ISOLATION OF AN ENCEPHALITOGENIC PHOSPHOLIPID-PROTEIN COMPLEX BY DIALYSIS OF MYELIN IN ORGANIC SOLVENTS

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Chemical studies of myelin have been complicated until recently by the difficulty of preparing this substance free from other tissue elements. However, Laatsch et al. (1962) devised an ultracentrifugal method for concentrating myelin and achieved a preparation containing minimal amounts of other cellular particles. A modification of this technique resulted in myelin completely soluble in chloroform:methanol (2:1), Laatsch (1963). The present report describes dialysis of chloroform-methanol solutions of myelin resulting in the isolation of a highly encephalitogenic lipid-protein complex.

Several reports on dialysis in organic solvents have been published but each has presented one or more of the following difficulties: prolonged dialysis time, lack of stability of the membranes in the solvents used, or use of relatively large quantities of solvents and solutes, Eberhagen and Betzing (1962), Legault-Demare and Faure (1951), Hakomori and Takeda (1961), Olmstead (1960), and van Beers et al. (1958). The simplicity and

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relative speed of the microtechnique described in this report suggest its potential usefulness in molecular and micellar studies of lipids and lipid-protein complexes.

The techniques described are analogous to those of Craig, who has thoroughly developed the method of film dialysis in aqueous systems, Craig and King (1962). The same techniques are capable of achieving reproducible kinetic studies in organic solvents. Visking cellophane dialysis tubing is used with the precautions suggested by Craig, namely, tubing for all comparable experiments is from the same roll of cellophane which is kept carefully wrapped and refrigerated; and when knots are tied, tightening is done from the end to be discarded, minimizing distortion of pores on the functional side. The knotted strips of tubing are soaked in water $\geq$ two days and then ethanol $\geq$ two days. For dialysis, a glass rod is inserted in the cellophane bag, still in the ethanol; then the whole unit is quickly transferred to a 165 x 20 mm glass tube filled with the appropriate solvent and fitted with a ground glass joint. The glass rod is removed and a 0.5-1.5 ml sample in the same solvent is placed in the bag for dialysis. The bag collapses, forming a film less than a mm in thickness. The part of the bag protruding from the test tube is slit, folded over the rim, and a glass stopper is inserted. Dialysis proceeds under nearly ideal conditions, with a large surface to volume ratio of retentate, promoting much more rapid separation than usual in organic systems. Diffusate may be changed simply by lifting the tubing, made semi-rigid by the effect of solvent, and placing it in a tube of fresh solvent. These changes must be made as quickly as possible since even partial drying of the membrane causes pore shrinkage. The apparatus is shown in Fig. 1.

Guinea pig myelin, prepared by the method of Laatsch et al. (1963), was made up to approximately 10 mg/ml in dry chloroform:

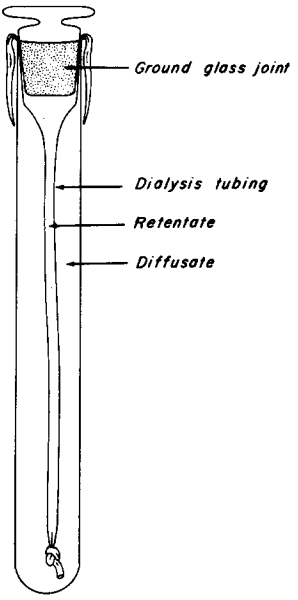

Fig. 1 - Apparatus for microdialysis in organic solvents.

methanol (2:1 v/v redistilled solvents). One ml aliquots were dialyzed in duplicate chambers versus a diffusate volume of 25-27 ml dry 2:1 chloroform-methanol. At appropriate intervals fresh chloroform-methanol was used to replace the diffusates, which were evaporated in tared glass tubes under nitrogen at 40-50° C. After twelve days total dialysis time, the final retentate was rinsed from the bag and similarly dried. All samples were put in a desiccator overnight. Fig. 2 shows the per cent retentate remaining at various time intervals.

As the figure indicates, the diffusion process can best be explained by assuming a complex mixture of substances of varying sizes. This curve can be approximated by an expression for sums of exponentials obtained by the curve-peeling technique, Solomon (1960): $r = 20.0e^{-0.173t} + 4.9e^{-0.034t} + 20.0e^{-0.00101t}$ where r = % original weight left as retentate. The interaction of the major components of myelin must be reflected in this expression. If all are molecularly disperse, the first term of the equation

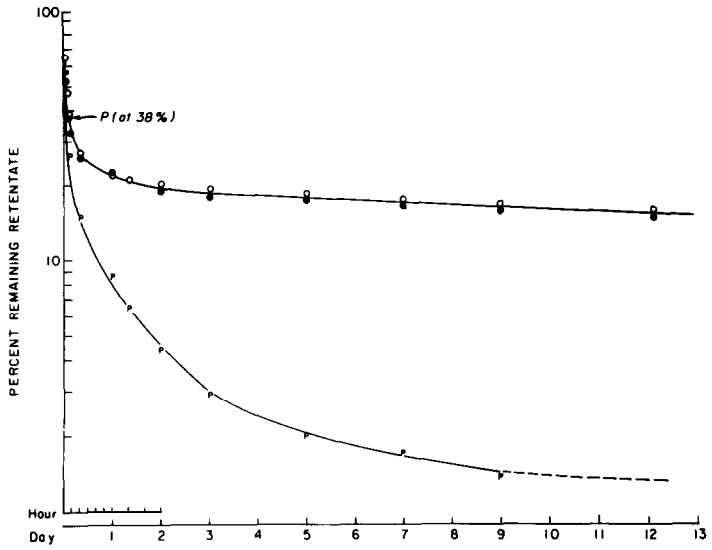

Fig. 2 - Dialysis of guinea pig brain myelin in 2:1 chloroform-methanol. Starting concentration, 12.9 mg/ml retentate in Visking dialysis tubing #20/32. Dialysis carried out at room temperature. Ordinate: per cent total weight solute remaining as retentate, calculated by differences from diffusates. Upper curve: o and ● are weights obtained in duplicate experiments. Total recovery (weight retentate and $\leq$ diffusate weights) was 95% for o and 99% for ●. Lower curve: Average of duplicate phosphorus values.

