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The properties of brain galactocerebroside monolayers

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Using a Langmuir film balance we have compared the properties of films of the brain galactocerebrosides at 37 °C. There are two types of cerebroside in brain, those with an α-hydroxy substituent on the acyl chain (HFA) and those without (NFA). At equivalent pressures the areas of both cerebroside films are significantly less than the areas of films of the brain glycerolipids, the choline and ethanolamine phosphatides.The isotherm of NFA galactocerebrosides has two discontinuities, one at low and one at high film pressure, while the isotherm of HFA galactocerebrosides is a smooth curve at all film pressures. Below the high-pressure transition the area of the NFA film is significantly larger than the area of the HFA film. When compressed beyond the high-pressure transition there is a marked hysteresis between compression and expansion isotherms of the NFA galactocerebrosides. The pressures of both films continue to rise steeply when they are compressed into areas which are too small for them to exist as simple monolayers. We conclude that under compression cerebroside films form bilayer structures; that bilayer formation starts at low pressure and occurs progressively as the HFA cerebroside monolayer is compressed, but occurs more abruptly in the NFA cerebroside monolayer at the high-pressure-transition region of the isotherm. A study of pure cerebrosides with a single defined acyl chain shows that there is a correlation between the relative volumes of the hydrophobic and hydrophilic parts of the molecule and the ease of bilayer formation. The larger the relative volume of the hydrophilic group the more readily the cerebroside forms a bilayer film. Other brain lipids added to cerebroside monolayers have sharply differing effects on their areas. The areas of films containing cholesterol are less than the areas calculated by adding the areas of the pure components multiplied by their mole fractions. On the other hand, the area of phosphatidylcholine-containing films is much larger than calculated.

Introduction

We have chosen to study the myelin galactocerebrosides because of their involvement in the disease multiple sclerosis. Multiple sclerosis (MS) is an autoimmune disease in which myelin, the insulating membrane surrounding nerve axons, is destroyed while the axons themselves are spared. Of all the biomembranes, myelin contains the highest proportion of lipid (75–80%), and of these lipids galactocerebrosides are both the most numerous and most characteristic. It has been shown that (a) antibodies against cerebrosides are present in sera from patients suffering from multiple sclerosis [1], (b) such antibodies demyelinate cerebellar tissue cultures [2] and (c) experimental

Abbreviations: NFA, HFA, galactocerebrosides with unsubstituted or α-hydroxy-substituted acyl chains, respectively; PVC, poly(vinyl chloride).

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allergic encephalomyelitis, a model for multiple sclerosis, can be induced in rabbits more readily if they are inoculated with galactocerebroside as well as myelin basic protein [3]. Epidemiological studies have shown that some individuals are more susceptible to this disease than others. Susceptibility is under multigenic control [4], and although at this time it is not clear how susceptibility is manifest at the molecular level, there is a body of opinion for a defect in membrane structure [5]. Such a defect could involve the galactocerebrosides.

Galactocerebroside isotherms have been reported in four previous publications [6–9]. However, in all these reports the subphase temperature was at or near 20 °C, far from the biologically relevant temperature, 37 °C, and in most cases the galactocerebroside films were a mixture of the unsubstituted and hydroxy-substituted species. Furthermore, mixtures of the cerebrosides with other brain lipids were not studied. We have used a Langmuir film balance to compare the properties of films of these lipids and their mixtures with cholesterol and brain sphingo- and glycerolipids.

Materials and Methods

Materials

Galactocerebrosides with unsubstituted (NFA) and α-hydroxy-substituted acyl chains (HFA) were purchased from Sigma. They had been prepared by chromatographic separation of lipids extracted from bovine brain. The contamination of each cerebroside fraction by the other was claimed to be less than 2% and the total cerebroside content of each fraction was stated to be 99%. An analysis of acyl chains in each lipid mixture was made by GLC. The cerebrosides were hydrolysed and the acyl chains converted to their methyl esters by a method described by Sweeley and Moscatelli [10]. Hydroxy fatty acids were trimethylsilylated according to the procedure of Carter and Gaver [11]. The acyl chain distributions are shown in Table I. The major difference between the two distributions is that a significantly higher proportion of unsubstituted chains contain a double bond. This difference has been shown to exist in all species on which analyses have been made, and the proportions of the acyl chains in bovine brain galactocerebroside samples used in this study are within the range of values found in galactocerebrosides from human myelin [12–16]. Average molecular weights calculated for NFA and HFA galactocerebrosides were 808 and 801, respectively. The melting temperatures (T_{max}) and enthalpies of NFA and HFA measured by differential scanning calorimetry were 78.9 °C, 90.3 J/g and 68.9 °C, 59.9 J/g, respectively.

TABLE I

ACYL CHAIN COMPOSITION OF CEREBROSIDES

Acyl chain	Galactocerebrosides	
	NFA	HFA
16:0	0.93	–
18:0	4.15	32.03
18:1	1.20	–
20:0	1.09	1.01
22:0	5.29	11.48
22:1	–	–
23:0	6.02	10.60
24:0	25.39	38.47
24:1	28.35	4.70
25:0	6.26	–
25:1	6.72	–
26:0	2.92	–
26:1	8.37	–
Others	3.31	1.74
Unsaturated chains	44.64	4.70

Synthetic nervonoyl, oleoyl and stearoyl galactocerebrosides were purchased from Sigma. These lipids were prepared by reacylating psychosine derived from bovine brain galactocerebrosides.

Sulphatides (galactocerebroside sulphate), phosphatidylcholines, sphingomyelins and ethanolamine phosphatides (phosphatidylethanolamines and phosphatidalethanolamines) were all purchased from Sigma and are bovine brain extracts. Average molecular weights have been calculated for them from published acyl chain analyses [13–16] and are 877, 762, 795 and 746, respectively.

Two ceramide mixtures prepared by removal of the galactose residue from bovine brain galactocerebrosides and the phosphorylcholine group from bovine brain sphingomyelins were purchased from Sigma. Their average molecular weights are 645 and 626, respectively.

