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# MONOLAYER CHARACTERISTICS AND CALCIUM ADSORPTION TO CEREBROSIDE AND CEREBROSIDE SULPHATE ORIENTED AT THE AIR–WATER INTERFACE

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SUMMARY

Cerebroside and cerebroside sulphate orient as monolayers at the air–water interface. The force–area curve of cerebroside is of a condensed type and cerebroside sulphate is typical of a liquid-expanded monolayer. There is no reduction in area per molecule when the force–area curve is measured on 1 M HCl and this is believed to indicate the presence of $H_3O^+$ hydrogen-bonded to the sulphate groups. Surface potential–subphase pH curves show a marked change in $\Delta V$ centered about pH 6.5 which is unrelated to any primary electrostatic effects. Acetylation or trimethylsilylation of some of the polar groups of the lipid results in a substantial reduction in the change in $\Delta V$. The results are interpreted as a change in the orientation of water molecules about the polar head group of the lipid. An additional change in $\Delta V$ exclusive to cerebroside sulphate is seen at pH's below 3 which is associated with the ionization of the sulphate. The actual p$K$ of ionization was approx. pH —0.3. Adsorption of $^{45}Ca^{2+}$ to monolayers of cerebroside sulphate as determined by a surface radioactivity technique also indicates that the lipid is fully ionized above pH 3.5. $H^+$ competes for absorbed calcium below pH 5.5. The preference of $Ca^{2+}$ as the counter ion is of the order of $2 \cdot 10^4$ times greater than for $Na^+$ or $K^+$. $K^+$ has a slight competitive advantage over $Na^+$ in displacing adsorbed $^{45}Ca^{2+}$.

The adsorption of $^{45}Ca^{2+}$ to cerebroside sulphate monolayers at 53, 85, 200 and 400 Å$^2$/molecule was related to the concentration of $Ca^{2+}$ in the subphase. At 85 and 200 Å$^2$/molecule the apparent association constant of the binding adhered strictly to the mass equation with each $Ca^{2+}$ interacting by two-point electrostatic attachment. Deviations from the mass equation could be accounted for by electrostatic effects at 53 and 400 Å$^2$/molecule of the lipid. The Boltzmann distribution concept of counter-ion binding solved by the Gouy equation does not coincide with the mass action approach of the Stern theory. The reduction in the proportion of calcium bound per lipid molecule when the area occupied by each counter ion exceeds twice the diameter of the hydrated calcium ion indicates a "fixed" position of $Ca^{2+}$ in close association with the sulphate as predicted by the Stern theory.

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## INTRODUCTION

Cerebroside and cerebroside sulphate are major constituents of myelin and comprise about 10 % of the total dry weight of adult rat brain[1]. Although the functional role of these lipids in myelin still remains uncertain the accumulation of cerebroside and cerebroside sulphate in Gaucher's disease and metachromatic leucodystrophy respectively has focused attention on the properties of these lipids. Both cerebroside and cerebroside sulphate have been characterized chemically and are composed of sphingosine, a fatty acid in amide linkage, and a galactosyl residue attached to O-1 of sphingosine. The fatty acid of the ceramide varies but they are generally long chain tetracosanoic and tetracosenoic acids and more than half are stable α-hydroxy fatty acids. Cerebroside sulphate contains in addition a sulphate group esterified to the 3-position of the sugar moiety. The chemical structure of these lipids indicate that the molecules are amphipathic and cerebroside sulphate alone or when mixed with other amphipathic lipids can be dispersed in water by ultrasonic radiation to form stable lamella structures[2,3]. The present study is concerned with a characterization of some of the physical properties of cerebroside and cerebroside sulphate when oriented as monolayers at the air–water interface. ABRAMSON *et al.*[2] have studied cation binding to cerebroside sulphate by measuring changes in turbidity of aqueous dispersions of the lipid on addition of various counter ions. The authors indicated that titration of released $H^+$ from the sulphate on counter ion binding was not possible because of the essentially complete acid dissociation of the sulphate compared with their earlier success in the case of metal ion binding to phospholipids. The conclusions from these experiments was that $Ca^{2+}$ was bound more tightly than monovalent ions and that $K^+$ binding was stronger than $Na^+$ which confirmed the observations of BREYER[4] using a two-phase chloroform–methanol–water system. Analyses of the coagulated lipids after the addition of $Ca^{2+}$ indicated a stoichiometric relation of lipid to $Ca^{2+}$ bound of 2 : 1 which was also the case for $^{45}Ca^{2+}$ binding to monolayers of phosphatidylserine and monophosphatidylinositol[5]. The surface radioactivity technique has been used in the following experiments to investigate the binding of $Ca^{2+}$ to cerebroside sulphate monolayers.

## MATERIALS AND METHODS

Cerebroside sulphate from beef brain was purchased from Applied Science Laboratories, Inc., Pa.. Cerebroside, supplied by Pierce Chemical Company, Ill., was a mixture of cerasin and phrenosin. Both cerebroside and cerebroside sulphate migrated as single spots on thin-layer chromatography. The lipids were trimethylsilylated[6] by reacting 100 μg of cerebroside or cerebroside sulphate dissolved in 50 μl anhydrous pyridine with 100 μl bis-trimethylsilyltrifluoroacetamide from a freshly opened bottle (Regis Chemical Company, Ill.). The mixture which was contained in a teflon-faced screw capped bottle was incubated at 55° for 30 min and then evaporated to dryness under nitrogen. The lipid derivatives were taken up in chloroform–methanol (1 : 1, by vol.) for spreading on the trough. Similar samples were acetylated by incubation with 200 μl of acetic anhydride–pyridine (1 : 1, by vol.) overnight at room temperature. The lipids were taken to dryness under nitrogen and aspirated *in vacuo* for 1 h before reaction with bis-trimethylsilylfluoroacetamide as described

above. The molecular weights were taken as 906 and 830 for cerebroside sulphate and cerebroside, respectively[7].

A rectangular plexiglass trough was constructed with a surface area of 112 $cm^2$ and a capacity of 75 ml. The contents were stirred by a glass-sheathed metal rod which was activated by a magnet from the underside at a rate of 15 strokes per min. Initial experiments showed that when a water-soluble dye was injected locally at one end of the trough it was evenly distributed in the subphase after about 2 min of stirring.

The trough was filled with about 80 ml of water which had been double distilled, deionized and freshly distilled from alkaline $KMnO_4$. These purification steps were sufficient to reduce the level of contaminating cations such that there was no interference with measurements of $^{45}Ca^{2+}$ adsorption to cerebroside sulphate monolayers at subphase concentrations of 5 nmoles. The surface was cleaned several times by passing a plexiglass barrier over the surface and aspirating surface material from the path of the barrier. The barrier was adjusted to the required area and cerebroside or cerebroside sulphate dissolved in chloroform–methanol (2:1, by vol.) was introduced slowly onto the cleaned surface from a microsyringe (Hamilton Co., Inc., Whittier, California). Inconsistent adsorption of $Ca^{2+}$ to cerebroside sulphate monolayers was observed if the lipid had been standing at room temperature for several days in chloroform–methanol (2:1, by vol.). Spreading solutions prepared freshly each day gave reliable results.

Surface pressure was measured by the Wilhelmy method using a sand-blasted platinum wire dipping into the surface and measuring the downward force by a sensitive torsion balance. When the dipping rod was flamed between experiments reproducibility of molecular areas for individually spread monolayers was $\pm 7$ % at 2 dynes/cm and decreased proportionally at higher spreading densities.

The interfacial junction potential was measured with a $^{226}Ra$ air-ionizing electrode in circuit with a standard calomel electrode and a high impedance Leeds and Northrup pH/mV meter. By convention $\Delta V$ is expressed in mV and related arbitrarily to the value recorded for a clean water surface. The pH was adjusted where required with a Radiometer pH-stat delivering appropriate volumes of HCl or NaOH to the subphase.

The adsorption of $Ca^{2+}$ to cerebroside sulphate monolayers was determined by a surface radioactivity technique similar to that used by HAUSER *et al.*[5].