might represent cholesterol, the second term phospholipids plus cerebrosides, and the third term lipid-protein complexes. Even weak interactions, however, would create a more complex situation. By studying dialysis rates of known systems, we hope to analyze the experimental curve in terms of its individual components.

The soluble myelin preparation used for these experiments consisted of 17% protein, 40% phospholipid (1.6% P) and 18% cholesterol, Laatsch et al. (in press). The remaining 25% probably consisted mainly of cerebrosides. Cholesterol, P, and protein determinations were carried out on all samples obtained in sufficient quantity. The data in Fig. 3 indicate that essentially all the cholesterol diffuses out in the first hour. Phosphorus, representing phospholipids almost exclusively, follows more slowly.

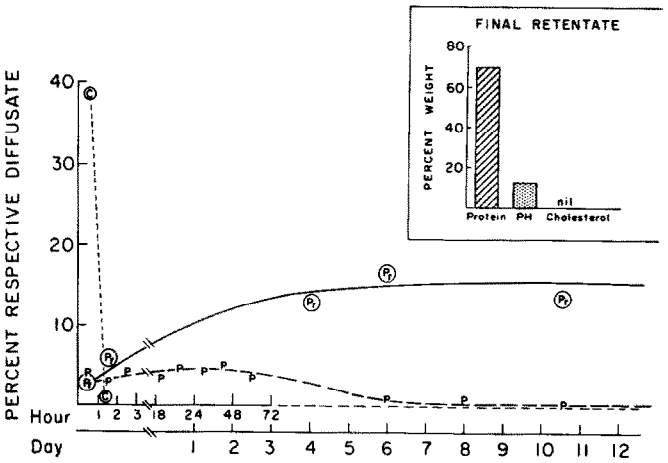

Fig. 3 - Composition of fractions obtained by dialysis of myelin in chloroform-methanol. For conditions of experiment see Fig. 2. Legend: C--cholesterol, method of McDougal and Farmer (1957); P--phosphorus, Bartlett (1959); Pr--protein, Lowry et al. (1951); PH--phospholipids (P x 25). Each point on the curves represents the value for an individual diffusate and was arbitrarily placed at the middle of the time period over which the sample was collected. The curves were drawn freehand to provide the smoothest fit.

Its concentration in the diffusate rises to a maximum at three hours, plateaus for three days, and then declines to a trace. Proteins appear slowly and reach a constant proportion of the diffusate after four to five days. The protein-like material in relatively high concentration in the early samples, e.g. 2.5% of the one hour diffusate, may be the phosphatidyl peptides originally described by Folch and LeBaron (1953). Most striking was the fact that the retentate, after twelve days of dialysis, contained 70% protein and 0.49% P. This very faintly opalescent solution inside the dialysis bag became crystal clear on dilution with an equal amount of 2:1 chloroform-methanol. Such preparations, which are believed to be solutions of phospholipid-protein complexes, have remained stable for several weeks at 4° C. Thin layer chromatography on silica gel in 95% 2:1 chloroform:methanol - 5% water showed that the retentate remained as a single, well-defined spot

at the origin, giving positive stains for phosphorus, protein, and choline, but not for inositol, cholesterol, reducing sugars, or plasmalogens. Myelin chromatograms have an identical spot as well as spots identified as various phospholipids, cerebrosides and cholesterol. Diffusate chromatograms had only a faint spot at the origin but the rest of the constituents of whole myelin were clearly visible. Full details of these results will be reported separately.

In his studies on proteolipids, Folch (1959) reasoned that bonding must exist between proteins and lipids because of their solubility in moist chloroform-methanol and because of the constant analytic values obtained on repeated preparations. Solubility of protein in moist chloroform-methanol, however, is not absolute proof of lipid-protein bonding. Although in the present experiments a classical proteolipid preparation has not been studied, the lipid-protein complex from chloroform-methanol soluble myelin, studied by dialysis combined with thin layer chromotography, has provided unequivocal proof that such bonds do exist.

As Fig. 2 indicates, dialysis for three days results in almost complete separation of free lipids from protein-complexed lipids. Such a retentate and diffusate were dried in a nitrogen stream, weighed, and uniformly suspended with a glass homogenizer in physiologic saline. Aliquots were combined with Freund's adjuvant and assayed for encephalitogenic activity in guinea pigs in the usual manner, Alvord and Kies (1959). The dose per animal was 22.5 μg retentate or 21 μg diffusate. Of those animals injected with retentate, four-fifths became paralyzed within three weeks and all five showed marked histologic evidence of allergic encephalomyelitis. None of the animals receiving diffusate showed clinical signs and only one of the five had equivocal histological evidence.

We conclude from these data that there exists in guinea pig myelin a chloroform-methanol soluble phospholipid-protein complex which can be separated from the accompanying free lipids by a dialysis technique which possesses the advantages of simplicity, relative speed, and extreme sensitivity. The entire encephalitogenic activity present in whole myelin resides in this non-dialyzable lipid-protein complex.

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