CHEMICAL AND ULTRASTRUCTURAL COMPARISON OF SYNTHETIC AND PATHOLOGIC MEMBRANE SYSTEM

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Characteristic membranous bodies have been previously reported in the cytoplasm of the cerebral neurons in Tay-Sachs disease (1) (Fig. 1). Subsequently (2) these lamellated particles were isolated from homogenized autopsy tissue by differential and density gradient centrifugation and subjected to extensive chemical analysis. The membranous cytoplasmic bodies (MCB) were found to consist of about 50 per cent (dry weight) gangliosides and could account for the accumulation of this complex sphingolipid which characterizes this lipid-storage disease. The appearance and composition of the MCB suggested: (1) that they might arise from an alteration of cytoplasmic organelles, or (2) that they were formed spontaneously by interaction of the component lipids in a fashion analogous to myelin figure formation (3). Chemicals approximating the known composition of the MCB were combined in proper proportion *in vitro* as a test of this latter hypothesis. The resultant aggregates were examined with light and electron microscopy.

MATERIALS AND METHODS

Values for the chemical composition of the isolated MCB (2) were averaged (Table I) and these figures used for composing the synthetic mixture. Bovine gangliosides (4) were substituted for the chloroform and water-soluble gangliosides of the Tay-Sachs material. A tryptic hydrolysate of casein ("Trypticase," Baltimore Biological Laboratories) was used to approximate the amino acid, peptide, and protein portion of the native material. The phospholipid

preparation[1] was isolated from beef brain (5). The cerebroside mixture[2] was also from this source (6) The cholesterol was commercial (Merck).

The artificial intracellular medium was prepared from the stock Krebs-Ringer—phosphate solutions (7) with the volume proportions adjusted to give high potassium to low sodium and high magnesium

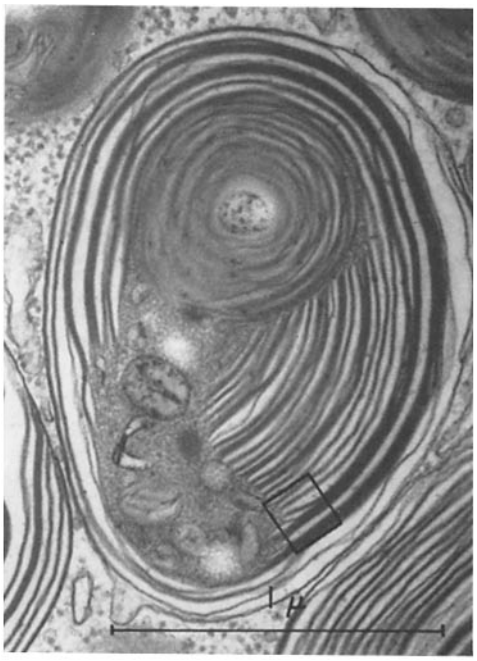

FIGURE 1 Membranous cytoplasmic body (MCB) of Tay-Sachs disease. Cortical biopsy, × 44,000. Epon embedded. Courtesy R. D. Terry. (Reprinted, with permission, from the *J. Neuropathol. and Exp. Neurol.*, 1963, **22**, 18).

to low calcium ratios. Additional potassium dihydrogen phosphate was added to bring the pH to 6.5. The ionic composition in millimoles per liter was: K, 136; Na, 51; Mg, 4.6; Ca, 1.5; Cl, 185; HPO_4, 3.5; SO_4, 4.6.

Except for the phospholipids, the organic constituents were combined as a dry mix. The phospholipids were then added in chloroform solution (50 mg/ml) which largely solubilized the other components. Addition of the phospholipids in this way was found to be quite critical. The suspending salt medium was then added to give a total organic concentration of 1 mg/ml. Mixing in a vortex-type stirrer was adequate to produce an opalescent suspension without any sediment. After 3 to 4 hours at room temperature, with occasional gentle agitation, the suspension was transferred to the 4°C cold room. Samples were taken at intervals for observation under the phase microscope. After 24 hours an aliquot was centrifuged and the pellet fixed in 2 per cent osmium tetroxide buffered to pH 7.3 with veronal acetate (8). It was then rapidly dehydrated in a graded series of alcohols and embedded in 9:1 butyl-methyl methacrylate with 1 per cent benzoyl-peroxide as catalyst. Thin sections mounted on Formvar-covered grids were stained with lead hydroxide and carbon coated (9, 10). They were examined with a Siemens Elmskop 1. This is the procedure used previously in the study of the isolated MCB (2).