Cholesterol (reputedly over 98% pure) and stearic acid (reputedly over 99.5% pure), both designated puriss grade, were purchased from Fluka, AG.

The purity of all lipids was checked by thin-layer chromatography (TLC) on silica gel plates. Various amounts of the lipids were spotted onto the base of the plate and its was eluted with a mixture of chloroform and methanol. The ratio of the two solvents was chosen so that each lipid had an R_F of 0.5. Cerebrosides were visualised by spraying their plates with orcinol solution. All other chromatograms were sprayed with a 0.1% solution of Rhodamine 6G in ethanol. No lipids were used in this study if anything other than a single well-developed spot appeared in their chromatograms.

In all experiments but one the subphases were pure water. Pure water was obtained by passing analytical grade water (from BDH, 'Analar', under 5 ppm residue on evaporation) through a Millipore Milli Q water purification system. This apparatus consists of a carbon filter, two ion-exchange cartridges and a 0.22×10^{-6} m paper filter in series. Water from the Milli Q system had a resistivity of over 18 MΩ. Its pH was 5.5 and its ultraviolet absorbance at 200 nm was always less than 0.01. Water was only drawn from the system when needed. The isotherm of the ethanolamine phosphatides was measured on phosphate-buffered saline (100 mM NaCl/20 mM phosphate (pH 7.4)). Sodium chloride, sodium dihydrogen orthophosphate and disodium hydrogen orthophosphate were 'Analar' reagents from BDH. When the barrier of the film balance was run across a subphase of phosphate-buffered saline no change in surface tension was observed. As a further precaution against the introduction of surface-active contaminants with the salts, the surface layer of water was removed with a pasteur pipette connected to a mains water-driven aspirator after the barrier had been run across the subphase. This compression-cleaning cycle was repeated three times before the film was spread.

Some difficulty was experienced finding a solvent which would both dissolve galactocerebrosides and spread them efficiently on a water surface. Eventually we settled on a mixture of 11 parts hexane, 5 parts chloroform and 4 parts ethanol by volume. The hexane (Uvasol) and ethanol (pro analysis) were from E. Merck and the chloroform (Gold Label) was obtained from Aldrich. Each of the solvents was of the best grade available commercially. They were checked for surface-active contamination by spreading ten times the volume normally used to apply a lipid to the surface of the subphase, $500 \cdot 10^{-6}$ l, advancing the barrier and monitoring the surface tension of the subphase. In all cases no change in the surface tension was detected.

All lipids except stearic acid were spread in the hexane/chloroform/ethanol mixture. Stearic acid was spread from pure chloroform.

Methods

The major consideration when attempting to measure monolayer properties is the exclusion of adventitious film-forming materials from the subphase surface. Great care was taken in the procedures adopted to minimise the chance of films being contaminated with foreign surfactant molecules. All the experimental work reported here was carried out in one laboratory devoted solely to the film balances and their associated equipment. The laboratory had no windows, only one door and a filtered air supply. The benches and floor were routinely swabbed down to prevent the accumulation of dust.

The glassware needed was not used for other experimental work. All glassware, after careful washing, was soaked in fresh chromic acid for 1 week. The strength of the acid was regularly checked by noting its ability to blacken filter paper. Once the acid lost the ability to immediately blacken the filter paper it was discarded. After acid treatment, glassware for handling purified water was repeatedly washed with this water before it was used. Volumetric flasks in which lipid solutions were stored, were also washed with pure water after chromic acid treatment, but further washings were made with first pure ethanol and then spectroscopic grade dichloromethane. Because dichloromethane is highly volatile, flasks can be air dried in a short period of time. We deliberately avoided oven drying because it is difficult to prevent glassware being contaminated when it is stood in an oven for several hours. All glassware had necks with ground glass sockets.

When not in use, these were tightly sealed with glass stoppers. No plastic or metal surfaces were allowed to come into contact with lipid solutions or subphase water.

Lipid samples were dried by standing them overnight in a vacuum desiccator containing phosphorus pentoxide. The lipids which were received as solutions first had the solvent removed in a stream of dry nitrogen. Approx. 10 mg of dry lipid were placed in a 25-cm ^{3} (± 0.02 cm^3) volumetric flask and its weight measured to an accuracy of ± 0.01 mg. with an Oertling analytical balance. Solutions containing two lipids were prepared in two ways. Either the lipids were added to the flask consecutively and the flask reweighed after each addition or calculated volumes of solutions of known concentration were metered into a fresh flask by pipette. After adding the appropriate solvent (see Materials) the solutions were stored in a freezer at $-20\,^\circ$C to prevent solvent evaporation. The lipid solution was metered onto the subphase surface with an SGE 0.1 cm^3 microsyringe. The microsyringe was washed thoroughly before and after spreading with pure spreading solvent. The volume of the solution spread was chosen so that the area of the film at collapse was approximately one-quarter of the area of the subphase. 15 min were allowed for evaporation of the solvent before the film was compressed. Films were compressed at rate of 68.5 cm^2/min. In a typical experiment, this would correspond to 8.0 Å^2 molecule per min. This rate is slow enough for the shape of isotherms of stearic acid and the phospholipids to be compression rate independent. In their case, kinetic hysteresis effects can be excluded. All isotherms were run at least twice in the direction of increasing pressure. Each run was performed with a fresh film and subphase.

