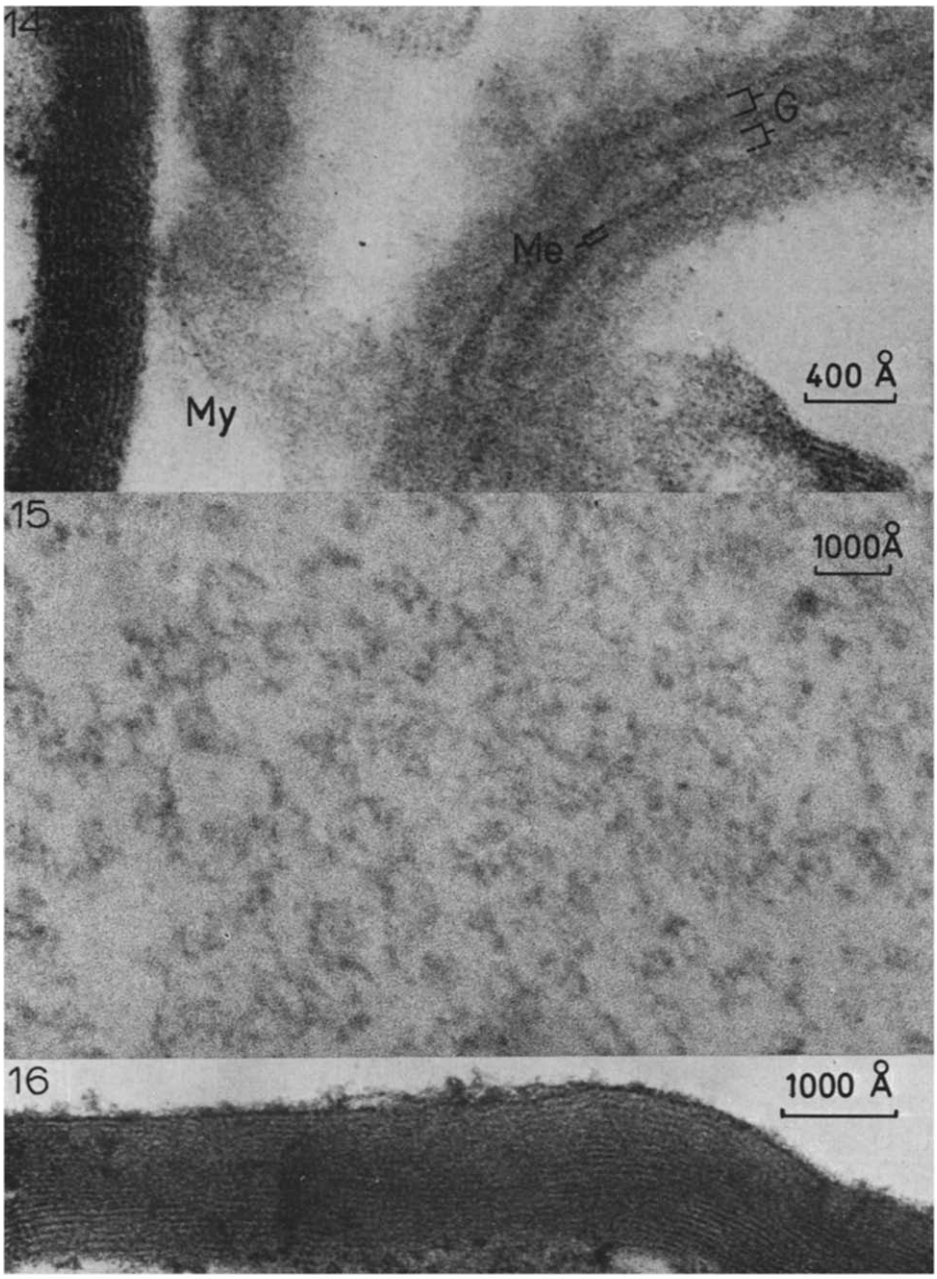


Fig. 14. Comparison of stacked membranes (Me) separated by gaps (G) with myelin figure (My) exhibiting regular repeating spacing in recombinate. 300 000×.

Fig. 15. Typical portion of a section of the lipid-free protein fraction. Note absence of any membrane-like structures. 100 000×.

Fig. 16. Protein-free lipid fraction appears as large masses of layered material with an identity spacing varying between 45 and 60 Å. 150 000×.

fraction is composed of large masses of strongly osmiophilic layered material with an average spacing of about 4–4.5 nm (Fig. 16).