TABLE I

Composition of Isolated Tay-Sachs Membranous Bodies and the Synthetic Mixture

	Tay-Sachs MCB*		Synthetic Mixture§
	Batch I	Batch II	
	per cent	*per cent dry wt.*	*per cent dry wt*
Gangliosides‡	58.6	53.9	56.6
Cholesterol	17.7	26.9	22.4
Phospholipids	8.8	12.8	11
Cerebrosides	4.9	3.9	4
Amino acids	2.0	2.5	6
Protein	8.0	0	

* Adapted from Samuels *et al.* (2).
‡ Calculated by difference. Structural uncertainties result in values of 40 to 70 per cent based on hexosamine, sialic acid, or hexose determinations.
§ See text for details.

OBSERVATION AND DISCUSSION

In samples taken after 2 hours' incubation, at room temperature, numerous particles could be seen, in phase contrast, of the same size range (0.5 to 2 μ) and optical density as MCB. In addition, they gave the same characteristic staining reaction with Laskey's thionin (11) at 60°C: a pale violet which appears intensely blue-green under phase contrast. In Tay-Sachs and normal tissue, only the nucleoli and, to a lesser extent, disrupted myelin also show this response.

Examination of the ultrastructure of the synthetic preparation revealed several spherical forms

[1] Lecithin, 50 per cent; phosphatidylethanolamine, 24 per cent; phosphatidylserine, 15 per cent; sphingomyelin (by difference), 11 per cent; phosphoinositides, 0 per cent.

[2] Cerasine, 63 per cent; phrenosine, 27 per cent; cerebroside esters, 4 per cent; phospholipids, 1.2 per cent.

closely resembling the intracellular MCB, as well as membranous arrays in large quantity (Figs. 2 and 3). The uniformity of the material was verified by the study of more than 100 grids cut at different depths from fifteen blocks prepared from two separate experiments. The center of these artificial bodies generally appeared rather amorphous with rudimentary lamellae discernible here at the white line. These arrays were mostly linear with only an occasional suggestion of a concentric arrangement. Revel, Ito, and Fawcett (13) formed myelin figures with an egg lecithin preparation. Their material consisted most commonly of multilayered tubes, but spherical multilaminar forms were also reported. However, these were quite gross, 10 μ or larger. The membranes were com-

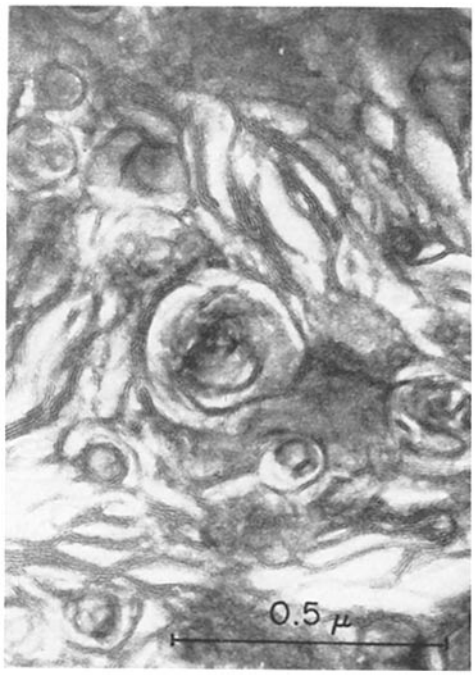

FIGURE 2 Artificial membranes. × 71,500.

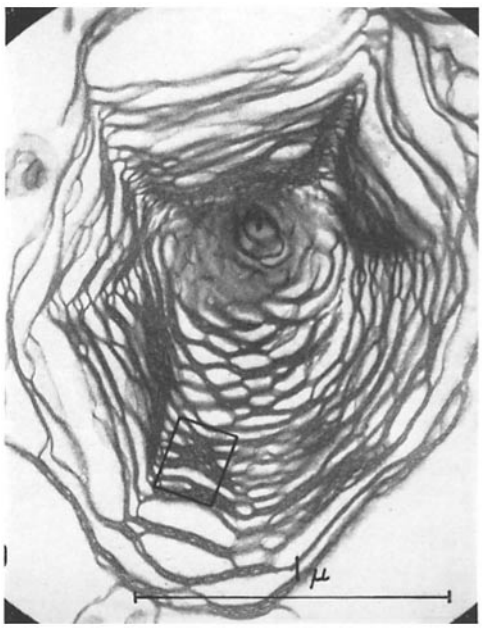

FIGURE 3 Artificial membranous body. × 41,000.

and there. On the average, the over-all period measured 60 to 72 A, with a dense line of 25 to 33 A and a light line of 22 to 36 A. These dimensions compare favorably with the corresponding values of the MCB: 50 to 60 A period, 26 to 31 A dense line, and 22 to 28 A light line (Fig. 4 *a*, *b*). As a further point of similarity, fusions of the unit membranes were found to proceed by the merging of adjacent dark lines to form a darker line in both the MCB and artificial bodies.

