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Characterisation of Cerebroside 3-Sulphate Dispersions

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Galactosyl ceramide 3-sulphate (cerebroside 3-sulphate) was tritiated using [^{3}H]$NaBH_4$ with $PdCl_2$ as catalyst. Quantitative purification of the three components of the product was achieved by chromatography on Florisil. Dispersions, prepared by either low energy sonication or by dilution from organic solvent, were compared by controlled pore glass chromatography, ultra-centrifugation and electron microscopy. Both dispersion techniques were shown to form unilamellar vesicles, the average size of vesicles produced by sonication being far larger than those produced by solvent dilution. The diameter of isolated vesicles produced by solvent dilution was in the range 10-80 nm.

The increasing use of dispersions of cerebroside 3-sulphate for the assay of cerebroside sulphatase (E.C.3.1.6.8., identical with sulphatase A, E.C.3.1.6.1.) necessitates a reappraisal of the dispersion techniques used and the physical nature of such dispersions. A variety of methods for the preparation of lipid vesicles has been described [1]. For sulphatides, dispersion by high-energy sonication has been widely used [2, 3] but lipid degradation, oxidation, aerosol formation and titanium contamination are inherent problems, as they are for phospholipid dispersion [4]. Prolonged low-energy sonication for phospholipid dispersion has been investigated in detail [5] but no such data is available for sulphatide dispersion.

An alternative approach has been developed where injection of an ethanolic solution of phospholipid into water, followed by concentration by ultrafiltration, gives single unilamellar vesicles, indistinguishable from those prepared by sonication [6-8]. This technique is attractive in that it is mild, reproducible and does not require large amounts of energy to break down lipid aggregates, a problem inherent in any ultrasonic technique.

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It has been suggested that highly charged lipids, such as sulphatides and phosphatidyl inositol, form micelles rather than bilayer structures on dispersion [9, 10] although they can be incorporated into bilayer structures when mixed with lecithin [11]. Other workers [3, 12] have assumed a micellar structure for sulphatide dispersions, although the possible formation of vesicular structures has been recognised [3]. Recent studies have shown that phosphatidyl inositol is able to form vesicles on dispersion by sonication [13].

We report here the preparation and purification of ^{3}H-cerebroside 3-sulphate and a comparison of two methods of dispersion in aqueous solution at pH 4.4, the optimum pH for cerebroside sulphatase activity. The dispersions have been characterised by chromatography on controlled pore glass beads, ultracentrifugation and electron microscopy.

Materials and Methods

Preparation of ^{3}H-Cerebroside 3-Sulphate

The procedure of Schwarzmann [14] as applied by Raghavan *et al.* [15] was adapted as follows. Cerebroside 3-sulphate (5 μmol, Supelco Inc., Bellefonte, PA, USA) in tetrahydrofuran (4.5 ml, freshly distilled from CaH_2) and $PdCl_2$ (150 μl, 25 mg/ml in water) were placed in a Thunberg tube. [^{3}H]Sodium borohydride (25 μmol at 2 Ci/mmol, Amersham International, Amersham, UK) in 125 μl of 1 M NaOH was placed in the side-arm and the assembled Thunberg tube connected through a three-way tap to a vacuum pump and a N_2-filled balloon. Alternate evacuation and filling with N_2 was repeated three times to displace all the air, the contents were mixed and the reaction allowed to proceed with vigorous stirring overnight. Addition of 300 μl of 0.5 M acetic acid in a fume hood destroyed any unreacted $NaBH_4$. The precipitated Pd was removed by centrifugation and washed three times with 2.5 ml portions of chloroform/methanol, 2/1 by vol. The pooled supernatant and washings were dried down under N_2, the residue dissolved in 5 ml chloroform/methanol, 2/1 by vol, and washed [16] to remove salts. Aliquots were counted by liquid scintillation counting to assess the degree of incorporation of tritium, correcting for quenching by an external standard method.