Densitometer tracings of membrane profiles from stroma and recombinate were recorded from several high-power electron micrographs from matched preparations. The distance of the midpoint of the two peaks was measured as well as the overall width of the two peaks, the latter being less reliable. In Fig. 17, a number of representative tracings have been superimposed; the mean peak distance was 4.14 nm for stroma as compared to 4.26 nm for recombinate; this difference is statistically not significant ($0.1 < P < 0.2$). The overal width of stroma profiles appeared to be slightly wider than that of recombinate, but again this difference was nog statistically significant. Thus it can be concluded that the thickness of the original membrane was reproduced in the recombinate.

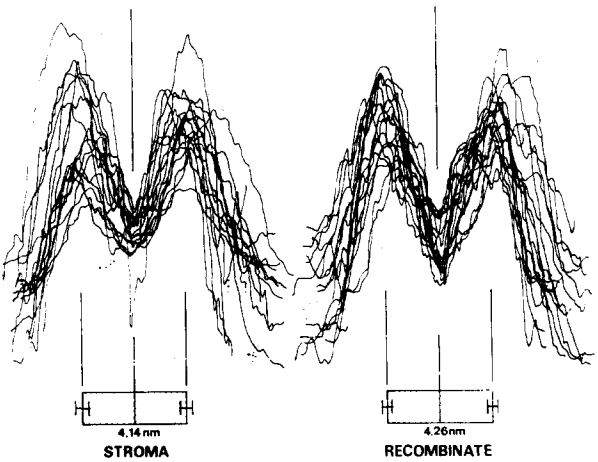


Fig. 17. Superimposed densitometer tracings of density distribution across stroma and recombinate membrane profiles. Bars and figures at bottom represent the mean peak distance; brackets indicate the standard deviation.

In high-power electron micrographs (Figs. 9–13), a granular substructure of the dense layers of the membranes is apparent in both types of preparations. The significance of this granularity is unknown, and it may be a mere artifact, or it may have its root in some molecular peculiarities of membrane structure. Whatever its meaning, it is striking that an identical granularity appears in both stroma and recombinate.

DISCUSSION

Conditions of solubilization and recombination

In the present work, 2-chloroethanol was used to dissolve red cell membranes, as this solvent offers the possibility to separate in solution the bulk of the membrane lipids from the lipid-free membrane proteins.

It has been shown that membrane proteins isolated by this method retain to a large extent their lipid-binding capacity, thus offering the possibility to study the recombination product with respect to chemical and morphological criteria.

Most investigators who have attempted lipoprotein reconstitution have added micellar lipids, obtained by sonication, in aqueous solution to lipid-depleted proteins[20,17,18,12,10]. Under these conditions the binding must occur between the protein molecules and smaller or larger lipid micelles. In the procedure employed in the present study, proteins and lipids were dissociated and fractionated in an organic solvent and were brought together in the same medium; hence they both remained dissociated until, by dialysis, the solvent was gradually replaced by water. This procedure thus offers more favorable conditions for the interaction of proteins and lipids at the molecular level than the above-mentioned method.

As already mentioned, a similar recombination technique using methanol as a solvent has recently been described by JI AND BENSON[13] who demonstrated the specific binding capacities of chloroplast protein.

Density gradient studies

KORN[40] and JI AND BENSON[13] have proposed the idea that membrane proteins possess rather precise information as to the quantity and nature of lipids they will bind. The similarity of recombinate and stroma with respect to the protein and lipid composition (Figs. 5 and 6) would indeed lend support to this hypothesis. It must be considered, however, that the lipid binding may also be a random process reflecting the proportions of the lipid classes present during dialysis with the lipid-free protein. The analyses of the lipid and the lipoprotein bands after density gradient centrifugation (Table I and Figs. 4 and 5) are in support of this idea. Experiments using lipid mixtures of varying composition to elucidate this question are under way.

The scattering of the densities and of the ratios between protein and main lipid classes in the recombinates may also point to a random process of reassociation, but it may also be the consequence of inclusion of varying amounts of pure lipid micelles within the clusters of membrane vesicles as seen in Figs. 10–13. This effect which as yet is uncontrollable would indeed explain the large band width of the recombinates in the density gradient as well as the variations of the protein/lipid ratios.