Radioactive $^{45}CaCl_2$ (New England Nuclear Corporation) of specific activity 15.4 mC/mg $Ca^{2+}$ was diluted to a concentration of 0.5 $\mu$mole $^{45}Ca^{2+}$/ml and aliquots were added to the subphase. Surface radioactivity was measured by a flow counter (Baird-Atomic, Inc., Model 821B) mounted at a fixed distance from the surface. The ultrathin end-window was $<100$ $\mu g/cm^2$ and with a purge gas of 5 % methane in argon the detector gave a plateau of several hundred V about the operating voltage of 2250 V. The pulses from a scaler pre-amplifier (Baird-Atominc, Inc., Model 132) was integrated in two stages, the signal was amplified in the first stage with a 5-sec integration time and in the second stage cathode follower integrated over 20 sec. The output from the second stage was recorded continuously on a pen recorder.

The relationship between the number of $Ca^{2+}$ adsorbed per $cm^2$ of surface was determined by spreading monolayers of cerebroside sulphate on aqueous subphases containing $^{45}Ca^{2+}$ and measuring the increase in radioactivity from the surface over the background radiation measured in the absence of the film. Samples of the sub-

phase (0.1 ml) were removed and diluted in 10 ml of 30 % (v/v) methanol in toluene containing 0.6 % (w/v) 2,5-diphenyloxazole and 0.001 % (w/v) 1,4-bis-2-(5-phenyloxazolyl-2)-benzene for scintillation counting in a Packard Tri-Carb Scintillation Spectrophotometer (Model 3780).

Incomplete recoveries of added $^{45}Ca^{2+}$ which was particularly evident when the concentration of the isotope in the subphase was low resulted from the binding of a proportion of the $^{45}Ca^{2+}$ to sites on the trough. All the $^{45}Ca^{2+}$ could be recovered when these sites were presumably saturated by the addition of 1 $\mu$mole of HCl to the subphase. There was a direct relationship between the calculated number of calcium atoms/cm$^2$ of surface and the surface radioactivity. No correction was found necessary for a decrease in background radioactivity due to the reduction of $^{45}Ca^{2+}$ in the subphase; where an appreciable proportion of the calcium was removed from the subphase the specific activity was reduced and the appropriate correction for calcium adsorption made for this dilution.

## RESULTS

### *Physical properties of cerebroside and cerebroside sulphate monolayers*

The force–area curves of cerebroside sulphate (Fig. 1A) are of the form typically shown by lipids in the liquid-expanded state[8]. This can probably be attributed to the presence of the sulphate group on the 3-position of the galactosyl moiety which would be fully ionized on a subphase of pH 10 and would give rise to an electrostatic repulsion between like charges of adjacent molecules. It would be expected that if the sulphate was discharged by spreading the lipid on a subphase at pH below the p$K$ of the ionization there would be a reduction of these repulsive forces and a consequent reduction in the area per molecule. Cerebroside sulphate, however, shows a slight increase in

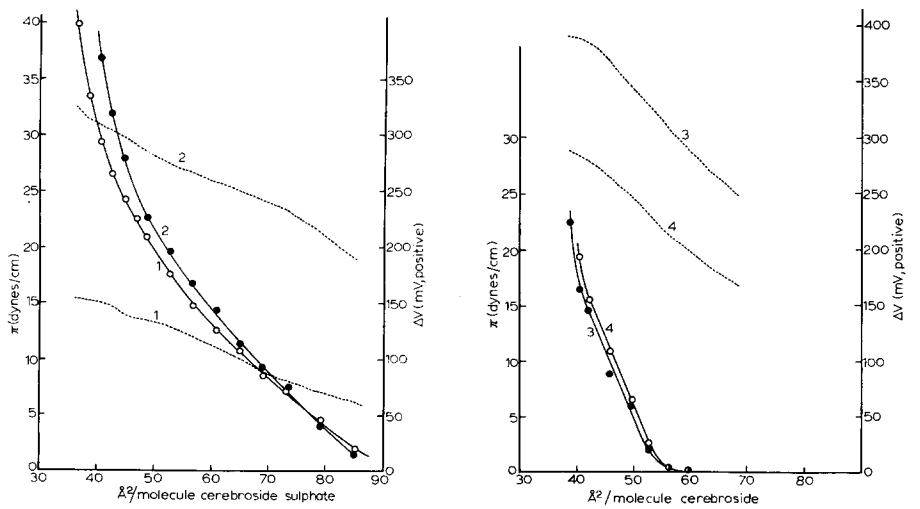


Fig. 1. A. Relationship between surface pressure ($\pi$) and potential ($\Delta V$) and area occupied by cerebroside sulphate molecules. Curves 1. Subphase 1 M HCl. ●—●, $\pi$; ·····, $\Delta V$. Curves 2. Aqueous subphase pH 10. ○—○, $\pi$; ·····, $\Delta V$. B. Relationship between surface pressure ($\pi$) and potential ($\Delta V$) and area occupied by cerebroside molecules. Curves 3. Subphase 1 M HCl. ●—●, $\pi$; ·····, $\Delta V$. Curves 4. Aqueous subphase pH 10. ○—○, $\pi$; ·····, $\Delta V$.

area per molecule at pressure above 8 dynes on a subphase of 1 M HCl compared with areas on an alkaline subphase. It is probable that the apparent p$K$ of the sulphate is above pH 0 (see later) but at this pH it should be substantially discharged. In any case, the use of more acidic subphases was avoided because the sulphate ester is acid labile. At surface pressures above 50 dynes/cm cerebroside sulphate monolayers were stable in that the lipid did not pass into the aqueous phase or exhibit any three-dimensional aggregation. When compressed films were expanded to 65 Å$^2$/molecule values of $\pi$ and $\Delta V$ were comparable with those of monolayers spread at lower density and compressed to the same area. No definitive determination of the limiting area at the collapse pressure could be obtained as when the pressure was higher than about 50 dynes/cm the films spilled over the edges of the trough and the film area became unreliable. From the slope of curve 1 of Fig. 1A the limiting area is probably in the region of 35–36 Å$^2$/molecule.

The force–area curve of cerebroside is that usually given by lipids in a more condensed state and could be predicted from the presence of long and predominantly saturated paraffinic groups on the molecule. The "lift off" area is 55 Å$^2$/molecule and the limiting area is slightly less than 40 Å$^2$/molecule. The limiting area could not be accurately determined because the monolayer became unstable above 20 dynes/cm so that further reductions in area per molecule resulted in a steady decrease in the surface pressure. The area per molecule of cerebroside does not vary with subphase pH nor does a 400-fold difference in ionic strength of the subphase appear to have any effect. There is a difference of approx. 100 mV in the surface potential which cannot conceivably be due to any ionization effects between pH 0 and 10.

The nature of this change in surface potential was examined by titrating monolayers of cerebroside and cerebroside sulphate spread on a subphase of pH 3.6 and recording changes in surface potential at equilibrium on the successive addition of more acid or alkali to the subphase. The results show two discrete changes in surface potential of cerebroside sulphate monolayers (Fig. 2A). The change in $\Delta V$ between pH 1 and 3 is presumably due to the ionization of the sulphate as there is no compar

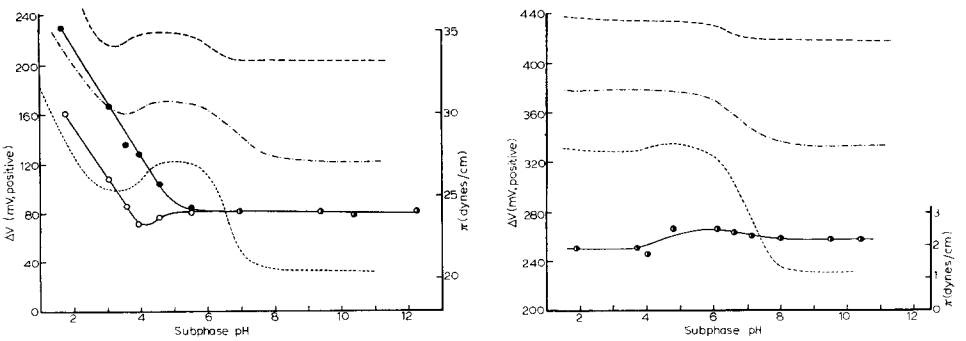


Fig. 2. Relationship between surface potential and pH of subphase below monolayers of cerebroside sulphate (A) and cerebroside (B). Monolayers ($2 \cdot 10^{16}$ molecules per film) were spread on subphase previously adjusted to pH 3.6 and recording changes on addition of NaOH or more HCl. -------, $\Delta V$ native lipids; –·—·–, $\Delta V$ trimethylsilylated derivatives; — — —, $\Delta V$ acetylated and trimethylsilylated derivatives. Initial surface pressures (●—●) of individual monolayers of cerebroside sulphate (A) and cerebroside (B) spread on subphases adjusted to the required pH were determined after allowing 1 min for the solvent to evaporate. Equilibrium surface pressures (○—○) were recorded when $\Delta V$ reached a steady value.