Two film balances were used. They were of similar design. A base plate/water jacket was formed by covering a rectangular poly(vinyl chloride) frame, top and bottom with a 5/16 inch brass sheet. The PVC frame was cut from 1.25 inch thick block. Water from a Haake circulator was passed through the base plate via nozzles cut into the PVC. Thick teflon sheet was attached to one side of the base late with double-sided adhesive tape. The balance was constructed so that only teflon parts would contact the subphase and film. The wall was cut from solid teflon with steps milled on the inside to locate the barrier. It was clamped lightly to the base plate with brass bolts. No adhesive was used. Although teflon has a large thermal expansion coefficient the trough remained free of leaks when operated in the ambient — $37\,^\circ$C temperature range. The barrier was also cut from solid teflon and it was supported by and reinforced with stainless steel brackets. The barrier sat on teflon rails on either side of the trough and was driven by a 1 mm pitch screw from a stepper motor (Model ID06, McLennan Servo Supplies, London). Pulses to drive the stepper motor were derived from a crystal oscillator. Film pressure was measured by a modern variation of the Wilhelmy plate system [17]. A strip of filter paper was dipped through the water surface and the force exerted vertically by surface tension transformed into a minute displacement by means of a spring. The displacement was measured by converting it into a d.c. voltage with a differential transformer (Model SE350, SE Labs (EMI), London). A displacement transducer constructed in this way has more than adequate sensitivity. Compared with the glass or metal plates originally used, a strip of filter paper has the advantage that it is perfectly hydrophilic and will maintain the necessary zero contact angle. Although film molecules will deposit on many substrates, they do not deposit on filter paper. After amplification, the output of the displacement transducer was recorded on a Servoscribe *x-t* chart recorded. The subphase temperature was monitored with a thermistor fitter with a teflon sheath. The troughs measured 39.9×18 cm and 19.6×12.0 cm. Their water capacities were 540 and 140 cm^3, respectively. Each was fitted with a water-jacketed perspex cover with sliding perspex doors.

After each set of measurements the paper Wilhelmy plate was removed and the trough filled with fresh chromic acid. The temperature of the acid was held at $40\,^\circ$C and it was allowed to remain in the trough for 3–4 h. After its removal the trough was refilled with Millipore water. The water was removed through a pasteur pipette connected to a mains water-driven aspirator. The trough was refilled and a fresh Wilhelmy plate fitted. Two further changes of water were made before the next series of experiments was started.

TABLE II

MEASUREMENTS OF THE AREAS OF STEARIC ACID MONOLAYERS AT TRANSITION PRESSURE AND 25 °C

Trough	Solution	Mean area (Å²/molecule)	Number of measurements	S.D.
Small	1	19.95	19	0.73
Small	2	20.19	19	0.44
Overall mean		20.07	38	
Large	1	20.58	4	0.30
Large	3	20.54	21	0.41
Large	4	20.26	12	0.49
Overall mean		20.45	37	

The output of the pressure transducer was calibrated by recording the isotherm of stearic acid, which has been well characterized at 25 °C [18]. This isotherm has a sharp phase transition. We were unable to find in the literature a suitable isotherm measured at 37 °C, the temperature at which the experiments reported here were carried out. This will introduce a small error to the calibration, but it is of little consequence since our primary objective is a comparison of the film-forming properties of the myelin lipids. Table II contains molecular areas calculated for stearic acid films at the point at which they undergo the phase transition. The average area per molecule (in Å²) at a specific surface pressure was calculated by using the following relationship

$$\text{area} = \frac{(\text{const. } 1 - (\text{const. } 2)\,x)(n_1 M_1 + \cdots + n_x M_x)}{(\text{vol.})\ (\text{concn.})}$$

where x = the distance the pen travelled on the chart recorder in centimetres, $n_1 \cdots n_x$ = mole fraction of components $1-x$ ($n_1 + \cdots n_x = 1.0$), $M_1 \cdots M_x$ = the molecular weights of the components, vol. = the volume of the lipid solution spread, in μl, concn. = the concentration of the lipids in the spreading solvent, in mg per cm³ and consts. 1 and 2 are constants determined by the chart recorder and barrier speeds, and the trough dimensions.

The measurements collected in the table were made progressively throughout the course of the work, and therefore are influenced by long-term as well as short-term changes in factors which influence the variability of the results. There appears to be a significant difference between the areas measured by the two troughs of about 0.4 Å per molecule. The isotherm for stearic acid shown in Gaines [18] (run on 10^{-4} M H_2SO_4) has a phase transition at 24.98 dyn/cm surface pressure and 19.85 Å² per molecule area. An isotherm run on water of pH 5.8 had a phase transition at a pressure and area of 25.73 dyn/cm and 20.48 Å² per molecule (Arnett, E.M., personal communication). We have used this value of surface pressure to calibrate our instruments. The reproducibility of the measurements will be seen to be sufficient for it to be possible to make comparisons between the properties of brain lipid films.

Results

Isotherms of brain lipids

Fig. 1 contains isotherms of cholesterol and the main brain sphingo- and glycerolipids measured at a subphase temperature of 37 °C. All the lipids form stable films. Films of glycerolipids occupy larger areas than those of sphingolipids and collapse at lower pressures. The high compressibility of the glycerolipids at all film pressures and the absence of discontinuities in their isotherms shows

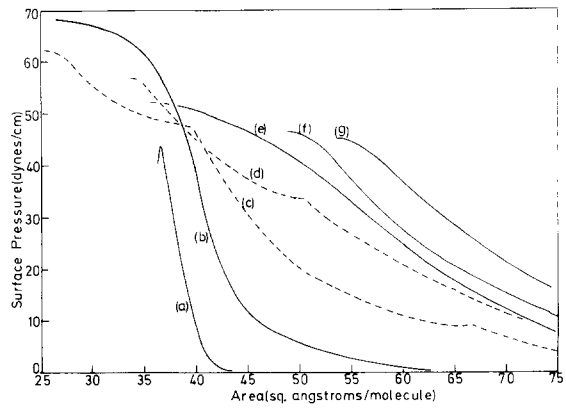

Fig. 1. Isotherms of the main brain lipids. (a) Cholesterol, (b) α-hydroxy galactocerebrosides (HFA), (c) galactocerebrosides (NFA), (d) cerebroside sulphate, (e) sphingomyelin, (f) choline phosphatides and (g) ethanolamine phosphatides. The cerebroside isotherms are the medians from five individual measurements.

that they exist in the liquid expanded state up to film collapse.