With regard to the cholesterol, it may be seen that red cell membrane looses this component during dialysis and density gradient centrifugation in higher proportions than, for example, the phosphatides. It is well known that cholesterol belongs to the loosely bound membrane lipids[41] and also that it shows the highest exchange rate[42]. It is therefore not surprising that in recombined membrane lipoprotein, this compound is present in smaller amounts than in native red cell membrane (Table I and Fig. 4). The lower values for glycolipids in the recombinate (both floating free lipid and protein-bound lipid, Fig. 6) is due to the technique of FOLCH *et al.*[26], as it is well known that during the washing procedure, glycolipids are extracted.

The fact that 30–40 % of the membrane proteins are not recovered in the lipoprotein band of the density gradient indicates that some of the proteins do not recombine or, alternatively, that alle proteins only partially recombine with lipids. The failure to show by disc electrophoresis (Fig. 6) dissimilarities of the proteins in stroma and in the recombined material is in favour of the latter possibility. From Table II it can be seen that during dialysis more than 90 % of the protein becomes insoluble when Tris or phosphate buffer is used at neutral pH. 50–70 % of this insoluble material, according to the density gradient centrifugation, is lipoprotein, and the remaining

amount therefore corresponds to lipoprotein with high or low lipid content or to lipid-free protein.

The question as to how much the structure of the proteins is influenced by the interaction with lipids can, as yet, not be answered. The electron microscopic findings, as well as previously reported results on different blood group activities of recombined erythrocyte membranes[1,2], support the possibility that lipids may act as structural cofactors of membrane proteins. In addition, preliminary results of measurements of circular dichroism and nuclear magnetic resonance of recombined material, which will be published elsewhere[9], are in favour of at least a partial restoration of the native protein structure during recombination. On the other hand, the membrane is certainly not fully restored since no enzymatic activity has as yet been found[4].

Structural findings

Electron microscopy of chemically fixed preparations revealed the reoccurrence of membrane-like structures in the recombinate, whereas these were missing in pure protein and lipid fractions. The recombined membranes closely resembled the original stroma membranes in terms of thickness and structure. Whereas the ghost membranes appeared as broad shells corresponding to partially collapsed emptied sacs of the red cells[39], the recombined membranes occurred in the form of vesicles of varying size. In some instances, though rarely, short pieces of membranes with free edges were observed. These membrane vesicles had a tendency to form concentric shells which could develop to huge multilayered whorls. Such formations could, however, be clearly differentiated from multilayers of phospholipids, as the membranes always remained separated by irregular gaps containing some amorphous material. These observations on the structure of recombined membranes are in agreement with the recent findings of RAZIN *et al.*[43] on reaggregated membranes of *Mycoplasma laidlawii* which also had a tendency to form side-by-side aggregation.

Larger aggregates of these recombined membranes often contained myelin figures showing a regular spacing of 4.5–5 nm; these were presumably composed of highly concentrated phospholipids. In some dense portions of larger aggregates (Fig.5), no membrane structure and no spacing of myelin figures could be discerned. This may be due to oblique sectioning; however, it cannot be excluded that this may represent aggregation of proteins which have partially or not at all recombined with lipids. These two observations may explain the wider variation in the density of recombinate as compared to stroma preparations (Table I and Figs. 3 and 4).

The restoration of a membrane structure which closely resembles the original stroma, by recombining proteins and lipids isolated from red cell stroma, is in line with the results of recombination experiments on membranes of mitochondria[20] and *Mycoplasma laidlawii*[21].

REFERENCES

1 P. H. ZAHLER, *Vox Sanguinis*, 15 (1968) 81.
2 P. H. ZAHLER AND E. R. WEIBEL, *Protides Biol. Fluids, Proc. Colloq.*, 16 (1968) 71.
3 P. H. ZAHLER AND D. F. H. WALLACH, *Biochim. Biophys. Acta*, 135 (1967) 371.
4 P. H. ZAHLER, D. F. H. WALLACH AND E. F. LÜSCHER, *Protides Biol. Fluids, Proc. Collog.*, 15 (1967) 69.
5 D. F. H. WALLACH AND P. H. ZAHLER, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 1552.
6 A. H. MADDY AND B. R. MALCOLM, *Science*, 150 (1965) 1616.