able change in $\Delta V$ for cerebroside monolayers (Fig. 2B). Both lipids show a change in surface potential of between 90–100 mV centered about pH 6.5. By inference again this change in surface potential which is of the same order of magnitude for both lipids would not be the result of any primary electrostatic effects. Since there is no change in $\Delta V$ in the more alkaline region of the titration curve or any change in the surface pressure of cerebroside monolayers the amide proton of the sphingosine moiety is probably undissociated up to pH 10.5.

To investigate the source of this change in surface potential the components which contribute to the measured value of $\Delta V$ were examined. Surface potential is the sum of the vertical dipole moments arising from the reorientation of water molecules about the adsorbed lipid ($\mu_1$), the chacteristic dipole moment of the particular lipid class or so-called group dipole moment ($\mu_2$) and finally the dipole moment about the terminal bond at the upper limit of the monolayer ($\mu_3$). When the lipid contains an ionizable group this gives rise to a vertical dipole proportional to the electrostatic potential in the plane of the charges relative to the bulk phase and denoted by $\Psi_0$. The relationship to the measured $\Delta V$ is obtained by applying the Helmholtz formula[9] for an array of $n$ dipoles per cm$^2$. Where the dipole moments are vectorially additive in the vertical direction then:

$$\Delta V = 4\pi n(\mu_1 + \mu_2 + \mu_3) + \Psi_0 \qquad (1)$$

assuming a value of unity for the dielectric constant.

It should be emphasized that individual dipoles cannot generally be considered as independent variables so that a reorientation of one or other of the dipoles will almost invariably result in some change in the overall orientation of the remainder. Thus in the situation where only one dipole is directly concerned in a reaction then the manifestation of the effect in the form of a change in the observed $\Delta V$ will be the net effect of the reorientation of the complete set of dipoles. For this reason no assignments to individual dipoles can be made unambiguously although in the present treatment only one of the dipoles is considered to be involved directly whereas the others may only be associated consequentially. The direct involvement of the terminal dipole can be precluded with relative certainty because of its remoteness from the aqueous phase. Secondly the group dipole seems an unlikely contender because the observed change in $\Delta V$ appears in the pH region about 6.5 in such widely diverse lipids as octadecanol, monophosphatidylinositol, dicetyphosphoric acid, phosphatidylethanolamine as well as the cerebrosides (see DISCUSSION). The one feature in common with all these amphipaths is a polar interaction with the aqueous subphase. To obtain some information about the interaction of hydrophilic groups of cerebroside and cerebroside sulphate with water molecules a number of polar groups of each lipid were blocked by forming the trimethylsilyl and acetylated derivatives and recording changes in $\Delta V$ with subphase pH maintaining the same area/mol in each case.

The results (Fig. 2A and B) show that reaction of cerebroside sulphate and cerebroside with bis-trimethylsilylfluoroacetamide under relatively mild conditions reduces the decrease in surface potential by about half for both lipids (from approx. 95 mV to 45 mV). Acetylation over a prolonged period followed by reaction with bis-trimethylsilylfluoroacetamide under the same conditions as above reduces the change in $\Delta V$ by about 80 % (from approx. 95 mV to 18 mV) of that for the unreacted

lipids. Between 5 and 6 acetyl groups react with cerebroside[10] (phrenosin possesses an extra hydroxyl residue on the 2-position of the fatty acid) leaving three other possible polar groups, namely, the pyran oxygen of the galactosyl ring, the carbonyl oxygen of the fatty acyl residue and the amide nitrogen of the sphingosine moiety. Although as stated previously, surface potential measurements do not permit any direct assignments of the interaction of water molecules with individual polar groups of the lipid, the results indicate that complete blocking of the hydroxyl groups by acetylation produces a substantial reduction in the change in $\Delta V$ in the pH region about 6.5, while the change in $\Delta V$ of cerebroside sulphate in the more acidic region remains essentially unaltered. The small change in $\Delta V$ even after complete acetylation could be attributed to the residual polar regions not amenable to acetylation. These regions would also assist in the orientation of the derivatives at the interface. The shift of the whole curve to more positive $\Delta V$ values could be substantially accounted for by a change in the group dipole moment ($\mu_2$) when the lipid is trimethylsilylated or acetylated. It should be noted, however, that the above comparisons were made with the area per molecule maintained at the same value for all films so that comparisons within individual experiments should remain valid. The trimethylsilyl derivative of the sulphate group appears to be labile when the lipid is spread on water. This is indicated by a retention of the increase in surface potential at pH $<3$. Under more rigorous conditions[10] it is possible to hydrolyse the sulphate ester and replace this with a trimethylsilyl residue in which case the change in $\Delta V$ associated with the ionization of the sulphate could be eliminated.

There was good general agreement between the surface potentials measured on the same monolayer as the pH was altered and that of individually spread monolayers at various subphase pH. The surface pressure of cerebroside was unchanged over the pH range indicated in Fig. 2B. The equilibrium surface pressures of cerebroside sulphate monolayers increased below pH 3.5 and as discussed later this is probably due to hydroxonium ion, hydrogen bonded to the ionized sulphate.

*The electrostatic properties of cerebroside sulphate monolayers*

In addition to changes about pH 6.5 in cerebroside sulphate monolayers there is the creation of a dipole associated with the counter ions absorbed to the film as the sulphate becomes fully charged about pH 3.5. The actual p$K$ of the ionization of the sulphate can be determined from the apparent p$K$ obtained from $\Delta V$ measurements by calculating the surface pH from the relationship $pH_s = pH_b + \varepsilon\Psi_0/2.3kT$ where subscripts s and b refer to the surface and bulk phases respectively; $\varepsilon$ = electronic charge; $k$ = Boltzmann constant and $T$ = absolute temperature. Since in general, the electrostatic potential ($\Psi_0$) approximates to $\Psi_G$ the value can be calculated by applying the Gouy theory of counter ion distribution. It should be noted that application of the Gouy model to the present system involves certain assumptions concerning the charge density[11] and discreteness of charge effects[12,13] and as will be demonstrated later is only reliable as a first approximation. However, with these reservations, calculations of $\Psi_G$ from the Gouy equation in the form of Eqn. 2 would seem justified.

$$\Psi_G = \frac{2\,kT}{\varepsilon Z} \sin h^{-1}\left[\frac{\sigma}{c^{\frac{1}{2}}}\left(\frac{500\pi}{DRT}\right)^{\frac{1}{2}}\right] \qquad (2)$$

where $\sigma$ = charge density; $c$ = total electrolyte concentration; $k$ = Boltzmann constant; $T$ = absolute temperature; $\varepsilon$ = electronic charge; $Z$ = valence; $D$ = dielectric constant of water; $R$ = gas constant. Hence for a monolayer of cerebroside sulphate containing $2 \cdot 10^{14}$ charged groups per cm$^2$ (bulk pH 3.5) the value of $\Psi_G$ is approximately 107 mV. The apparent p$K$ of the sulphate is therefore about pH 1.5 (Fig. 2A) and the actual p$K$ of ionization is pH $-0.3$.