The sphingolipid isotherms are a more diverse group. At low pressures the areas of the sphingomyelin and cerebroside sulphate films, which have ionic polar groups, are larger than the neutral galactocerebroside films. The sphingomyelin film is like those of the glycerolipids, compressible and without a discontinuity in its isotherm. It exists in a fully expanded liquid state right up to the point at which it collapses. By contrast, the isotherm of cerebroside sulphate has a discontinuity at 34 dyn/cm pressure and 50.5 $Å^2$ per molecule area. The magnitude of the change in compressibility of the film at this point indicates that it has undergone a transition from a liquid expanded to a liquid condensed state [19]. The isotherm of the galactocerebroside with unsubstituted acyl chains (NFA) has a similar transition at 9.5 dyn/cm pressure and 66.5 $Å^2$ per molecule area. However, on continued compression films of this lipid undergo a second transition at 43.5 dyn/cm pressure and 39.5 $Å^2$ per molecule area. It is difficult to measure a true collapse pressure for NFA galactocerebrosides because at very high pressures the film becomes so rigid that it ceases to flow around the Wilhelmy plate and the plate is pushed away from the vertical.

Of all the brain lipids, the cerebrosides with the hydroxy substituent on the alpha carbon of the acyl chain (HFA) have the isotherm which lies at the lowest molecular area. In fact, the isotherm of this lipid very nearly overlaps that of cholesterol, which forms an incompressible film in which the molecules are very tightly packed. The isotherm has no discontinuities and, like NFA, the film becomes rigid before a collapse point is reached.

The most striking difference between sphingolipids and glycerolipids is the very low molecular area to which sphingolipid films can be compressed and the pressure of the film continue to rise. At this point, the area available to molecules in cerebroside films is approximately half that of molecules in glycerolipid films.

Isotherms of galactocerebrosides with a single defined acyl chain

Fig. 2 contains the isotherms of stearoyl (18 : 0), oleoyl (18 : 1, 9 *cis*) and nervonoyl (24 : 1, 15 *cis*)

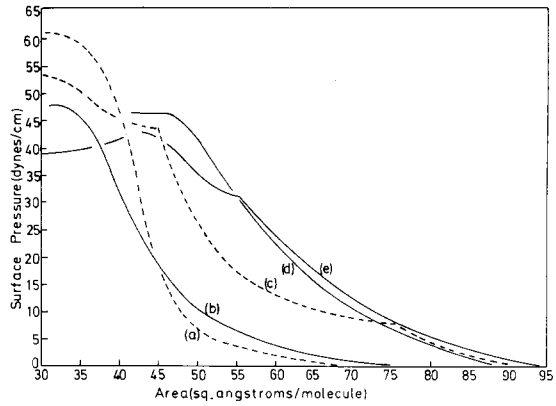

Fig. 2. Cerebroside isotherms. (a) α-Hydroxy galactocerebrosides (HFA), (b) *N*-stearoyl galactocerebroside, (c) galactocerebrosides (NFA), (d) *N*-oleoyl galactocerebroside, (e) *N*-nervonoyl galactocerebroside. The isotherms shown are the medians of five individual measurements.

galactocerebrosides. The isotherms of NFA and HFA galactocerebrosides are shown for reference. The isotherms of the unsaturated oleoyl and nervonoyl galactocerebrosides lie close to each other at relatively large molecular area near the isotherm of NFA. The stearoyl galactocerebroside isotherm lies at a markedly lower area than the isotherms of the unsaturated lipids, overlapping the isotherm of HFA galactocerebrosides. However, there are significant differences in the shape of each isotherm. The isotherm of oleoyl galactocerebroside is a smooth curve, with no discontinuities. There is a discontinuity in the isotherm of nervonoyl galactocerebroside, but it occurs at a much higher pressure, 31 dyn/cm, and lower molecular area, 55 $Å^2$ per molecule, than the initial discontinuity in the NFA isotherm. Both oleoyl and nervonoyl galactocerebroside collapse near the point at which NFA undergoes its second transition. In the case of these two cerebrosides, and only these two of all the cerebrosides studied, collapse occurs before the film becomes sufficiently rigid to displace the Wilhelmy plate.

The HFA and stearoyl galactocerebroside isotherms are smooth curves, with no discontinuities. However, stearoyl galactocerebroside films displace the Wilhelmy plate at approx. 45 dyn/cm pressure, a pressure at which oleoyl and nervonoyl galactocerebroside films collapse. Of all the cerebrosides examined that formed rigid mono-

layers, stearoyl galactocerebroside did so at the lowest film pressure.

Monolayer characteristics of ceramides

Isotherms of ceramides derived from both bovine brain galactocerebrosides and sphingomyelins are very similar to each other (data not shown). Areas per molecule at 25 dyn/cm are 38.1 and 38.8 Å^2 per molecule, respectively. The ceramides form highly incompressible films which cease to flow and displace the Wilhelmy plate at lower pressures than any of the natural or synthetic galactocerebrosides, 37.6 dyn/cm for galactocerebroside and 41.2 dyn/cm for sphingomyelin ceramides.

Influence of reduced compression rate on galactocerebroside isotherms

Fig. 3 shows portions of isotherms of the NFA and HFA galactocerebrosides obtained at a relatively slow compression rate (1/7). Compared to isotherms measured at higher compression rates, it can be seen that the discontinuity at high pressure in the isotherm of the NFA galactocerebrosides has broadened into a plateau, the pressure remaining constant while the area per molecule is reduced by approx. 14 Å^2. The low-pressure transition in the isotherm is unaffected by compression rate (data not shown). The extent of the plateau region varied significantly between isotherms run consecutively with the same lipid solution and the isotherm shown is the one which had the broadest plateau. In this isotherm, the high-pressure transition starts at an area of approx. 42 Å^2 per molecule. After the constant pressure region which follows, the pressure of the film rises steadily and the next break in the isotherm, just at the point where the film becomes rigid, occurs at approx. 20 Å^2 per molecule. Thus, the film rigidifies at an area approximately half that at which the plateau region in the isotherm begins.

At this compression speed a small discontinuity appears in the isotherm of the HFA galactocerebrosides.