Purification of Cerebroside Sulphate on Florisil

Florisil (100-200 mesh, Sigma Chemical Co., Poole, UK) was freed of fines by decantation from 35% (v/v) methanol and, after drying, deactivated with water [19]. A column (0.8 × 14 cm) of deactivated Florisil was washed extensively with chloroform/methanol/water, 95/5/0.25 by vol, and crude ^{3}H-cerebroside sulphate (5 μmol) dissolved in 10 ml of the same solvent was applied. The column was eluted successively with (I) 80 ml chloroform/methanol/water, 95/5/0.25 by vol; (II) 60 ml chloroform/methanol/water, 92/8/0.5 by vol; (III) 90 ml chloroform/methanol/water, 88/12/1.4 by vol; (IV) 30 ml chloroform/methanol/water, 87/13/1.5 by vol; and (V) 60 ml chloroform/methanol/water, 86/14/1.7 by vol, collecting 5 ml fractions. The radioactivity of each fraction was determined and radioactive fractions were examined by TLC.

Thin Layer Chromatography

Cerebroside sulphate samples (10 μl), before and after reduction by $NaBH_4$, were applied to silica gel 60 plastic plates (Merck, Darmstadt, W. Germany) and developed in chloroform/methanol/water, 56/24/4 by vol [17]. Glycolipids were visualised by spraying with α-naphthol reagent and heating at 100°C for 10 min [18]. The radioactivity of developed plates was determined using a Packard Radiochromatogram Scanner.

Dispersion by Solvent Dilution

The method developed for the preparation of phospholipid vesicles [6] was modified as follows. To 50 ml of 10 mM sodium acetate buffer (pH 4.4) were added, by rapid injection with vigorous stirring, 2 ml of a methanolic solution of ^{3}H-cerebroside sulphate (0.4 μmol, 5 mCi/mmol). The resulting dispersion was concentrated to 2 ml by ultrafiltration with vigorous stirring on an XM-50 filter (Amicon Ltd., Stonehouse, Glos., UK) and the radioactivity of the filtrate and retentate determined in order to assess recoveries. Typical recoveries were 85-90% in the retentate, the loss in the filtrate being less than 1%. Non-specific absorption on the membrane filter was minimised by the vigorous stirring. The dispersion was stable for up to six weeks at room temperature.

In some experiments 25 mM flavin mononucleotide (FMN) was included in the dilution medium and excess FMN removed from the dispersions by diluting the concentrate to 50 ml with 10 mM sodium acetate buffer, pH 4.4, and reconcentrating; this process being repeated three times.

Dispersion by Sonication

^{3}H-Cerebroside sulphate in methanolic solution (0.2 μmol; 15 mCi/mmol) was dried *in vacuo* in a conical glass tube and dispersed for periods up to 1 h at low power (30 watts) in 1 ml of 10 mM sodium acetate buffer (pH 4.4) using a Sonicator Cell Disruptor W 200F fitted with a standard tapered microtip (Heat Systems-Ultrasonics Inc., Farmingdale, NY, USA). To achieve temperature control throughout sonication, the tube was placed in a jacketed vessel connected to a constant temperature water bath. The extent of the dispersion was assessed by measurement of the absorbance at 300 nm at suitable time intervals. The preparation was less stable than that produced by dilution, sedimenting at room temperature within 48 h of dispersion.

Chromatography on Controlled Pore Glass Beads

Controlled pore glass beads (CPG-10-500; mean pore size = 48.6 nm, Electronucleonics Inc., Fairfield, NJ, USA) were thoroughly washed with 10 mM sodium acetate buffer (pH 4.4) to remove trapped air and the slurry poured to produce a 1.5 × 20 cm column. A 1 ml sample of dispersed cerebroside sulphate was applied to the column, which was eluted with equilibrating buffer at 24 ml/h collecting 10 drop (280 μl) fractions. The radioactivity of the eluted material was determined by scintillation counting and when FMN was present the fluorescence of the fractions was measured with excitation at 465 nm and emission at 520 nm. To minimise non-specific adsorption on the glass beads, columns were prewashed with dilute non-radioactive sulphatide dispersions, so allowing recoveries in excess of 90% to be achieved.