The ionic properties of cerebroside sulphate monolayers were also investigated by observing the adsorption of counter ions. The adsorption of $Ca^{2+}$ was chosen because the radioactive isotope of the metal could be determined specifically in the surface phase by utilizing a surface radioactivity technique. To follow the course of ionization of a monolayer of cerebroside sulphate by the adsorption of calcium as a counter ion a film was spread on a subphase containing $^{45}Ca^{2+}$ adjusted to pH 2.8 (Fig. 3, Curve A). A small amount of calcium was adsorbed to the film at equilibrium and this increased with increasing subphase pH up to pH 6. There was a small but steady increase in the $Ca^{2+}$ binding between pH 6 and 10 and a sharp reduction at higher subphase pH. Calcium adsorption to individual monolayers of cerebroside sulphate spread on $^{45}Ca^{2+}$ containing subphases adjusted directly to the required pH (Fig. 3, Curve C) showed a marked increase in the extent of $Ca^{2+}$ binding to the film. Furthermore when the subphase pH was below 5.5 there was a marked decrease in the adsorption of $Ca^{2+}$ to the film at equilibrium compared with the initial value obtained within 1 or 2 min of spreading the film. The initial value of $Ca^{2+}$ adsorbed was obtained by extrapolation of the radioactivity–time curve back to the point where the film was spread on

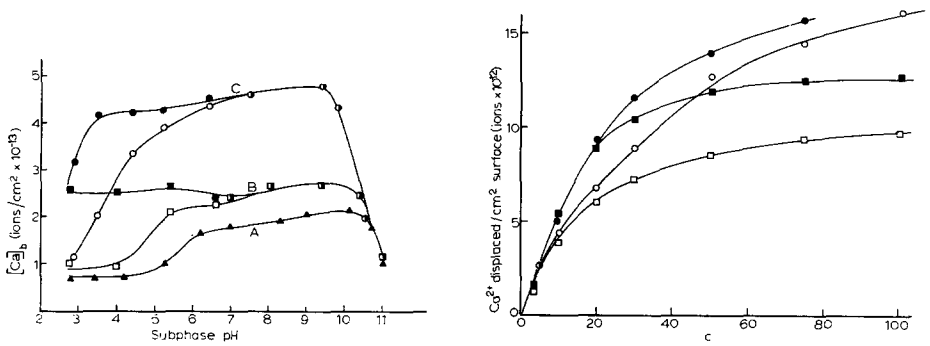


Fig. 3. The adsorption of $^{45}Ca^{2+}$ to monolayers of cerebroside sulphate on subphases of various pH. Each monolayer contained $18.75 \cdot 10^{15}$ molecules spread on 100 cm$^2$ of the surface and the subphase contained 0.2 $\mu$moles of calcium. A. ▲—▲, Calcium adsorption to a monolayer equilibrated on a subphase adjusted to pH 2.8 and the pH increased gradually. B. Calcium adsorption to individual cerebroside sulphate monolayers spread on the calcium containing subphase initially at pH 2.8 and adjusted to the required pH by adding appropriate amounts of NaOH. ■—■, Calcium adsorbed initially, obtained by extrapolation of the surface radioactivity to the point of film spreading. □—□, Calcium adsorption to the same monolayer at equilibrium. C. Calcium adsorption to individual monolayers of cerebroside sulphate spread on subphases adjusted directly to the required pH using the minimum amount of HCl or NaOH. ●—●, Calcium adsorption initially, obtained by extrapolation of the surface radioactivity to the time of film spreading. ○—○, Calcium adsorption to the same monolayer at equilibrium.

Fig. 4. Plot of the displacement of $^{45}Ca^{2+}$ adsorbed to monolayers of cerebroside sulphate as a function of univalent ion concentration in the subphase. Circles refer to cerebroside sulphate monolayers spread at a surface concentration of 70 Å$^2$/molecule and squares to monolayers of density 47 Å$^2$/molecule. $c$ represents the concentration of $Na^+$, open symbols and $K^+$, solid symbols expressed in $\mu$moles. The $[^{45}Ca^{2+}]_{total}$ was 10 nmoles.

the subphase. The rate at which the initial calcium was removed increased also with decreasing pH which would suggest that $H^+$ is competing with calcium as counter ions to the sulphate. From the extent of calcium binding initially to cerebroside sulphate monolayers it can be inferred that the sulphate is fully ionized above pH 3.5, the decrease in $Ca^{2+}$ binding above pH 9.5 is interpreted as a competition for the available binding sites on the film between $Ca^{2+}$ and $Na^{2+}$ from NaOH used to adjust the pH.

The difference in the extent of calcium binding to a single monolayer in which additional NaOH was added to the subphase and that of individually spread monolayers was reconciled by repeating the latter experiments on subphases initially at pH 2.8 and then adjusted to the required pH before spreading the film (Fig. 3, Curve B). The adsorption characteristics over the range of subphase pH investigated was similar to Curve C but the total amount of $Ca^{2+}$ adsorbed was reduced to values slightly above that of Curve A of Fig. 3. The presence of increasing $Na^+$ concentration in the subphase would appear to be responsible for the reduction in $Ca^{2+}$adsorbed. When the subphase was 0.1 M NaCl there was no adsorption of calcium onto monolayers of cerebroside sulphate over the pH range 2.8–10.5.

*The displacement of adsorbed $Ca^{2+}$ by $Na^+$ and $K^+$*

The competition between $Ca^{2+}$ and the monovalent ions $Na^+$ and $K^+$ for binding to cerebroside sulphate monolayers is shown in Fig. 4. Cerebroside sulphate monolayers were spread at areas of 70 and 45 Å$^2$/molecule on aqueous subphases containing 10 nmoles $^{45}Ca^{2+}$. When the radioactivity had reached a maximum after about 2 min from the time of spreading a calculated amount of NaCl or KCl was added to the subphase and the resulting decrease in radioactivity recorded. The calculated value of $Ca^{2+}$ displaced from the film was corrected for the displacement by $H^+$ by determining the loss of $Ca^{2+}$ from films without $Na^+$ or $K^+$ present in the subphase.

Both $Na^+$ and $K^+$ can displace adsorbed $Ca^{2+}$ from monolayers of cerebroside sulphate albeit only at relatively high concentrations. Thus in the presence of 20 $\mu$moles of either $Na^+$ or $K^+$ only about 1 nmole of $Ca^{2+}$ was displaced from the monolayer. $Ca^{2+}$ appears to be displaced more readily from monolayers spread at higher areas per molecule. $K^+$ is more effective than $Na^+$ at equivalent concentrations in the subphase and the difference increases with higher concentrations in the subphase.

*The binding characteristics of $Ca^{2+}$ to cerebroside sulphate monolayers*

The next series of experiments were concerned with the determination of the apparent association constant between $Ca^{2+}$ and cerebroside sulphate monolayers. The procedure involved spreading monolayers of the sulphatide at either 53, 85, 200 or 400 Å$^2$/molecule on aqueous subphases containing 1 $\mu$mole of HCl and varying amounts of $^{45}Ca^{2+}$. The radioactivity–time trace from the recorder was extrapolated back to the time of spreading to obtain the number of calcium ions adsorbed to the surface layer. Although monolayers spread at areas greater than 200 Å$^2$/molecule would be expected to exhibit heterogeneity, subphase stirring appears to distribute the molecules evenly on the surface. No variation in surface radioactivity was observed when the flow counter was placed at various points on the trough and the end window was sufficiently large to eliminate differences in distribution on the molecular scale.

According to the law of mass action for the reaction, $Ca^{2+}$ + cerebroside sul-

phate $\rightleftharpoons$ Ca–cerebroside sulphate, the apparent association constant of the reaction can be obtained from Eqn 3:

$$K_a = \frac{[\text{Ca–CS}]}{[Ca^{2+}]_f [CS]_f} \tag{3}$$

The square brackets refer to concentrations of $Ca^{2+}$ and cerebroside sulphate (CS) and the subscripts, f refer to free form of the ions. Since calcium bound to the monolayer can be determined from surface radioactivity measurements the value $[\text{Ca–CS}] = [Ca]_b$ where subscript b refers to the bound form of calcium.