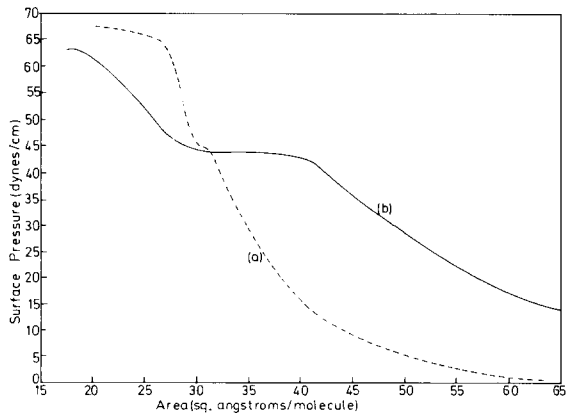

Fig. 3. Isotherms of (a) α-hydroxy galactocerebrosides (HFA) and (b) galactocerebrosides (NFA) run at 1/7 standard compression speed.

Compression-expansion isotherms of NFA and HFA galactocerebrosides

Fig. 4 contains compression-expansion isotherms of both NFA and HFA galactocerebrosides. The rates of compression and expansion were identical. It can be seen that the compression and expansion isotherms of HFA galactocerebrosides are very similar. The first compression isotherm does lie at slightly larger area, but thereafter the isotherms, both expansion and compression, occupy similar positions. There is little or no hysteresis.

The behaviour of an NFA galactocerebroside film subjected to repeated expansion-compression cycles was very different. On the first cycle, if the compression was stopped short of the second discontinuity, a degree of hysteresis was evident, but it was no greater in extent than that observed on the first HFA cycle. However, on the second compression the isotherm lies at larger area rather than smaller, as found with HFA films, and the second phase transition begins at slightly lower pressure and larger film area than normal. The transition is broad and resembles that seen at the slow compression rate. If the barrier is reversed as the film pressure starts to rise again, the pressure drops very rapidly as the barrier traverses the region corresponding to the plateau in the compression isotherm. Thus, there is marked hysteresis in the second compression-expansion cycle. Such behaviour is observed on all subsequent compression-expansion cycles. Rather than recompress the film, it is also possible to demonstrate hysteresis by reversing the barrier after the plateau region, found when films are compressed slowly.

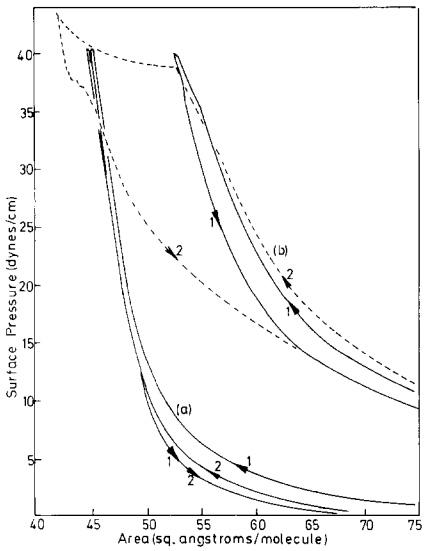

Fig. 4. Compression-expansion isotherms for (a) α-hydroxy galactocerebrosides (HFA) and (b) galactocerebrosides (NFA).

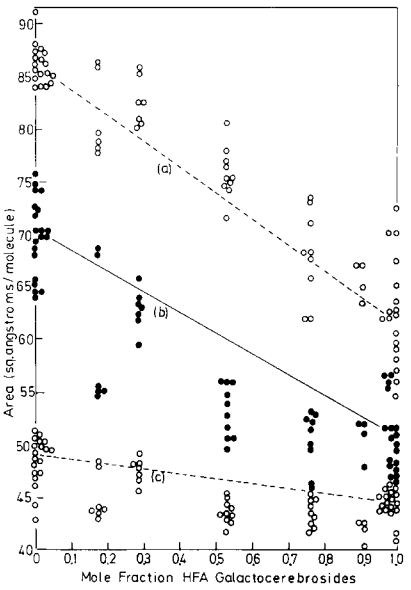

Fig. 5. Plots of the areas of mixed films of galactocerebrosides (NFA) and α-hydroxy galactocerebrosides (HFA) versus the mole fraction of HFA at film pressures of (a) 2, (b) 10 and (c) 35 dyn/cm.

At very large areas expansion and compression isotherms meet. If the barrier is stopped at an intermediate area, 55 $Å^2$ per molecule say, then the film pressure is observed to increase with time, exponentially approaching the compression isotherm area. The breadth of the plateau region in the second compression isotherm depended on the position of the isotherm. Those isotherms which lay at the upper end of the area range measured had the broadest plateau region. It is one of these which is shown in Fig. 4.

Mixed films of cerebrosides and other brain lipids

Mixed films of HFA and NFA galactocerebrosides. Fig. 5 contains plots of area per molecule versus film composition for mixed films of HFA and NFA galactocerebrosides at three film pressures, 2, 10 and 35 dyn/cm. It is clear from this figure that measurements of the area of galactocerebroside monolayers are inherently more variable than the monolayers of the other lipids. The standard deviation of the NFA measurements is approx. 5-times that found for monolayers of stearic acid at 25 °C. While the varibility of the area of NFA galactocerebroside films shows no dependence on film pressure, the variability of the area of HFA films decreases as the film pressure rises.

At 2 dyn/cm film pressure, and when HFA is a major film component, the experimental points fall near a straight line joining the pure component areas. At this line, the area of the mixed film is equal to the sum of the areas of the pure components multiplied by their mole fractions in the mixture, and the film exhibits ideal behaviour. When NFA, is the major component, the points tend to lie above this line. As the pressure is increased the experimental points fall with respect to the ideal behaviour line. At low HFA content the film behaves ideally, while at high HFA content there is a marked negative departure from ideality.

