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MIXTURES OF GANGLIOSIDES AND PHOSPHATIDYLCHOLINE IN AQUEOUS DISPERSIONS

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SUMMARY

The interaction of ox brain gangliosides and pure egg phosphatidylcholine has been studied by taking aqueous dispersions of the two lipids in all proportions and centrifugating the dispersion at 100000 × *g* for half an hour and measuring the amount and composition of the pellet and supernatant. The first way of making the mixtures was to mix measured amounts of the lipids in organic solvent, dry them and then suspend them in buffer. The second way was to put measured amounts of each pure lipid into two separate tubes and dry each down and suspend each one in buffer. The two pure suspensions were then mixed. To investigate what charge effects the gangliosides would have on membranes, monolayer layers were studied at low (5 %) ganglioside concentrations. The centrifugation studies show that when the lipids are suspended concurrently in 10 mM buffer, pH 6.5, below a lipid composition of 30 % ganglioside, 95 % of the lipid could be sedimented, while above 65 % the lipid composition of the supernatant was identical to that of the initial suspension although the total amount of the lipid in the supernatant varied with the composition. Thus it can be concluded that when the lipids are suspended in this way, they can exist in either a lamellar structure or in micellar solution, depending on the concentration. When, on the other hand the lipids are suspended separately all the lecithin is sedimented out whereas most of the ganglioside remains in suspension, indicating that the ganglioside could adsorb into the outer monolayer of the lamellar structures but not penetrate further and destroy them.

Detailed studies of the surface potential of 5 % mixtures of the mono- and disialogangliosides, showed that the gangliosides were stable in the monolayer and produced a charge that was concordant with the number of charged groups in the surface. The results were in agreement with those obtained with phosphatidic acid and with the Gouy–Chapman theory, it is also indicated on energetic considerations that it is most probable that the head groups of the gangliosides are not fixed in the plane of the air–water interface.

INTRODUCTION

The gangliosides were first discovered by Klenk[1] during an investigation of some peculiar lipidoses which specifically affect the brain. They were found in large

quantities in Tay–Sachs disease and in a smaller amount in normal brain. As the name implies this class of lipid is mainly to be found in the ganglion rich fraction, and upon further fractionation of the normal brain, the largest amounts appear to be found in plasma membranes from the synaptosomal fraction[2].

Gangliosides are a class of glycosphingolipids which contain at least one sialic acid residue which provides a corresponding number of carboxylic acid groups. The function of these lipids is unknown in normal brain although the concentration in grey matter is about 6 % of the total lipid whereas the white matter contains only 0.6 %[3] and a trace or nil has been found in myelin[4,5]. On the other hand in the diseases associated with an accumulation of gangliosides, the concentrations are increased many times and give rise to a gross disruption of neurological function including both demyelination and a failure to myelinate. A morphological characteristic of Tay–Sachs disease is the presence of so-called membranous cytoplasmic bodies, which are aggregates of phospholipid, cholesterol and gangliosides together with a small fraction of protein.

Chloroform–buffer dispersions of these constituents when fixed, dried and stained in the same way as the natural membranous cytoplasmic bodies yield electron micrographs with similar appearance to those of the natural membranous cytoplasmic bodies[6].

The properties of the gangliosides in aqueous dispersions have been measured by Gammack[7], who showed that they exist in micellar solution above a critical micelle concentration of about 0.02 g/100 ml and can form mixed micelles in association with lysolecithin. Contrasted to this are the normal membrane lipids such as phosphatidylcholine which characteristically form a smectic mesophase or lamellar structure (liposomes) in aqueous dispersions. The properties of gangliosides and phosphatidylcholine together as mixtures might, therefore, elucidate the role of the ganglioside in normal brain and also in the pathogenesis that occurs in Tay–Sachs disease.

When two such contrasting lipids are mixed in all proportions there should be a region of concentration of gangliosides above which the lecithin fits into the ganglioside micelles, and below which the gangliosides fit into the lamellar structure of the lecithin, hence there should exist a concentration where there is a micellar lamellar phase transition. This transition has been discussed by Ohki and Hono[8] and the work of Parsegian[9] is also relevant to this system. Ohki concludes that for the transition to occur from a lamellar to a cylindrical micellar phase, each molecule must have between 1.25 and 2 units of charge per molecule, but neither this analysis nor that of Parsegian takes into account the effect of the size of the head groups. The head groups of gangliosides are as large as the hydrocarbon tails, being about 30 Å long and they are very hydrophilic because they contain a large number of hydrogen bonding groups. The presence of such large head groups could be one of the reasons why the gangliosides, themselves, form micelles. The system which has the closest similarity is that of the general detergent–amphiphile–water system discussed by Winsor[10]. The gangliosides being the detergent and the lecithin being the amphiphile. Winsor shows that there are a series of states of the system depending on relative concentrations. At high water concentrations, a spherical micelle can exist at low amphiphile concentration, and lamellar phases at high amphiphile concentrations, between these, various micellar shapes including a cylindrical micelle can also exist,

the structure depending on the relative attractive forces between the head groups and water and between the hydrocarbon tails on either side of the interface.

Studies were carried out to determine the form that the suspension of ganglioside phosphatidylcholine would take in mixtures over the concentration range 0–100 %. This was done by mixing the lipids together in organic solvent, drying and suspending them in buffer and observing the resultant suspension.

As it was also important to determine whether the gangliosides could disrupt preformed membranes, and so act as a true detergent, the experiment of suspending the lipids separately and then mixing the micellar and lamellar solutions was also performed.