With a known amount of calcium ($[Ca^{2+}]_{total}$) and cerebroside sulphate, ($[CS]_{total}$) present in the system. Eqn. 3 can be expressed in the terms of Eqn 4:

$$K_a = \frac{[Ca]_b}{([Ca^{2+}]_{total} - [Ca]_b)\,(n[CS]_{total} - [Ca]_b)} \tag{4}$$

where $n$ is the fraction of cerebroside sulphate molecules acting as binding sites for calcium.

Several expressions can be derived from Eqns. 3 and 4 which enables a graphical analysis of the data and from which values of both $K_a$ and $n$ can be obtained. Thus defining $r = [Ca]_b/[CS]_{total}$ then $[CS]_f = n\,[CS]_{total} - r\,[CS]_{total}$ and Eqn. 3 can be written as:

$$\frac{1}{K_a} = \frac{n[Ca^{2+}]_f}{r} - [Ca^{2+}]_f \tag{5}$$

If monolayers of cerebroside sulphate can be thought of as a single set of binding sites for $Ca^{2+}$ each with the same affinity for the metal then by rearranging Eqn. 5 in the form of Eqn. 6

$$\frac{r}{[Ca^{2+}]_f} = nK_a - rK_a \tag{6}$$

SCATCHARD[14] demonstrated that a plot of $r/[Ca^{2+}]_f$ *vs.* $r$ should yield a straight line provided that there are no electrostatic interactions between the binding sites resulting in a change in the $K_a$. In the case of adsorption to monolayers it would be expected that these interactions are likely to be related to the distance separating the charged groups in the surface layer. For this reason adsorption of $Ca^{2+}$ onto monolayers of cerebroside sulphate at four different areas per molecule were determined and the data are plotted in Fig. 5A. The intercept with the ordinate gives $nK_a$ and with the abcissa, where the limit $r/[Ca^{2+}]_f \rightarrow 0$, yields the value of $n$ directly. When the area per molecule is large a straight line is obtained indicating negligible interaction between binding sites. As the area per molecule decreases then there is a deviation from linearity which is especially evident in the most compressed film (53 Å$^2$/molecule). The values of $K_a$ and $n$ obtained from the plot are given in Table I and were derived by fitting a straight line to the values by a least squares analysis.

Another graphical method for analyzing the data is to rearrange Eqn. 5 to give:

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{K_a n[Ca^{2+}]_f} \tag{7}$$

As pointed out by HUGHES AND KLOTZ[15], the value of $n$ obtained from the abcissa of Fig. 5A can be more confidently determined although the slope of the reciprocal

relationship should give a better estimate of $K_a$. The value of $n$ in Eqn. 7 is obtained from the intercept with the ordinate. The reciprocal plot of the data is shown in Fig. 5B and the values of $K_a$ and $n$ tabulated in Table I.

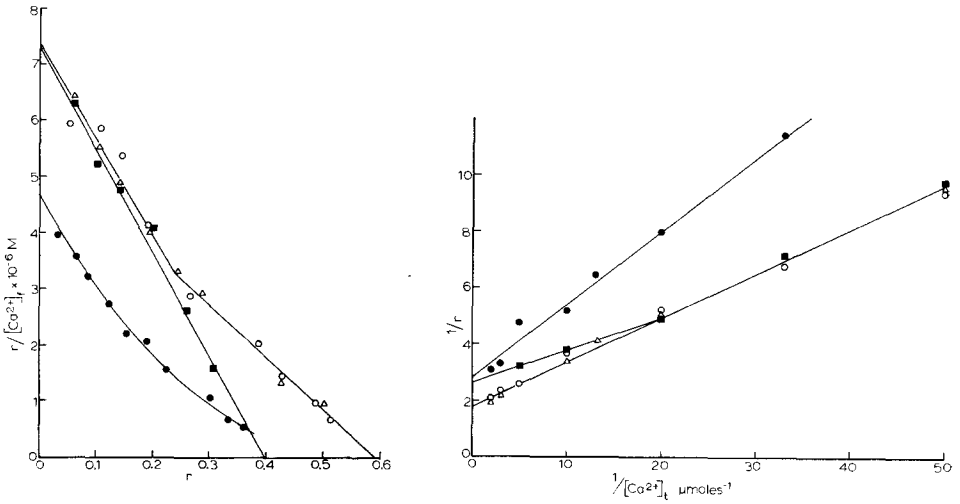


Fig. 5. A. Hughes–Klotz plot of the adsorption of $^{45}Ca^{2+}$ to monolayers of cerebroside sulphate at various areas per molecule. The plot gives the ratio $r/[Ca^{2+}]_f$ as a function of the ratio of $Ca^{2+}$ adsorbed per molecule of cerebroside sulphate ($r$). $[Ca^{2+}]_f$ represents the bulk concentration of metal ion, *i.e.*, $[Ca^{2+}]_{total} - [Ca^{2+}]_{surface}$ in $\mu$moles. ■—■, 400 Å$^2$/molecule; △—△, 200 Å$^2$/molecule; ○—○, 85 Å$^2$/molecule; ●—●, 53 Å$^2$/molecule. B. Reciprocal plot of the data for adsorption of $^{45}Ca^{2+}$ to monolayers of cerebroside sulphate. The concentration of calcium, $[Ca]_{total}$ is expressed in $\mu$mole and $r$ is the number of calcium ions bound/molecule of cerebroside sulphate. ■—■, 400 Å$^2$/molecule; △—△, 200 Å$^2$/molecule; ○—○, 85 Å$^2$/molecule; ●—●, 53 Å$^2$/molecule.

TABLE I

VALUES OF $K_a$ AND $n$ FROM THE GRAPHICAL ANALYSES OF CALCIUM ADSORPTION TO CEREBROSIDE SULPHATE MONOLAYERS

| *Treatment of data* | | *Area/molecule of cerebroside sulphate (Å$^2$)* | | | |
|---|---|---|---|---|---|
| | | *400* | *200* | *85* | *53* |
| Eqn. 6 | $K_a \times 10^{-7}$ | 1.85 | 1.20 | 1.48 | 1.03 |
| | $n$ | 0.40 | 0.54 | 0.51 | 0.40 |
| | $K_a n \times 10^{-6}$ | 7.40 | 6.50 | 7.55 | 4.10 |
| Eqn. 7 | $K_a \times 10^{-7}$ | 1.58 | 1.41 | 1.02 | 1.12 |
| | $n$ | 0.43 | 0.49 | 0.59 | 0.35 |
| | $K_a n \times 10^{-6}$ | 6.80 | 6.90 | 5.95 | 3.89 |
| Eqn. 9 | $K_a \times 10^{-7}$ | 2.29 | 1.02 | 1.12 | 0.87 |
| | $n$ | 0.37 | 0.59 | 0.55 | 0.42 |
| | $K_a n \times 10^{-6}$ | 8.47 | 6.00 | 6.15 | 3.65 |
| Eqn. 10 | $K_a n \times 10^{-6}$ | 7.95 | 7.22 | 6.15 | 4.17 |
| Average | $K_a \times 10^{-7}$ | 1.91 | 1.21 | 1.21 | 1.01 |
| | $n$ | 0.40 | 0.54 | 0.55 | 0.39 |
| | $K_a n \times 10^{-6}$ | 7.66 | 6.62 | 6.45 | 3.95 |
| Eqn. 4 ($n = 1$) | $K_a \times 10^{-6}$ | 7.80 | 7.31 | 7.44 | 4.40 |

The second approach is to solve Eqn. 4 for $[Ca]_b$ assuming that the amount of calcium bound to the film is negligibly small compared with the other terms in the equation. The solution is given as Eqn. 8:

$$[Ca]_b = \frac{[Ca^{2+}]_t \, n[CS]_t}{1/K_a + n[CS]_t + [Ca^{2+}]_t} \tag{8}$$

HAUSER *et al.*[5] showed that the adsorption of calcium to phospholipid monolayers conformed with a Langmuir-type adsorption isotherm. If Eqn. 8 is rearranged in the form:

$$\frac{[Ca^{2+}]_t}{[Ca]_b} = \frac{1}{K_a n[CS]_t} + \frac{[Ca^{2+}]_t}{n[CS]_t} \tag{9}$$

then a straight line with a slope proportional to $1/n$ should be obtained by plotting the ratio $[Ca^{2+}]_t/[Ca]_b$ as a function of $[Ca^{2+}]_t$. The apparent association constant ($K_a$) is obtained from the intercept with the ordinate. The plot (Fig. 6) gives straight lines for the adsorption of calcium to cerebroside sulphate monolayers at the 4 different areas/molecule and there is little indication of any deviation from linearity even at high film densities or high concentrations of calcium in the subphase. The values of $K_a$ and $n$ obtained from the line of best fit passing through the experimental points is given in Table I.