7 A. H. MADDY, *Biochim. Biophys. Acta*, 117 (1966) 193.
8 A. F. REGA, R. I. WEED, C. F. REED, G. G. BERG AND A. ROTHSTEIN, *Biochim. Biophys. Acta*, 147 (1967) 297.
9 P. H. ZAHLER AND D. F. H. WALLACH, in preparation.
10 S. SCANU, *J. Biol. Chem.*, 242 (1967) 711.
11 S. H. RICHARDSON, H. O. HULTIN AND D. E. GREEN, *Proc. Natl. Acad. Sci. U.S.*, 50 (1963) 821.
12 S. H. RICHARDSON, H. O. HULTIN AND S. FLEISCHER, *Arch. Biochem. Biophys.*, 105 (1964) 254.
13 T. H. JI AND A. A. BENSON, *Biochim. Biophys. Acta*, 150 (1968) 686.
14 J. W. CALLAHAN AND G. W. KOSICKI, *Can. J. Biochem.*, 45 (1967) 839.
15 P. C. CHAN, *Biochim. Biophys. Acta*, 135 (1967) 53.
16 D. E. GREEN AND S. FLEISCHER, *Biochim. Biophys. Acta*, 70 (1963) 554.
17 G. MAYER AND H. STAUDINGER, *Z. Physiol. Chem.*, 348 (1967) 599.
18 G. G. DE PURI AND F. D. CALLIUS, *Chem. Phys. Lipids*, 1 (1966) 1.
19 T. M. TERRY, D. M. ENGELMAN AND H. J. MOROWITZ, *Biochim. Biophys. Acta*, 135 (1967) 391.
20 D. G. MCCONNELL, A. TZAGOLOFF, D. H. MCLENNAN AND D. E. GREEN, *J. Biol. Chem.*, 241 (1966) 2373.
21 D. M. ENGELMAN, T. M. TERRY AND H. J. MOROWITZ, *Biochim. Biophys. Acta*, 135 (1967) 381.
22 R. F. A. ZWAAL AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 150 (1968) 323.
23 J. T. DODGE, C. MITCHELL AND D. J. HANAHAN, *Arch. Biochem. Biophys.*, 100 (1963) 119.
24 E. A. AZEN, S. ORR AND O. SMITHIES, *J. Lab. Clin. Med.*, 65 (1965) 440.
25 O. H. LOWRY, N. J. ROSEBROUGH, L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 19 (1951) 265.
26 J. FOLCH, M. LEES AND G. H. SLOAN-STANLEY, *J. Biol. Chem.*, 226 (1957) 497.
27 G. R. BARTLETT, *J. Biol. Chem.*, 234 (1959) 466
28 H. JATZKEWITZ, *Z. Physiol. Chem.*, 326 (1961) 61.
29 N. CHIAMORI AND R. J. HENRY, *Am. J. Clin. Pathol.*, 31 (1959) 305.
30 O. RENKONEN, T. U. KOSUNEN AND O. V. RENKONEN, *Ann. Med. Exptl. Biol. Fenniae Helsinki*, 41 (1963) 375.
31 D. M. NEVILLE, *Biochim. Biophys. Acta*, 133 (1967) 168.
32 G. E. PALADE, *J. Exptl. Med.*, 95 (1952) 285.
33 M. G. FARQUHAR AND G. E. PALADE, *J. Cell Biol.*, 26 (1965) 263.
34 J. H. LUFT, *J. Biophys. Biochem. Cytol.*, 9 (1961) 409.
35 H. D. COULTER, *J. Ultrastruct. Res.*, 20 (1967) 346.
36 A. FUKAMI AND K. ADACHI, *J. Electron Microscopy*, 14 (1965) 112.
37 L. J. MOLENAAR AND B. SCHOTANUS, *Sci. Ind.*, 9 (1962) 24.
38 E. S. REYNOLDS, *J. Cell Biol.*, 17 (1963) 208.
39 V. T. MARCHESI AND G. E. PALADE, *J. Cell Biol.*, 35 (1967) 385.
40 E. D. KORN, *Science*, 153 (1966) 1491.
41 B. ROELOFSEN, J. DE GIER AND L. L. M. VAN DEENEN, *J. Cellular Comp. Physiol.*, 63 (1964) 233.
42 I. M. LONDON AND H. J. SCHWARTZ, *Clin. Invest.*, 32 (1953) 1248.
43 S. RAZIN, Z. NE'EMAN AND I. OHAD, *Biochim. Biophys. Acta*, 193 (1969) 277.
44 S. P. L. SÖRENSEN, *Ergeb. Physiol.*, 12 (1912) 393.
45 G. S. WALPOLE, *J. Chem. Soc.*, 105 (1914) 2501.