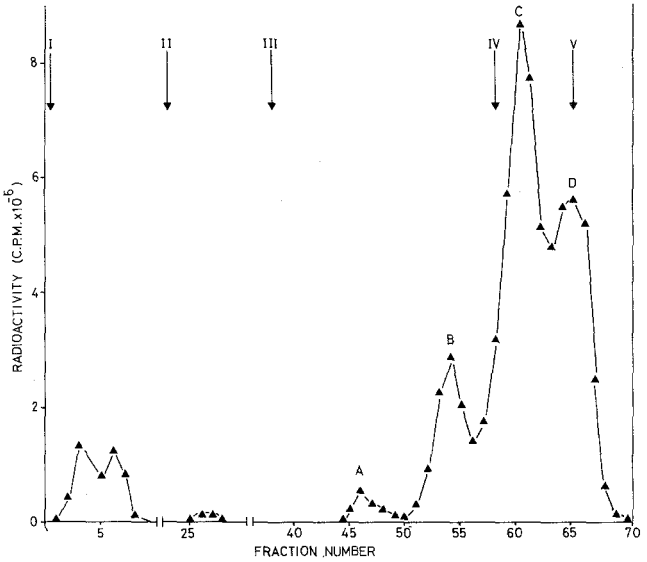

Figure 1. Purification of ^{3}H-cerebroside sulphate by chromatography on Florisil. Full details are given in the text. ^{3}H-Cerebroside sulphate (5 μmol, 4 mCi) in 10 ml of solvent I was applied and 5 ml fractions of the eluate collected. The arrows indicate change of eluting solvent.

Ultracentrifugation

Equilibrium sedimentation [20] and sedimentation velocity were studied in a Spinco Model E ultracentrifuge at temperatures controlled close to 20°C. Schlieren or interference optics were used as appropriate. Dispersions prepared by dilution were dialysed against repeated changes of 10 mM NaCl at the ultracentrifugation temperature prior to analysis. The apparent specific volume, v, of cerebroside sulphate was taken to be 0.94 [3].

Electron Microscopy

A variety of negative-staining methods were tested including coating of the support film with silica [21]. The best preservation of vesicles was obtained when freshly-prepared ultra-thin carbon films were floated from the mica substrate on to the surface of the dispersion and fine mesh copper grids were placed on the surface of the carbon film and collected with absorbent paper [22]. Negatively-stained dispersions were prepared by dilution of a methanolic solution of cerebroside sulphate into 10 mM ammonium molybdate followed by concentration by ultrafiltration. After allowing the grids to stand for 5 min, surplus liquid was carefully blotted and the grids examined and photographed at 100 kV in a Jeol Jem 100B transmission electron microscope.

Results

Tritium Incorporation

Typically 86% of the maximum possible incorporation into cerebroside sulphate was seen, the crude preparation having a specific activity of 860 mCi/mmol. Both standard cerebroside and cerebroside sulphate separate into three components on TLC, the R_F values being 0.57 (weak), 0.62 and 0.67 for cerebroside and 0.25 (weak), 0.27 and 0.30 for sulphatide. After tritiation by reduction with [^{3}H]$NaBH_4$, the crude sulphatide also shows three major components, either by staining or by scanning for tritium. The R_F values of the reduced sulphatide are 0.31, 0.34 and 0.42 and a trace of material in the cerebroside region is also observed.

Purification on Florisil

The elution profile of radioactivity is shown in Fig. 1. A small amount of ^{3}H-cerebroside, formed by desulphation, is eluted with solvents I and II. Four further peaks of radioactivity are seen eluting in solvents III, IV and V. Peak A is similar to the unidentified material previously reported [19], while peaks B, C and D have R_F values on TLC of 0.42, 0.34 and 0.31, respectively. Peak C, which gave a single band on TLC, was used for dispersion studies.