Another form of Eqn. 8 has been derived by MILDVAN AND COHN[16] in their mathematical treatment of the binding of $Mn^{2+}$ to albumin. Thus in the limit of $[Ca^{2+}]_t \rightarrow 0$ then

$$\mathrm{Lim}_{[Ca^{2+}]_t \rightarrow 0} \frac{[Ca]_b}{[Ca^{2+}]_t} = \frac{n[CS]_t}{1/K_a + n[CS]_t} \tag{10}$$

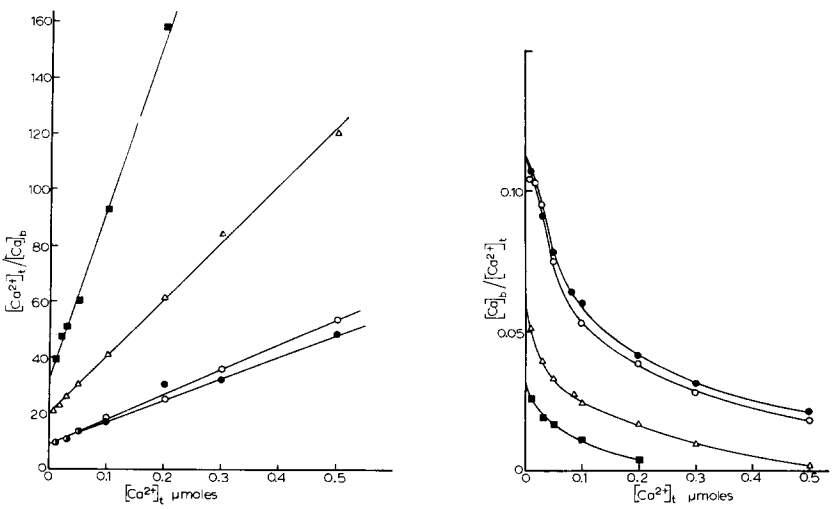


Fig. 6. Plot of the ratio of the total $[Ca^{2+}]$ to $[Ca^{2+}]$ adsorbed to monolayers of cerebroside sulphate as a function of the total $[Ca^{2+}]$ in the system expressed in $\mu$moles. ■—■, 400 Å²/molecule; △—△, 200 Å²/molecule; ○—○, 85 Å²/molecule; ●—●, 53 Å²/molecule.

Fig. 7. Plot of the relationship between the ratio of calcium bound to monolayers of cerebroside sulphate/total calcium added and the total $Ca^{2+}$ concentration expressed in $\mu$mole. ■—■, 400 Å²/molecule; △—△, 200 Å²/molecule; ○—○, 85 Å²/molecule; ●—●, 53 Å²/molecule.

and if the number of binding sites is small in the appropriate limits compared with the apparent association constant all of the calcium will be bound to the lipid on extrapolation to zero calcium. This appears to be a correct assumption as the extrapolated values obtained from the plot of $[Ca]_b/[Ca^{2+}]_t$ *vs.* $[Ca^{2+}]_t$ (Fig. 7) give a value of $K_a n$ close to that for the other graphical analyses (Table I).

In the preceding treatment of the data, no assumptions have been made regarding the proportion of cerebroside sulphate molecules acting as binding sites for calcium. Since each sulphatide molecule carries one negative charge then each should be capable of binding up to one $Ca^{2+}$. From the known values of $[Ca^{2+}]_t$, $[CS^-]_t$ and $[Ca]_b$ and a value of $n$ given as 1 then for each experimental point a value of $K_a$ can be calculated from Eqn. 4. The $K_a$ values obtained from these calculations are plotted in Fig. 8 as a function of $r$. The association constants show a linear decline with increasing amounts of calcium adsorbed to the film. The values of $K_a$ given in Table I were obtained by extrapolation at the limit of $r \to 0$. The $K_a$ values obtained in this way show reasonable congruency with the mean $K_a n$ values obtained from the four graphical approaches. The $K_a$ of $Ca^{2+}$ binding increases as the subphase $[Ca^{2+}]$ decreases for each spreading area. This would be expected since the adsorption is of the form of a Langmuir adsorption isotherm. The number of cerebroside sulphate molecules acting as a single binding site for each $Ca^{2+}$ ($n$) indicates that at 85 and 200 Å$^2$/molecule this is by a strict 2-point electrostatic attachment. At areas per molecule of 63 and 400 Å$^2$ the ratio of lipid to calcium increases although at low charge density the apparent association constant is higher than at the intermediate areas used and the value at high charge density is lower.

In the final experiments the relationship between the number of $Ca^{2+}$ bound per cerebroside sulphate molecule ($r$) and the area per molecule of lipid was examined

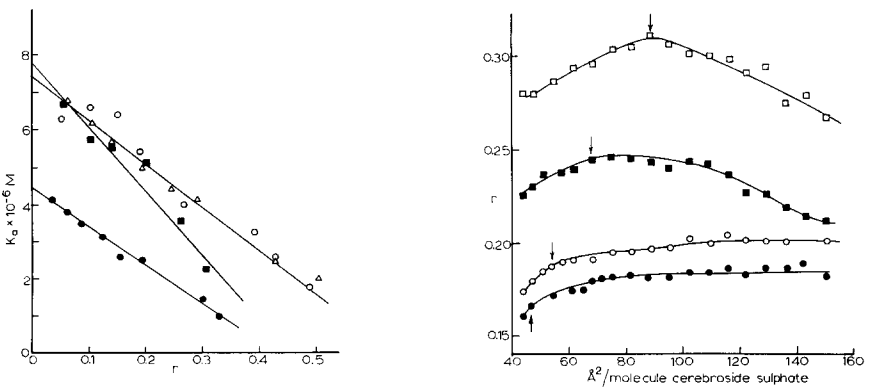


Fig. 8. Plot of the apparent association constant $K_a$ as a function of the number of calcium ions bound to each cerebroside sulphate molecule ($r$) of monolayers at various areas/molecule. The value $K_a$ has been calculated for each point using Eqn. 4. ■—■, 400 Å$^2$/molecule; △- -△, 200 Å$^2$/molecule; ○—○, 85 Å$^2$/molecule; ●—●, 53 Å$^2$/molecule.

Fig. 9. Relationship between the area per molecule of sulphatide and the ratio ($r$) of calcium bound to each lipid molecule. $7.5 \cdot 10^{15}$ molecules of sulphatide were spread on a water subphase containing 5 $\mu$moles NaOH and various amounts of calcium: ●—●, 7.5 nmoles $Ca^{2+}$; ○—○, 25 nmoles $Ca^{2+}$; ■—■, 75 nmoles $Ca^{2+}$; □—□, 250 nmoles $Ca^{2+}$. The surface radioactivity was recorded at equilibrium after adjusting the area occupied by the film. The arrows indicate the position where the concentration of $Ca^{2+}$ in the surface phase reaches approx. $3.55 \cdot 10^{13}$ molecules per cm$^2$.

in a system where $[Ca^{2+}]_t$ and $[CS]_t$ were kept constant. Cerebroside sulphate monolayers were spread at low density on aqueous subphases containing 5 $\mu$moles NaOH to adjust the pH to approx. 8. The subphase also contained varying amounts of $^{45}Ca^{2+}$ (7.5–250 nmoles) and when an equilibrium adsorption was achieved the monolayer was compressed in stages and the $[Ca]_b$ recorded with each step. The results (Fig. 9) show that in the presence of low concentrations of $Ca^{2+}$ in the subphase (7.5 and 25 nmole) the value of $r$ remains relatively constant at areas greater than about 55 Å$^2$/molecule of cerebroside sulphate. At higher film densities the amount of $Ca^{2+}$ bound to the monolayer decreases. With higher concentrations of $Ca^{2+}$ in the subphase (75 and 250 nmole) there is a gradual increase in $Ca^{2+}$ bound reaching a maximum about 90 Å$^2$/molecule and decreasing when the surface concentration of $Ca^{2+}$ exceeds approx. $3.55 \cdot 10^{13}$ ions/cm$^2$.

