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Isolation of lipid-free plasma membrane proteins by gel filtration on Sephadex LH-20 using 2-chloroethanol–water as solvent

The purification and characterization of the proteins of cellular membranes have been severely hindered by their high lipid affinity and by their very limited solubility in the aqueous and organic solvents hitherto employed. However, in the course of spectroscopic investigations or protein conformations in cellular membranes[1], we observed that the plasma membranes and endoplasmic reticulum membranes of Ehrlich ascites carcinoma and of a number of other cell types dissolve readily in 2-chloroethanol–water (9:1) at pH <3. This solvent system promotes the formation of both α-helix and β-conformation in membrane proteins and, in effecting their dissociation from membrane lipids, allows separation of these membrane components by gel permeation chromatography on the organophilic polydextran Sephadex LH-20.

Plasma membrane and ribosome-free endoplasmic reticulum were isolated as described previously[2,3]. 2-Chloroethanol (b.p. 127–129°) was selected to be transparent to 280 mμ and to have an absorbance at 260 mμ not exceeding 0.15. Most lots of "highest purity" 2-chloroethanol do not meet these specifications and must be purified by fractional distillation. Freshly redistilled 2-chloroethanol has an apparent pH of about 4 (glass electrode), but this drops to about pH 2 within a few days (due to release of HCl), even when stored in the dark at 0°. 2-Chloroethanol is highly toxic and one must guard against skin contact and inhalation. Protein determinations were by the ninhydrin procedure[4] using crystalline bovine serum albumin as reference standard. However, both proteins and aminophosphatides contribute to the ninhydrin value of the membranes. The ninhydrin color of both standards and samples was developed at a final 2-chloroethanol concentration of 10% (v/v) and was about 5% lower than without this solvent. Storage of samples and standards in acid 90% 2-chloroethanol for 12 h at room temperature did not affect their color yields detectably. Total phosphorus was by the method of LOWRY *et al.*[5], proportioned to a final volume of 1 ml. Lipid phosphorus was estimated in an identical manner on extracts prepared by the method of FOLCH, LEES AND SLOAN-STANLEY[6]. Thin-layer chromatography was according to WAGNER *et al.*[7], L-α-(dipalmitoyl)-cephalin, L-α-(dipalmitoyl)lecithin and purified beef brain sphingomyelin all from Mann Research Laboratories Inc. and cholesterol from Eastman Kodak Chemicals were used as reference standards.

Membrane solutions in 2-chloroethanol were prepared as follows: Membrane suspensions in 0.25 M sucrose were diluted to a concentration of about 2 mg protein per ml with 0.01 M Tris–HCl (pH 8.0) and centrifuged for 45 min at 50 000 rev./min at 4° (Spinco-L-2 ultracentrifuge; rotor SW50). The packed membranes were homogenized in 5 ml deionized water and resedimented. The new pellets were dispersed in water to give a suspension of about 10 mg protein per ml. This was mixed vigorously with 5 vol. of 2-chloroethanol, leading to rapid solution of the membrane material. The membrane solutions were then concentrated five-fold under a stream of nitrogen at room temperature. After this step the proportion of 2-chloroethanol is about 95% and the apparent pH (glass electrode) about 2. (Further concentration leads to precipitation of membrane protein.)

The membranes are poorly soluble at apparent pH above 5. Neutralization of acid membrane solutions leads to precipitation of the membrane protein. Solubility in 2-chloroethanol–water mixtures increases markedly below pH 5 and is greatest at pH 2 and below, where the membrane proteins carry a net positive charge[8]. Unlike myelin[9], the membranes have no protein components soluble in neutral or acid chloroform–methanol (2:1). Complete solution of the membranes is already observed in 2-chloroethanol–water (1:1, v/v) at acid pH, but only at low protein levels. Moreover, at the high proportions of 2-chloroethanol the membrane lipids are in true solution (rather than micellar suspension) as evidenced by their retardation on Sephadex LH-20, which excludes particles with molecular weights in excess of 1000.

For gel filtration Sephadex LH-20 was packed into columns after swelling in 2-chloroethanol–water for 16 h. Column dimensions were 2.5 cm × 58 cm. When applied in a volume of 1 ml, excluded material (india ink) was eluted between 95 and 105 ml and included substances (fluorescein) between 175–205 ml. The flow rate was 20 ml/h at 25° and 12 ml/h at 0°. Elution patterns were identical at these two tem-