Dispersion by Sonication and Solvent Dilution

The absorbance changes with time at both 0°C and 37°C for sonicates of peak C material, prepared as described in the Methods section, are shown in Fig. 2. At 0°C the absorbance is still decreasing after 1 h, suggesting that dispersion of aggregated material is still proceeding, whereas a stable absorbance value is reached after 6 min at 37°C. In five independent experiments at 37°C the final absorbance at 300 nm of sonicates (0.2 mM; 0.2 mg/ml) was 0.310 ± 0.008, while, in six experiments on dispersions, produced by dilution and adjusted to the same concentration, the absorbance was 0.148 ± 0.012.

Controlled Pore Glass Chromatography

There is a marked difference in behaviour on controlled pore glass chromatography of sonicated dispersions and dispersions produced by solvent dilution (Fig. 3). Sonicated preparations elute in a broad asymmetric peak with a maximum at the void volume (determined by passing tobacco mosaic virus through the column) while solvent dilution preparations show two peaks, one at the void volume and the other intermediate between the void volume and void volume + inner volume (tryptophan marker). When FMN is included in the dilution medium, although considerable amounts are totally included in spite of repeated washing, some fluorescence corresponding to the two radioactive peaks is seen.

Ultracentrifugation

The heterogeneity of dispersions of cerebroside sulphate is confirmed by their behaviour in the ultracentrifuge. Centrifugation of such a dispersion (sample 2 in Table 1) at

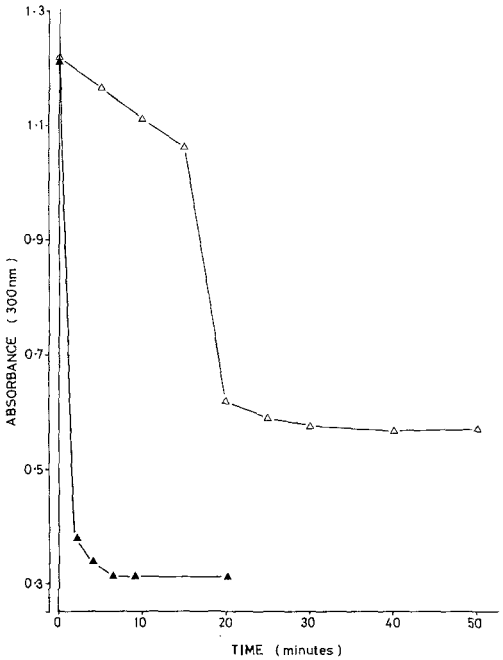

Figure 2. Dispersion of cerebroside sulphate by sonication. The turbidity of a 0.2 mM suspension of peak C material, measured at 300 nm, was determined after various times of sonication at low power (see text for details) at 0°C (△) and at 37°C (▲).