## DISCUSSION

The force–area characteristics of cerebroside and cerebroside sulphate are essentially compatible with the structure of these lipids. The condensed state of cerebroside monolayers is contrasted with the expanded form of the same molecule differing only in the presence of a dissociable group. The coherence of cerebroside monolayers between a "lift off" at about 55 Å$^2$/molecule and the limiting area of about 40 Å$^2$/molecule is probably due to the steric arrangement of the bulky polar head groups[17]. The limiting area is slightly less than that obtained with a conventional Langmuir balance (H. HAUSER AND M. C. PHILLIPS, personal communication) and probably arises from errors inherent in recording measurements on a smaller area.

The results of the present investigation emphasize the importance of adopting several approaches to characterize the state of ionization of monolayers at the air–water interface. For example, the degree of dissociation of ionic amphipaths has often been determined by observing changes in surface potential of the film when the subphase pH was altered and as indicated by Eqn. 1 the change should be proportional to $\Psi_0$ between the fully ionized and completely discharged states. The pH at which ionization is observed is generally higher for anionic amphipaths than for the same charged groups in free solution and can be accounted for by the attraction of $H^+$ to the surface. However, changes in $\Delta V$ about pH 6.5 have been observed in monolayers of monophosphatidyl inositol, dicetyl phosphoric acid and octadecanol (QUINN AND DAWSON, unpublished) and also for phosphatidylethanolamine and phosphatidic acid monolayers[18] which are unlikely to arise from primary electrostatic effects. PAPAHADJOPOULOS[18] noted groups titrating between pH 6 and 8 in monolayers of phosphatidylinositol after exposure to high pH which he interpreted as ionizable groups arising from hydrolytic products. A notable exception to any change in $\Delta V$ on subphase pH values between 2 and 11 is the zwitterion, phosphatidylcholine[19] but in this case the presence of an internal salt linkage between the charged phosphate group and the quaternary nitrogen[20] is likely to modify the polar interaction with water.

Although no direct analogies are possible between phospholipids and sulpholipids there is a possibility that ionization effects on changing the subphase pH are not exclusively involved in the observed changes in surface potential as indicated by the present experiments. This is obvious in the case of cerebroside in which there is a

change of about 100 mV in surface potential although the molecule has no ionizable groups between pH 1 and 11. The comparable change in $\Delta V$ at the same pH with cerebroside sulphate would also indicate that primary electrostatic effects can be excluded even in the presence of a fully ionized sulphate group although this may be responsible for the slight shift of the curve to the more acid region. Further evidence that the sulphate is fully ionized above pH 3 is seen in the adsorption of calcium to individual monolayers of cerebroside sulphate on subphases at various pH containing $^{45}Ca^{2+}$ (Curve C, Fig. 3). The initial values show little change in the amount of calcium adsorbed with increasing subphase pH above 3: the loss of calcium below pH 5.5 with time is increased with decreasing pH and probably results from competition with $H^+$ for the binding sites.

Recent dielectric measurements of myelin preparations[21] have indicated a considerable amount of water associated with the lipoprotein which is bound more tightly than would be expected from interactions between bulk water molecules. The binding of water to various sugars through hydrogen bonding to hydroxyl groups has been known for some time[22] and it is likely that water dipoles interacting with galactosyl hydroxyls of cerebrosides play an important part in the structuring of water in myelin. A change in the polar interaction of cerebrosides with water molecules when the pH changes from acid to alkaline may be related to a difference in dipole forces dependent on the pH. The strength of the dipoles would be related to the electronegativity of the oxygens involved[23] thus it would be expected that $H_3O^+$ would induce considerably stronger dipoles in the lipid than $H_2O$ or $OH^-$. There is no evidence that steric hindrance precludes a colinear orientation required for hydrogen bonding and a competition between dipole–dipole and hydrogen bonded water to the polar groups of the lipid is likely. Some indication of a decrease in ordered water structure with increasing pH from 5 to 8.5 has been obtained from measurements of proton relaxation times of water associated with viable microorganisms[24]. In the case of cerebrosides it is likely that any change in the orientated water structure about the galactosyl moiety in the pH region about 6.5 would have profound biological implications concerning the properties of myelin where these lipids occur in relative abundance.

Another apparent anomaly is the increase in surface pressure when a force–area curve of cerebroside sulphate on a subphase of 1 M HCl is compared with a monolayer spread at high pH where the sulphate would be completely ionized (Fig. 1A). It would be anticipated from the equation of state for ionized monolayers[25] that the surface pressure would decrease with increasing electrolyte concentration in the subphase and decreasing $\Psi_0$ as the sulphate becomes discharged. A more pronounced effect has been reported for octadecylsulphate on acid subphases[26] and it was suggested that the monolayer was stabilized in an expanded state by hydrogen bonden hydroxonium ion. The presence of $H_3O^+$ hydrogen bonded to the sulphate group of cerebroside sulphate would provide a plausible explanation of the higher surface pressures observed in the force–area curve. In contrast to octadecylsulphate there was no indication of a reduction in the collapse pressure of cerebroside sulphate monolayers when compressed on acid subphases. The change in surface potential between pH 3 and 5 may be related to water hydrogen bonded directly to the sulphate: there are no comparable changes in cerebroside monolayers and the pressure remains constant over the whole range of pH.

Calcium clearly has a much more specific affinity for cerebroside sulphate mono-

layers than monovalent ions. In the low concentration range this preference for calcium is in the order of $2 \cdot 10^4$ times greater than for sodium or potassium and potassium appeared to have a competitive advantage over sodium. The advantage, however, was somewhat less than the 1:2 ratio of $Na^+$ to $K^+$ reported for sulphatide in aqueous dispersions[2] although much higher concentrations of monovalent ions were used in these experiments. Applying a selectivity coefficient to compare the binding affinity of cations relative to $Na^+$ these authors showed that $Ca^{2+}$ is approx. 35 times more tightly bound than $Na^+$ but as in the previous case the ionic concentration of the system was many fold greater than in the present experiments. As previously noted for the displacement of calcium from phosphatidylserine monolayers by sodium and potassium[27] more calcium was displaced from cerebroside sulphate monolayers at low compared with films at higher density. This was interpreted as a reduction in the physical space available to the competing ion about the adsorption site as the packing density increased. From the order of magnitude of the competition between sodium and potassium for calcium binding to cerebroside sulphate it would seem unlikely that the effects have any strategic importance in terms of selective binding of monovalent ions.

The graphical analysis of the association of calcium with cerebroside sulphate monolayers indicates good agreement between predictions from the mass equation and experimental results. Calcium binding to monolayers, at least at areas per molecule of 200 Å$^2$/molecule or less, appears to be strictly by two-point electrostatic attachment. There is, however, some uncertainty as to the correct expression of the apparent association constant. If a single binding site is considered to be two adjacent molecules the value of the apparent association constant will be twice that when $K_a$ is expressed in terms of each individual molecule binding calcium independently.