The experimental values at 0.173 mole fraction HFA are especially variable, there appearing to be two separate populations of results. Typical isotherms from which each of these two populations of results were derived are shown in Fig. 6. The isotherms which gave the larger area values have a broad high pressure transition similar to that observed when NFA galactocerebroside films were subject to repeated compression-expansion cycles or very slow compression. The isotherms from which the lower area points derive have only one

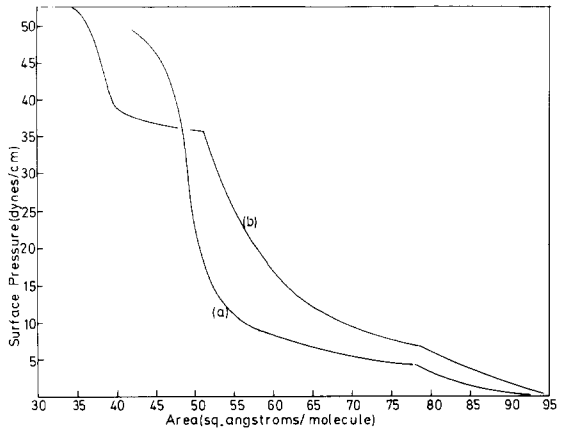

Fig. 6. Isotherms of mixed films of galactocerebrosides (NFA) and α-hydroxy galactocerebrosides (HFA) with an HFA mole fraction of 0.167. From population of results at (a) small area/molecule and (b) large area/molecule.

discontinuity, there is no evidence of a phase transition of any sort at high film pressure.

Mixed films of galactocerebrosides and cholesterol. Fig. 7 contains plots of the area per molecule versus film composition for mixed films of NFA galactocerebrosides and cholesterol at three film pressures, 2, 10 and 35 dyn/cm. The

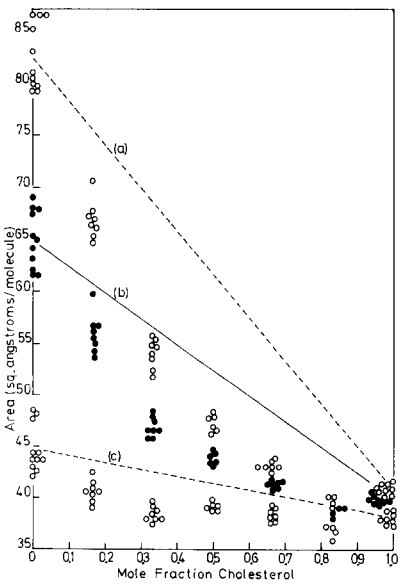

Fig. 7. Plots of the areas of mixed films of galactocerebrosides (NFA) and cholesterol versus the mole fraction of cholesterol at film pressures of (a) 2, (b) 10 and (c) 35 dyn/cm.

variability of measurements made on pure cholesterol is less than those made on the galactocerebrosides. It can also been seen that the variability of the mixture areas decreases as the percentage of cholesterol in the film is increased.

At all film pressures and compositions the areas of the mixtures lie below a line joining the pure component areas. This negative departure from ideal behaviour is most marked at low film pressures and for film compositions near equimolar. Isotherms of the mixtures (cholesterol at least 0.166 mol/%) do not have a second transition at 45 dyn/cm or show any evidence of hysteresis. Fig. 8 contains the data for mixtures of the other galactocerebroside, HFA, with cholesterol. As with NFA, the pressure of cholesterol reduces the variability of the measurements. However, the cholesterol-HFA galactocerebroside films behave more like ideal mixtures. Only at the lowest pressure, 2 dyn/cm, do the mixture areas show a negative departure from the line joining the pure component areas.

The presence of cholesterol in the films of both galactocerebrosides decreases their compressibility, yet even at the lowest cholesterol mole fraction the films collapse and do not form rigid solid phases.

Mixed films of NFA galactocerebrosides with brain phosphatidylcholines and sphingomyelins. The areas of sphingomyelin and phosphatidylcholine mixtures with NFA galactocerebrosides are shown

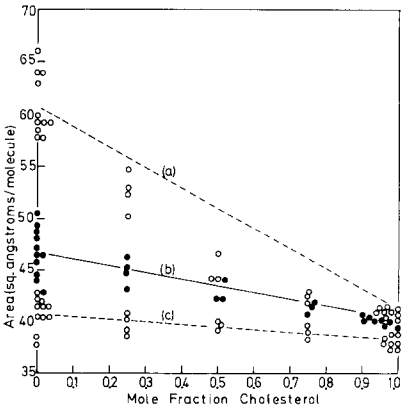

Fig. 8. Plots of the areas of mixed films of α-hydroxy galactocerebrosides (HFA) and cholesterol versus the mole fraction of cholesterol at film pressures of (a) 2, (b) 10 and (c) 35 dyn/cm.

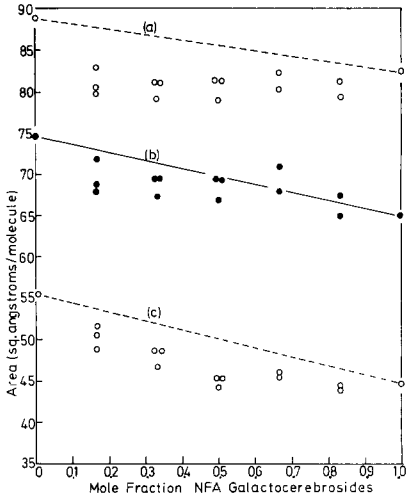

Fig. 9. Plots of the areas of mixed films of galactocerebrosides (NFA) and sphingomyelin versus the mole fraction of NFA at film pressures of (a) 2, (b) 10 and (c) 35 dyn/cm.