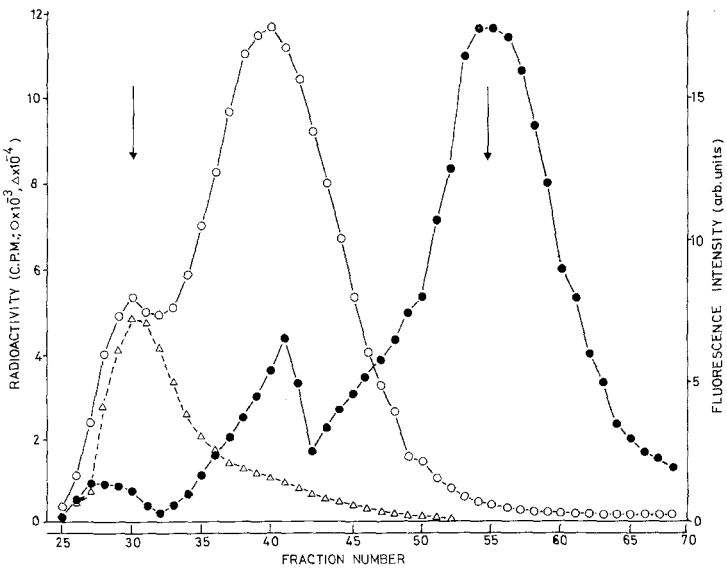

Figure 3. Chromatography of dispersions of cerebroside sulphate on controlled pore glass. Samples (1 ml) of 0.2 mM dispersions of peak C material applied to columns (1.5 × 20 cm) of controlled pore glass beads (mean pore size 48.6 nm), eluted with 10 mM sodium acetate (pH 4.4) and 280 μl fractions collected. △; sonicated preparation, radioactivity profile: ○; solvent dilution preparation, radioactivity profile: and ●; fluorescence of FMN. The arrows show the elution volumes of tobacco mosaic virus and of tryptophan.

Table 1. Particle weights[a] of the smallest sedimenting species in dispersions of cerebroside sulphate prepared by solvent dilution.

Sample	Initial conc. (mg/ml)	Speed (rev/min)	Cell length (cm)	$M \times 10^3$		
				$v=0.92$	$v=0.94$	$v=0.96$
1	0.2	18000	3	160	210	320
1	0.2	30000	3	140	200	300
2	1.0	20000	1.2	230	310	460

[a] Calculated from the least-squares slope of the linear portion of plots of $\ln\Delta y$ against $r^2/2$ as in Fig. 4.

44 000 rpm in 0.01 M NaCl (c_o approximately 0.8 mg/ml, cell length 3 cm) caused considerable amounts of material to move rapidly to the bottom of the cell, but a single asymmetric boundary, tailing forwards was also apparent. This had an $s_{20,w}$ (measured from the rate of movement of the maximum ordinate of the Schlieren pattern) of 3.4 S: $s_{20,w}$ (measured from the rate of movement of the square root of the second moment of the entire Schlieren pattern) was 3.2 S, which suggested that some dissociation was occurring on the trailing side of the boundary. As previously noted with dispersions produced by sonication [3], the area under the Schlieren curve decreased steadily during sedimentation, again suggesting dissociation. Linear extrapolation to zero time gave, assuming a specific refractive increment of 1.1×10^{-3} l/g [3], an initial concentration of cerebroside sulphate of 0.2 mg/ml. The sedimenting boundary therefore represents only about 25% of the dispersed material.

Similar results were obtained using equilibrium ultracentrifugation by the method of Yphantis [20]. Three experiments with two dispersions of cerebroside sulphate were carried out and particle weights of the smallest sedimenting species are given in Table 1. As v is not known precisely, three values covering the experimental range [3] were used. Again the presence of material of a much higher particle weight was obvious by the great increase in concentration of solute at the base of the cell (Fig. 4) where the concentration gradient was too steep to allow measurement. Integration of the plot of c versus r, corresponding to the plot of $\ln \Delta Y$ versus $r^2/2$ in Fig. 4, showed that the mass of cerebroside sulphate centripetal to $r = 7.134$ cm (the limiting value in the evaluation of the slope in Fig. 4) was 26 μg. As the total mass of cerebroside sulphate loaded in the cell was 98 μg, again the sedimenting species amounted to only 27% of the total. The agreement between the sedimentation velocity and sedimentation equilibrium results is satisfactory.

Electron Microscopy

Typically, clusters of unilamellar vesicles are seen at relatively low magnification (Fig. 5a). The diameter of the individual vesicles ranged from approximately 100 nm to 350 nm. Isolated vesicles, having a typical liposomal appearance are seen in some areas of the grid under high magnification and these range from 10-80 nm diameter (Fig. 5b).