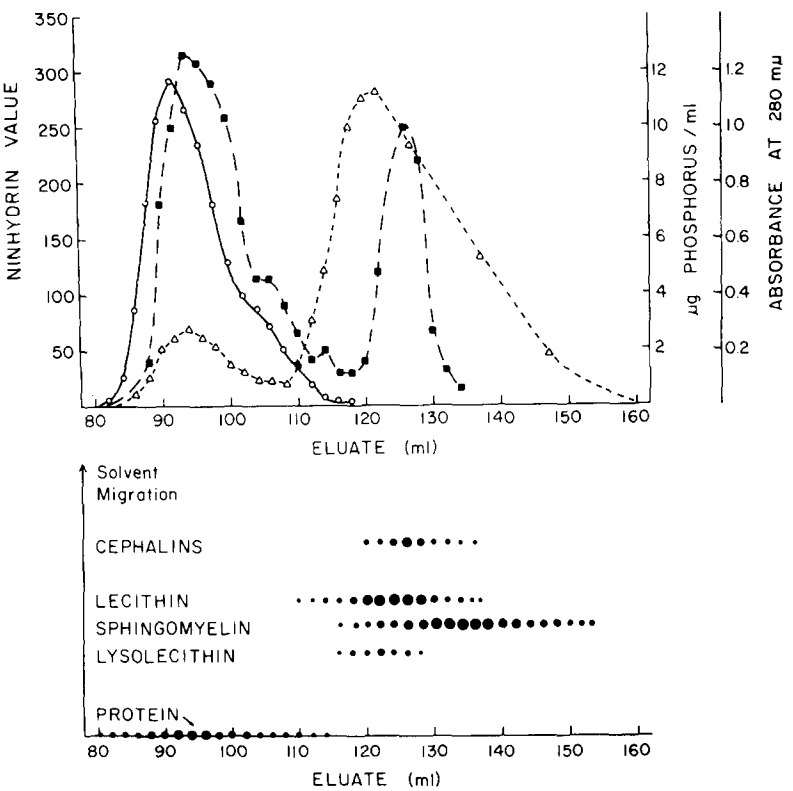

Fig. 1. Gel filtration of plasma membrane on Sephadex LH-20 using 2-chloroethanol–water (9:1) as solvent. 11 mg membrane protein were applied in a volume of 1 ml unto a 2.5 cm × 58 cm column. Details of column operation in text. *Upper panel:* ○—○, $A_{280\ m\mu}$; ■—■, ninhydrin value in mg "protein" per ml using crystalline serum albumin as reference standard; △—△, total phosphorus. *Lower panel:* Schematic representation of thin-layer chromatography of eluted fractions on Silica Gel G (E. Merck, Darmstadt). Solvent: chloroform–methanol–water (60:35:8). Coloring agents: General-I_2 vapor; proteins and amino-phosphatides–ninhydrin spray.

peratures. For membrane fractionation the columns were washed with 2-chloroethanol–water (9:1) until the absorbance of the eluate was equal to that of the solvent. Samples were applied in a volume of 1 ml and 2.0-ml fractions were collected automatically. LH-20 is hydrolysed slowly in acid solvents. Therefore, if the gel is to be re-used, it is extruded into water after elution of the lipid. The slurry is then neutralized, washed with water, then with acetone and finally dried in air.

Fig. 1 illustrates a fractionation typical of 3 experiments each with plasma membrane and endoplasmic reticulum of Ehrlich ascites carcinoma. As shown by the absorbance at 280 mμ and by the ninhydrin reaction, membrane proteins were eluted mainly within the excluded volume, but there was slight trailing of the protein peak. Thin-layer chromatography of the fractions comprising the excluded volume showed only non-lipid material (at the application sites). Phosphatides appeared as a broad peak starting immediately after the protein. The ninhydrin reaction in this peak is due to the amino-phosphatides. Cholesterol was eluted last (after 170 ml) and was fully separated from the phosphatides.

On the basis of absorbance at 280 mμ, 95% of the protein applied was eluted prior to the appearance of lipid. 42% of the ninhydrin-reactive material was recovered in the protein peak and 53% in the pooled lipid fractions. 12% of total phosphorus was in the protein peak. This could not be extracted into chloroform–methanol (2:1) and is in part attributed to the traces of non-ribosomal RNA which are consistently found in the tumor membrane fractions, but not in erythrocyte ghosts[10]. All of the phosphorus in the lipid fraction could be extracted into chloroform.

We have studied the solubility characteristics of the plasma membrane protein fraction with the aim of finding aqueous solvent systems suitable for further fractionation. These experiments were performed as follows: 1-ml samples of the pooled membrane protein in 2-chloroethanol–water (9:1)(about 0.5 mg/ml) were transferred into various solvents by equilibrium dialysis. Thereafter the absorbances at 400 mμ (turbidity) and 280 mμ were determined. The samples were then centrifuged at 16 000 rev./min and 4° for 45 min (Lourdes Betafuge, rotor 9 RA 24) and the absorbances of the supernatants measured.

The results (Table I) indicate that the lipid-free plasma membrane proteins are extensively soluble in 3 M KI, 7 M guanidine–HCl and 8 M urea at neutral pH. There appears to be appreciable solubility also in 0.01 M NaOH and somewhat less in

TABLE I

ABSORBANCES AT 400 mμ (TURBIDITY) AND 280 mμ OF LIPID-FREE PLASMA MEMBRANE PROTEIN, BEFORE AND AFTER CENTRIFUGATION FOLLOWING EQUILIBRIUM DIALYSIS AGAINST VARIOUS AQUEOUS SOLVENTS

Solvent	*Before centrifugation*		*After centrifugation*	
	$A_{400\ m\mu}$	$A_{280\ m\mu}$	$A_{400\ m\mu}$	$A_{280\ m\mu}$
H_2O	Flocculated		0.000	0.013
KI (3 M)	0.014	0.417	0.007	0.400
Guanidine–HCl (7 M)	0.006	0.345	0.006	0.350
Urea (8 M)	0.001	0.340	0.000	0.335
NaOH (0.01 M)	0.055	0.488	0.024	0.496
HCl (0.01 M)	0.025	0.410	0.023	0.283

0.01 M HCl, but the residual turbidity in these cases points to the presence of molecular aggregates.

We are now evaluating the suitability of such solvent systems for the fractionation of membrane proteins by gel filtration, ion-exchange chromatography and electrophoresis and also the reversibility of the conformational and other changes wrought by 2-chloroethanol. In this connection we note that the native conformation and activity of ribonuclease can be fully recovered after exposure to 100% 2-chloroethanol at acid pH[11].

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