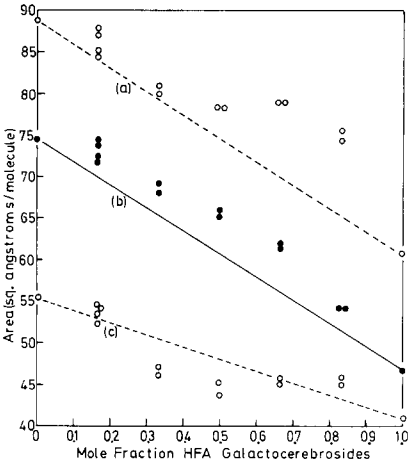

Fig. 11. Plots of the areas of mixed films of α-hydroxy galactocerebrosides (HFA) and sphingomyelin versus the mole fraction of HFA at film pressures of (a) 2, (b) 10 and (c) 35 dyn/cm.

in Fig. 9 and 10, respectively. Both mixed films display non-ideal behaviour. However, in the case of the sphingolipid mixture, the experimental points either lie on or below the line joining the pure component areas, while in the case of the phosphatidylcholine mixture, without exception they lie above it. There is no clear relationship between the deviation from ideal behaviour and film composition in the phosphatidylcholine mixtures. Non-ideality in sphingomyelin mixtures is dependent on film composition and occurs to the greatest extent at high sphingomyelin mole fractions.

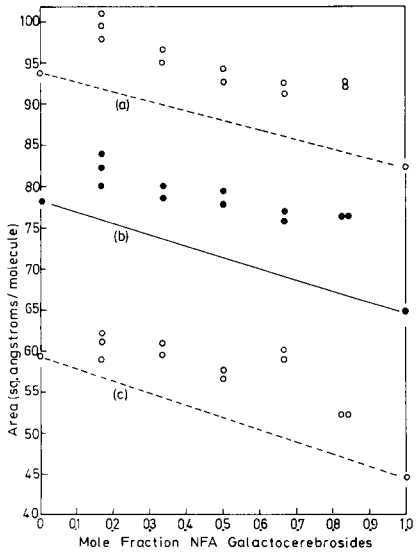

Fig. 10. Plots of the areas of mixed films of galactocerebrosides (NFA) and choline phosphatides versus the mole fraction of galactocerebrosides at film pressures of (a) 2, (b) 10 and (c) 35 dyn/cm.

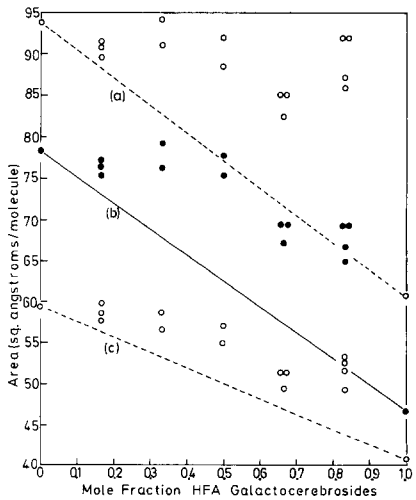

Fig. 12. Plots of the areas of mixed films of α-hydroxy galactocerebrosides (HFA) and choline phosphatides versus the mole fraction of HFA at film pressures of (a) 2, (b) 10 and (c) 35 dyn/cm.

Mixed films of HFA galactocerebrosides with myelin phosphatidylcholines and sphingomyelins. The interactions between HFA galactocerebrosides and these lipids are more complex than those found with NFA. At the lower film pressures, 2 and 10 dyn/cm, the areas of the sphingomyelin mixtures exhibit a positive departure from ideality (see Fig. 11). This is more pronounced at the lower sphingomyelin mole fractions. At the higher film pressure shown (35 dyn/cm), the experimental points scatter about the line joining the pure component areas. In the case of the phosphatidylcholine mixtures (Fig. 12) all experimental points lie above this line. At the largest HFA mole fraction studied, 0.83, the departure from ideality is inversely related to the film pressure and the value at 2 dyn/cm, approx. 23 $Å^2$/molecule, is extremely large.

Discussion of results

The structure of galactocerebroside films

The pressure of natural cerebroside films continues to increase when they are compressed into areas which are too small for the existence of a simple monolayer. The fact that the pressure continues to increase at these small areas argues against wholesale collapse and loss of order among the film molecules. In addition, at very small areas the molecules must be very highly ordered indeed, for they are capable of forming a film which is sufficiently rigid to physically move the Wilhelmy plate. It is evident from Fig. 1 that this occurs when the area of the cerebroside films is approximately half the area at which glycerolipid films collapse, indicating that the rigid cerebroside phase is a bilayer. The pressures of films of the other sphingolipids, sphingomyelin and cerebroside sulphate, also continue to rise below the area at which glycerolipid films collapse.

At slow compression speeds, there is a broad plateau in the isotherm of the NFA galactocerebrosides within which the film pressure is constant. A transition of this extent, over 14 $Å^2$ per molecule, starting at 42 $Å^2$ per molecule, cannot be caused by a change in the packing of the lipid molecules in the monolayer. The dependence of the extent of this transition on compression speed indicates that the process which gives rise to it is significantly slower than the rate at which the area of the film is reduced at the faster barrier speed. At the slower speed more of the film molecules are involved. This has the effect of reducing the area at which the film rigidifies and displaced the wilhelmy plate. In the isotherm shown in Fig. 3 this area is close to 20 $Å^2$ per molecule, an area half that at which the transition commences. Therefore, we conclude that the high-pressure transition in NFA galactocerebroside isotherms is caused by the formation of a bilayer.

Compared to NFA films, less pressure is required to compress HFA films below the area at which the film could exist as a monolayer (40–45 $Å^2$ per molecule, see Fig. 3). This is despite the fact that cohesive forces between the HFA galactocerebroside molecules are less than those between NFA galactocerebrosides (T_{max} HFA, 68.9°C; T_{max} NFA, 78.9°C). We conclude that HFA molecules form a bilayer more readily. The lack of hysteresis in compression-expansion istherms of HFA galactocerebrosides shows that transition from bilayer to monolayer is as rapid as the initial formation of the bilayer. By contrast, marked hysteresis in the NFA galactocerebroside isotherms shows that the NFA galactocerebroside bilayer does not rapidly reform a monolayer when the area available to it is increased.

Mixed films

The ares of NFA and HFA galactocerebroside films were more variable than the areas of the glycerolipid or cholesterol films. The variability was particularly marked in the case of the mixed film of the two cerebrosides which had the smallest fraction of the HFA lipid. The isotherms fell into two groups, examples of which are shown in Fig. 6. At this composition the films are unstable and the isotherms of the mixture can take the form of the isotherm of either of the components.