HAUSER *et al.*[5] in their analysis of the adsorption of calcium to phosphatidylinositol monolayers concluded that calcium formed a Stern layer in a position below

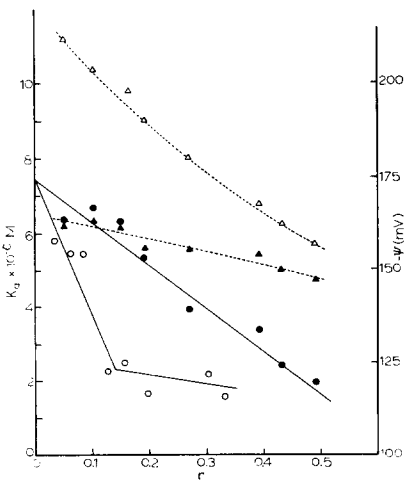


Fig. 10. Variation in apparent association constant ($K_a$) calculated from Eqn. 12 with the ratio of calcium bound per lipid molecule. ●—●, 85 Å$^2$/molecule; ○—○, 53 Å$^2$/molecule. The relationship between the Gouy potential ($\Psi_G$), △···△; the Stern potential ($\Psi_S$) ▲···▲ and $r$ for monolayers of cerebroside sulphate at 85 Å$^2$/molecule.

the plane of the charged phosphate groups but adjacent to the film. A similar comparison of changes in the Gouy and Stern potentials has been undertaken in the case of calcium adsorption to cerebroside sulphate monolayers. The Gouy potential ($\Psi_G$) of cerebroside sulphate monolayers at 85 Å$^2$ per molecule have been calculated from Eqn. 2 and the relationship to the ratio ($r$) of ions bound to the lipid is shown in Fig. 10. The Stern potential can be obtained from the expression

$$\frac{\theta}{1-\theta} = [Ca^{2+}]_f \, e^{\lambda_p + W/kT} \cdot e^{-Z\varepsilon\Psi_S/kT} \qquad (11)$$

where $\theta = [Ca]_b/n\,[CS]_t$, $k$ = Boltzmann constant and $T$ = absolute temperature. The exponent $\lambda_p + W/kT$ is an expression of the total specific adsorption energy between calcium and the sulphate oxygens and is resolved into an electrostatic polarization term ($\lambda_p$) and a term related to Van der Waals forces[9] ($W$). The factor $\lambda_p$ is a measure of the tendancy of the bond between the adsorbing ion and the charged group to be covalent and is related in absolute terms to the difference in electronegativities between $Ca^{2+}$ and the sulphate oxygen. The value of the exponent is generally obtained from charge reversal experiments but to the authors knowledge this has not been done for calcium adsorption to sulphate. As a first approximation, the value has been determined from electronegativity differences given by PAULING[23] and the Van der Waals forces are considered to be negligible. The value of $\lambda_p + W/kT$ used was 2.8.

The second exponent of Eqn. 11 is an electrostatic term where $Z$ = valence of $Ca^{2+}$; $\varepsilon$ = electronic charge and $\Psi_s$ is the potential at the plane of the first layer of adsorbed counter ions. The values calculated from calcium adsorbed to cerebroside sulphate monolayers at 85 Å$^2$ per molecule is shown in Fig. 10. Although the absolute values of $\Psi_s$ may vary depending on the accuracy of the value for the specific adsorption term (a doubling of this term shifts the whole curve down by about 35 mV) the change in $\Psi_s$ with $r$ is considerably less than the calculated values of $\Psi_G$. The values for monolayers of cerebroside sulphate at 53 200 and 400 Å$^2$/molecule are not included in Fig. 10 but the relationship between $\Psi_s$ and $\Psi_G$ does not change substantially with surface density of the lipid molecules.

The relationship of the Stern equation (11) to the association constant of the reaction is apparent from Eqn. (12) which can be derived from the mass equation (4)[28*].

* The derivation of Eqn. 12 from Eqn. 4 is as follows: by inverting Eqn. 4 and multiplying out the numerator of the right hand side of the expression the apparent dissociation constant of the reaction can be written:

$$K_d = \frac{1}{K_a} = \frac{n[Ca^{2+}]_t\,[CS]_t - n[Ca]_b\,[CS]_t - [Ca^{2+}]_t\,[Ca]_b + [Ca]^2_b}{[Ca]_b} \qquad (13)$$

Cancelling the appropriate terms, Eqn. 13 can be expressed as follows:

$$K_d = \frac{n[Ca^{2+}]_t\,[CS]_t}{[Ca]_b} - \frac{n[Ca]_b\,[CS]_t}{[Ca]_b} + [Ca]_b - [Ca^{2+}]_t \qquad (14)$$

Rearranging Eqn. 14 gives:

$$K_d = (n[CS]_t/[Ca]_b - 1)\,([Ca^{2+}]_t - [Ca]_b) \qquad (15)$$

Since $\theta = [Ca]_b/n[CS]_t$ and $[Ca^{2+}]_t - [Ca]_b = [Ca^{2+}]_f$ then Eqn. 15 on substitution can be rearranged to give:

$$K_d/[Ca^{2+}]_f = 1/\theta - 1 \qquad (16)$$

When Eqn. 16 is expressed in terms of the apparent association constant the expression is identical to Eqn. 12.

$$\frac{\theta}{1-\theta} = [Ca^{2+}]_f K_a \qquad (12)$$

$K_a$ can be resolved into the intrinsic association constant $e^{(\lambda_p + W)/kT}$ and an electrostatic term given by the second exponent of Eqn. 11, $e^{-Ze\Psi_s/kT}$. Substitution of the experimental values into Eqn. 12 for the adsorption of calcium to cerebroside sulphate monolayers of 85 200 and 400 Å²/molecule as would be expected give curves identical with those of Fig. 8 since the same experimental values were used to calculate $-\Psi_s$. It is also evident that variation in the value of $\lambda_p$ will be compensated for in Eqn. 12 by the value of $\Psi_s$ calculated from Eqn. 11 but the magnitude of either of the components of $K_a$ will depend on the value of $\lambda_p$ chosen. Further experiments are needed to determine $\lambda_p$ directly so that values can be correctly apportioned between the two terms.

Substitution of the calculated values of $\Psi_G$ (Fig. 10) assuming that calcium penetrates the monolayer up to the plane of the negative charges, give much larger values of $K_a$ although when the concentration of calcium in the surface is high the values approach the same order of magnitude of the experimental results. From the evidence thus presented it would seem that it is not a question of whether the results conform to a Stern type of arrangement as the Stern potential itself is derived from the experimental values but how closely the results reflect the true ionic state of the monolayer. In so far as obeyance of the mass equation is relevant as a description of the total electrostatic forces when calcium adsorbs to cerebroside sulphate monolayers then calculation of $\Psi_s$ from Eqn. 11 would be closer to the actual electrostatic potential then $\Psi_G$ calculated from the Gouy equation 2.

Finally, mention should be made of the cases where the adsorption of calcium appears to deviate from the mass action law. These effects probably arise primarily from electrostatic conditions at the interface. HAUSER *et al.*[5] showed that a higher proportion of calcium was bound to phospholipid monolayers as the distance between the charged head groups approached the diameter of the hydrated $Ca^{2+}$. This is observed in Fig. 9 where calcium adsorption increases when in equilibrium with high concentrations of calcium in the subphase. When the area per molecule is reduced steric conditions are more favorable for the formation of a divalent binding site. The decrease in calcium binding when the surface concentration exceeds $3.55 \cdot 10^{13}$ ions per cm² is most likely due to charge repulsion between calcium ions in the surface. A reduction in $K_a$ of calcium binding to monolayers at 53 Å²/molecule (Figs. 8 and 10) can also be interpreted in this way as except for $r$ values of less than 0.08 the concentration of calcium in the surface exceeds $3.55 \cdot 10^{13}$ ions per cm². At this point each calcium occupies an area of radius 9.4 Å which is twice that of the hydrated ion. Thus in an array of adsorbed counter ions separated by repulsive electrostatic forces of adjacent ions additional energy must be required for further ions to adsorb when the space available is less than that required for the hydrated ion. This would also suggest that the distance between the charged group of the lipid and the calcium counter ion does not approach the Van der Waals radius of the charged groups but maintains a distance consistent with the diameter of the hydrated ion. The retention of the hydration shell about $Ca^{2+}$ is probably related to the weaker polarization of sulphate relative to water[29]. The hydration of protons acting as counter ions was noted previously (Fig. 1A) where $H_3O^+$ linked to adjacent molecules through hydrogen bonds maintained an expanded force–area curve of cerebroside sulphate on a subphase of

pH 0. The decrease in binding at the critical density of adsorbed counter ions would suggest that calcium adsorbed initially is juxtaposed with the binding sites rather than forming a more diffuse electrical double layer.

ACKNOWLEDGEMENTS

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