These membranes bear only a superficial resemblance to the brain-phospholipid myelin figures prepared by Stoeckenius (12). The phospholipid preparations of Stoeckenius had a period of 35 to 53 A with an 18 to 20 A dark line and a 20 to 22 A light line. Furthermore, it was reported that splitting of adjacent membranes always occurred posed of 25 to 30 A dark lines with light lines of the same size, but adjacent membranes were often widely spaced.

The formation of these particles contradicts the proposal of Lazarus *et al.* (14) that the MCB are lysosomes. The lack of protein in one batch of MCB (2) also argues against this view. The presence of myelin figures within acid phosphatase-positive cytoplasmic organelles, characterized by a surrounding unit membrane (lysosomes), has been reported elsewhere (15, 16). The MCB differ from these organelles in the absence of a separate, surrounding unit membrane, although some of them do show associated acid phosphatase activity (17), perhaps as a stage in their degradation.

It is of interest to consider the relevance of this spontaneous process to cellular pathology. Since

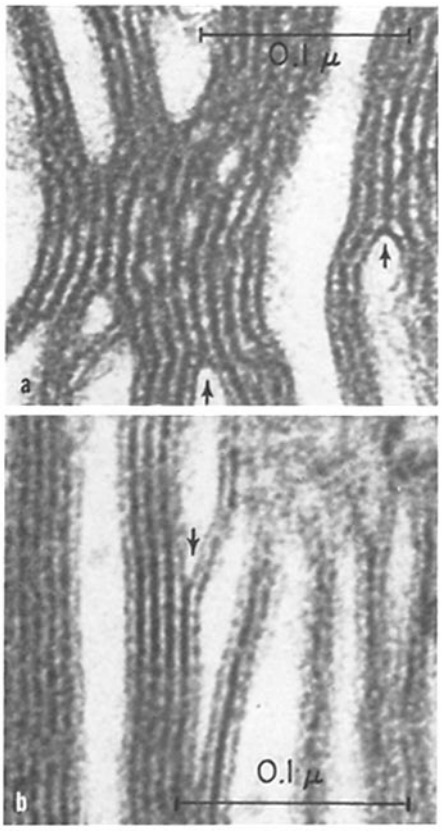

FIGURE 4 *a* Detail of artificial membranes. × 270,000
FIGURE 4 *b* Detail of MCB membranes. Epon embedded. × 300,000.

the components of the synthetic mixture are normal neuronal constituents, some mechanism prevents the formation of membranous lipid aggregates within healthy cells. Metabolic utilization and normal membrane formation may keep the concentration of free lipids at a low level. The rapid *in vitro* aggregation of phospholipids suggests that molecules such as lipoproteins may serve as "carriers" within the cell to transfer the various lipids from their site of cell entry or synthesis to their site of utilization. A similar process is believed to be operating in the bloodstream (18). A defect in this mechanism would be as injurious as the more typical enzymatic deficit with its consequent metabolic block.

Although the formation of structured aggregates by purified lipid preparations has been known for some time (19), alteration of the membrane dimension from that of the usual phospholipid period (35 to 53 A) had been previously achieved only by the addition of a large protein, globin, to the outermost layer (12). Recently, however, Stoeckenius found that the small protein, cytochrome *c*, could be incorporated into myelin figures with a thickening of all the dark lines and an average period of 68 A (20). In the present study, a defined mixture of gangliosides, phospholipids, cholesterol, cerebrosides, and amino acids and peptides, approximating the composition of the membranous cytoplasmic bodies of Tay-Sachs disease, has been shown to form membranous arrays with a period of 60 to 72 A. These are comparable to the measurements of the MCB. *In vitro* duplication of pathologic intracellular structures has not been reported before. The spontaneous assemblage of lipid molecules has been implicated as a factor in cell pathology, as previously suggested by Miller (21), and has been specifically indicated as an explanation for the membranous cytoplasmic bodies in the neurons in Tay-Sachs disease.

The authors express their thanks to the late Dr. Saul Korey and Drs. R. Katzman and W. T. Norton of the Department of Neurology, and to Dr. R. D. Terry of the Department of Pathology (Neuropathology), Albert Einstein College of Medicine, for providing the analyzed lipid mixtures, and for their encouragement and support.

Dr. Samuels is a Postdoctoral Fellow of the National Multiple Sclerosis Society. Dr. Gonatas is a Postdoctoral Fellow of the National Institute of Neurological Diseases and Blindness.

This research has been supported by grants from the United States Public Health Service (2B-5275) (C2), and (NB-02255-05) and the Cerebral Palsy Foundation (R-145-61).

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Received for publication, November 7, 1963.

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