The areas per molecule of mixed films made from brain phosphatidylcholines and both NFA and HFA galactocerebrosides lie above the line joining the two component areas at all film compositions and pressures (see Fig. 10 and 12). A positive departure of the mixture areas from the line joining the pure component areas is usually taken to indicate that the components are miscible

but that attractive forces between their molecules are less than the average force between molecules of the pure components. However, at least in one instance, the 1/6 phosphatidylcholine, 5/6 HFA galactocerebrosides mixture, the expansion is too large to be explained in these terms. In the case of the NFA galactocerebroside mixtures the break in the isotherm and hysteresis associated with the formation of a bilayer are eliminated when the mixture contains more than 1/6 phosphatidylcholine. Therefore, we attribute at least a part of the non-ideal behaviour of these films to the presence of phosphatidylcholine hindering the compression-induced conversion of the monolayer to a bilayer.

Mixtures of cholesterol with HFA galactocerebrosides show near ideal behaviour, while the areas of the NFA galactocerebroside mixtures fall well below the ideal behaviour line. This is the reverse of the deviations from non-ideality observed with phosphatidylcholine mixtures. Previous work has shown that cholesterol and certain phosphatidylcholine mixtures show a negative departure from ideal behaviour [20]. This is because cholesterol increases the order parameter of the acyl chains of the phospholipid. A similar mechanism may be in operation here. There is also the possibility that cholesterol increases the propensity of NFA galactocerebrosides to form a bilayer.

Neither of the cerebroside mixtures with sphingomyelin show a consistent departure from ideal behaviour at all film compositions and pressures. Therefore, sphingomyelin does not appear to significantly enhance or hinder the process of bilayer formation characteristic of each of the two galactocerebrosides.

The relationship between lipid shape and film structure

Stoffel et al. [21] have studied the film properties of none *N*- and *O*-acylated sphinganines and sphingenines (sphingosine) and found that some of these compounds collapse to stable trilayers when compressed to 40–45 dyn/cm pressure. They concluded that trilayer formation occurs when the hydrophilic part is distinctly larger than the hydrophobic part of the lipid molecule. The measurements on brain ceramides and cerebrosides reported here have many similarities with those reported by Stoffel et al. Although cerebrosides form bilayers rather than trilayers, it is clear that the stability of the monolayer decreases as the size of the hydrophilic part of the lipid increases with respect to the hydrophobic part. Ceramides, derived from sphingomyelin and galactocerebrosides by removal of the polar group, form stable monolayers at all film pressures. The NFA galactocerebrosides, which have a high proportion of long and bulky unsaturated acyl chains, can form bilayer structures, but conversion to the bilayer from the monolayer is complete only when the film is compressed slowly to very high pressure. Note also that nervonoyl and oleoyl galactocerebroside (single unsaturated acyl chain) monolayers are not converted to bilayer structures by compression. The substituted galactocerebrosides have a larger hydrophilic region due to the presence of the α-hydroxy group and the acyl chains of these lipids are less bulky than those of NFA, since on average they are shorter and virtually fully saturated. Monolayers of these lipids convert readily to bilayers at low film pressures and judging from the lack of hysteresis in compression-expansion isotherms, conversion back to the monolayer is equally rapid when the pressure is reduced. Stearoyl galactocerebroside, although it does not possess an α-hydroxy group, has a comparatively small and unsaturated acyl chain and its monolayer also readily converts to a bilayer under compression.

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References

1 Arnon, R., Crisp, E., Kelley, R., Ellison, G.W., Myers, L.W. and Tourtellotte, W. (1980) J. Neurol. Sci. 46, 179–186.
2 Dubois-Dalcq, M., Niedieck, B. and Buyse, M. (1970) Pathol. Eur. 5, 331–337.
3 Raine, C.S. Traugott, U., Farooq, M., Bornstein, M.B. and Norton, W.T. (1981) Lab. Invest. 45, 174–182.
4 Waksman, B. (1985) Nature 318, 104–105.
5 Boggs, J.M. and Moscarello, M.A. (1980) Neurochem. Res. 5, 319–336.

6 Quinn, P.J. and Sherman, W.R. (1971) Biochim. Biophys. Acta 233, 734–752.
7 Maggio, B. and Lucy, J.A. (1975) Biochem. J. 149, 597–608.
8 Oldani, D., Hauser, H., Nichols, B.W. and Phillips, M.C. (1975) Biochim. Biophys. Acta 382, 1–9.
9 Maggio, B., Cumar, F.A. and Caputto, R. (1978) Biochem. J. 171, 559–565.
10 Sweeley, C.C. and Moscatelli, E.A. (1959) J. Lipid Res. 1, 40–47.
11 Carter, H.E. and Gaver, R.C. (1967) J. Lipid Res. 8, 391–395.
12 Hoshi, M., Williams, M. and Kishimoto, Y. (1973) J. Neurochem. 21, 709–712.
13 Gerstl, B., Eng. L.F., Tavaststjerna, M., Smith, J.K. and Kruse, S.L. (1970) J. Neurochem. 17, 677–689.
14 De Vries, G.H. and Norton, W.T. (1974) J. Neurochem. 22, 251–257.
15 Mac Brinn, M.C. and O'Brien, J.S. (1969) J. Neurochem. 16, 7–12.
16 Norton, W.T. (1977) in Myelin (Morell, P., ed.), p. 176, Plenum Press, New York.
17 Fromherz, P. (1975) Rev. Sci. Instrum. 46, 1380–1385.
18 Gaines, G.L. Jr. (1966) Insoluble Monolayers at Gas-Liquid Interfaces, p. 220, Interscience, New York.
19 Gaines, G.L. Jr. (1966) Insoluble Monolayers at Gas-Liquid Interfaces, p. 178, Interscience, New York.
20 Demel, R.A., Bruckdorfer, K.R. and Van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 255, 321–330.
21 Stoffel, W., Pruss, H.D. and Sticht, G. (1974) Chem. Phys. Lipids 13, 466–480.