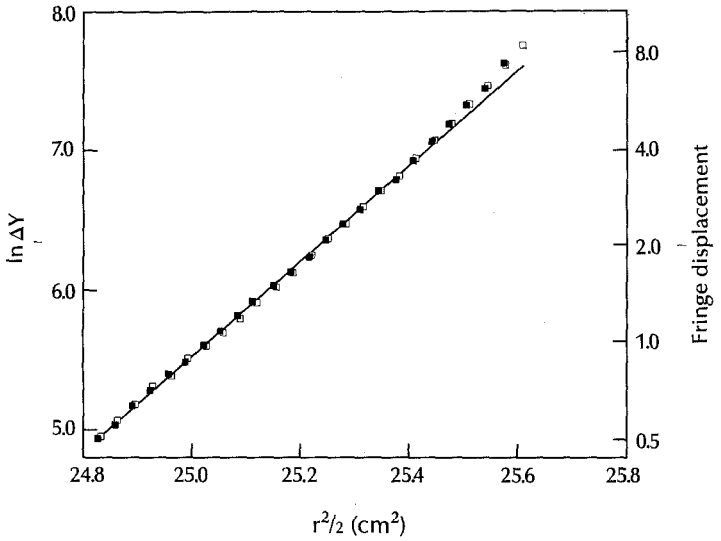

Figure 4. Fringe displacements in the equilibrium sedimentation of a 0.2 mM dispersion of cerebroside sulphate (peak C) prepared by solvent dilution. Distribution in 0.01 M NaCl after 14 h (□) and 15 h (■) centrifugation at 20 000 rpm in an An-D rotor at 20°C. Cell length 1.2 cm, sapphire windows.

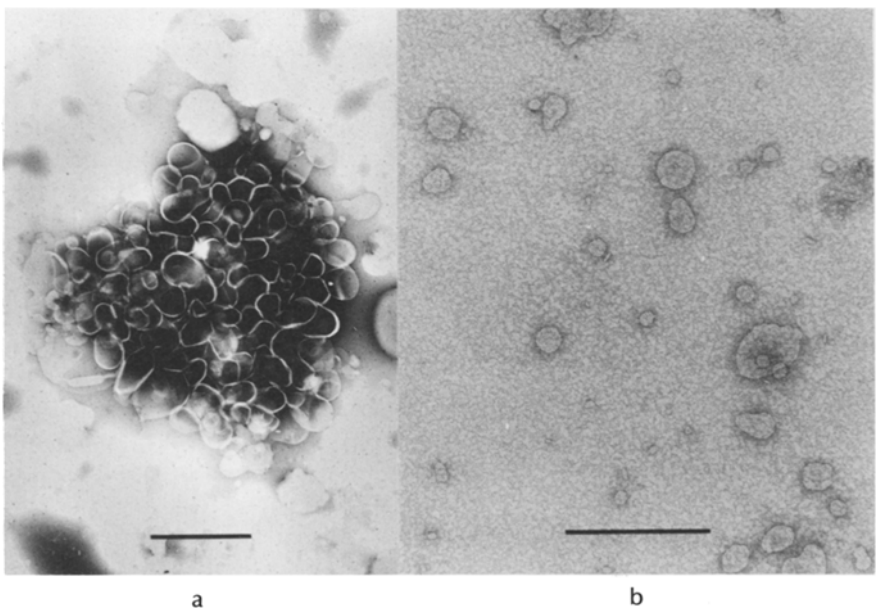

Figure 5. Electron micrographs of dispersions of cerebroside sulphate prepared by solvent dilution. Dispersions of peak C material were negatively stained with ammonium molybdate and examined as described in the Methods section. a; line represents 1.0 μm: b; line represents 0.5 μm.

Discussion

The use of [^{3}H]$NaBH_4$ with $PdCl_2$ as catalyst has proved a simple and very reproducible system for the introduction of tritium into cerebroside 3-sulphate. Assuming reduction of a single double bond in the ceramide moiety as demonstrated previously [15], 86% of the possible label is incorporated and insignificant hydrolysis to cerebroside is observed. The demonstration of three components in both the reduced and non-reduced sulphatide is unusual, most workers having reported only two components, corresponding to the hydroxylated and non-hydroxylated forms of ceramide [17]. It is possible that the minor component observed is sulphated lactosyl-ceramide, but this was not investigated further. Certainly cerebroside produced by desulphation of this particular sample of cerebroside sulphate also shows three components. The increased mobility on TLC after reduction has been observed previously [23]. Other workers who have claimed an unchanged mobility on reduction of cerebroside sulphate have used solvents which gave high R_F values [15, 24, 25]. Chromatography on Florisil confirms the presence of three radioactive components and allows their quantitative separation. The elution profile is similar to that previously reported [19] but again the reduced components elute ahead of the non-reduced material suggesting that they are somewhat more hydrophobic.

Turbidity measurements and chromatographic behaviour on controlled pore glass beads show that the average size of structures produced by sonication is far greater than that produced by dilution from organic solvent. This is not surprising since in the former technique large aggregates are being dispersed while in the latter technique individual molecules are being allowed to come together to form macromolecular structures. The simplicity and reproducibility of the solvent technique make it preferable for subsequent enzymatic studies.

The elution profile of dispersions produced by dilution is biphasic, some material eluting at the void volume and hence having a diameter greater than 48.6 nm, while the major component has a peak elution volume corresponding to a diameter of approximately 40 nm although there is considerable material of lower size. The incorporation of FMN is very similar to the elution profile of radioactivity which suggests that there are bilayer structures present since FMN is water-soluble and not incorporated into micelles.

The centrifugation of dispersions of cerebroside sulphate prepared by dilution from organic solvents confirms their heterogeneity. They contain smaller particles, $s_{20,w}$ 3.4 S, than the sonicated preparations studied previously ($s_{20,w}$ 6 S) [3] but, on the other hand, the latter did not contain the very large particles seen in the solvent preparations. It should be noted that the sonicated dispersions used in the previous studies [3] were prepared at a power of approximately 300 W and so are not directly comparable to the present dispersions prepared at 30 W.

If it is assumed that the material of $s_{20,w}$ 3.4 S corresponds to that having a particle weight of 300 000, as is suggested by the data in Table 1, then the apparent diffusion coefficient of the particles would, from the simple Svedberg relationship, be 0.4×10^{-7} cm^2/s. This is of the correct order of magnitude and so provides further justification for the assumption that both types of centrifugation reveal the same sedimenting species. Such particles would contain approximately 300 molecules of cerebroside sulphate. Taking a

cross-sectional area per molecule of 60 $Å^2$ [26], then a spherical particle formed from a bilayer of 300 molecules of cerebroside sulphate would have a diameter of about 5 nm. The sedimenting particles are therefore not those seen in the electron micrographs. Whether or not they are micelles cannot be decided but, if they are, they are rather atypical [3].

Particles of diameter of the order of 50 nm, as seen in electron micrographs, would consist of a bilayer of about 25 000 molecules of cerebroside sulphate and so have a particle weight of 25×10^6 and an *s* value very much greater than 100. These particles would not have been detected in the sedimentation experiments.

Further substantiation of the vesicular nature of these dispersions is provided by electron microscopy. The light band with dark outer halo seen on negative staining with ammonium molybdate is similar to that observed for phosphatidyl inositol [13] and is characteristic of bilayer structures. Although considerable aggregation has taken place during the preparation of material for electron microscopy, liposomal structures having a size (40 nm) comparable to that predicted by chromatography are observed but it is likely that many such structures have fused during preparation.

It has been reported recently [27] that mixed dispersions of phosphatidyl choline and sulphatide form micelles when the sulphatide content exceeds 70% (w/w). However, the dispersion was carried out at 55-60°C at pH 7.4 using high energy sonication. Previous studies [28] have shown that micelle formation is favoured by enhanced temperature and this may explain the discrepancy. Although micelles may be present in our dispersions, the major structures are undoutedly unilamellar vesicles.

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