If gangliosides adsorb into or exist in membranes, it is relevant to know what sort of properties they can confer on membranes and in particular any charge contribution. As large amounts of the pure mono- and disialogangliosides were not available it was necessary to work with a monolayer system rather than with microelectrophoresis as would have been preferred, to investigate the charge effect of the gangliosides.

The aim of these experiments was to determine the surface charge density of an insoluble monolayer of a known mixture of phosphatidylcholine–ganglioside and relate this to the number of known charged groups added. The surface potential of a monolayer with respect to a clean water surface ΔV is the sum of two terms, ψ_d, due to the difference between the fixed dipoles in the monolayer used and that of a clean water surface and it is here assumed to be independent of salt concentration[11]. The other term ψ_g is due to the electrical double layer set up by the fixed charges in the monolayer and their mobile counter ions in solution. With a bulk salt concentration of c moles/l,

$$\Delta V = \psi_d + \psi_g(c) \tag{1}$$

An approximation of the dependence of ψ_g on the salt concentration and the charge density is given by the Gouy–Chapman equation.

$$\psi_g = \frac{2kT}{e} \sinh^{-1} \frac{\sigma\ 500^{\frac{1}{2}}}{c^{\frac{1}{2}}\ \varepsilon RT} \tag{2}$$

where ε is the dielectric constant of the medium in the double layer, assumed constant; e, the charge on the electron; and k, R and T having their usual meaning. At 20 °C this equation reduces to:

$$\psi_g = 50.54 \sinh^{-1} \frac{136}{Ac^{\frac{1}{2}}} \tag{3}$$

where A is the area per charge in Å^2. If this is differentiated with respect to ln c we get:

$$\frac{\partial \psi_g}{\partial \ln c} = -25.27 \tanh \frac{136}{Ac^{\frac{1}{2}}} \tag{4}$$

or

$$\frac{\partial \psi_g}{\partial \log c} = -58.2 \tanh \frac{136}{Ac^{\frac{1}{2}}} \tag{5}$$

Thus for values of $136/Ac^{\frac{1}{2}} > 3$, $\partial\psi_g/\partial \log c = -58.2$ mV per decade of salt.

It can be seen from this that $\partial\psi_g/\partial \log c$ is a function of the surface charge only if $136/Ac^{\frac{1}{2}} < 3$. If we take the ΔV of a given monolayer at a standard reference point, say for $c = 100$ mM, then its difference from the ΔV at another concentration $\Delta(\Delta V)$ will be equal to the difference in ψ_g between these concentrations. It is therefore possible to obtain a predicted plot of $\Delta\psi_g$ against ln c calculating an area per charge from the known lipid concentrations and comparing this with the measured $\Delta(\Delta V)$ taking 100 mM as a reference point in both cases.

The head groups of the gangliosides are very large and in certain cases can have a charged group at a distance of 30 Å from the hydrophobic portion of the molecule, also in the case of the disialogangliosides the two charges would be 15 Å apart. Thus it is possible to have the charges in planes other than that of the air–water interface. In the Appendix we have calculated the effect on the measured values of $\Delta\psi_g$ if the charges were in two planes, one at the air–water (density δ_0) interface and one a distance a Å from this (density δ_a). In this case we obtain for $\psi_g < 25$ mV from Eqn A10 (see Appendix).

$$\psi = C(\sigma_0 + e^{-\kappa a}\sigma_a) \tag{6}$$

In the case of a single plane of charges with surface charge density δI a distance a from the interface (the monosialogangliosides) $\delta_0 = 0$ in Eqn 6

$$\psi = C\, e^{-\kappa a}\sigma_a \tag{7}$$

As κ is a function of the concentration the affect will be dependent on the concentration and for high values of κa (high salt), approaches zero, while a low value of κa (low salt) approaches the value it would have if the charges were in the interface. Although for $c = 1$ mM and $A = 1200$ Å^2 the value of the potential is 100 mV, much above the condition $\psi < 25$ mV used in the analysis, this is the region where the effect of the separation is minimal, so the error in assuming the same form will be small. If we take a value of a to be 15 Å, at 1 mM the reduction in potential will be 14 % and at 100 mM the reduction will be about 80 %. This turns out to give no measurable change in $\Delta(\Delta V)$ and so would not be detectable.

In the case of disialoganglioside, if we predict two planes of charge, one in the interface and one 15 Å away,

$$\psi = C\sigma_0\left[1 - \frac{1 - e^{-\kappa a}}{2}\right]$$

giving a 39 % reduction at 100 mM and 7 % reduction at 1 mM. This again gives no significant change in $\Delta(\Delta\psi)$. We cannot therefore distinguish by this method the configuration of the head groups and the measured values of ΔV are not accurate enough to detect the 2 % change in that parameter that would be predicted.

Eqn A12 of the Appendix gives the electrostatic part of the free energy of charging the two double layers. It can be seen that there is a free energy gain in the case of the separate double layers and the gain is of the order of kT per charge or less, decreasing with increasing salt concentration. The thermal energy would then tend to smear the charges out over the region between the interface and the maximum extremity of the charge. This would also lower the free energy, as now there would

be effectively a large number of planes of charge. Again this would not be noticeable as a change in $\Delta(\Delta V)$.

The method was checked with phosphatidic acid–phosphatidylcholine monolayers with the same charge density and compared to the results of McDonald and Bangham[12].

METHODS AND MATERIALS

Egg phosphatidylcholine and phosphatidic acid were prepared by established chromatographic procedures[13–16] and stored under nitrogen in chloroform at −20 °C. Pure mixed gangliosides were prepared by partition and dialysis[7] and the mixtures were separated on an Anasil S column, crystallized and recrystallized from warm methanol, and the purity checked according to the procedure of Penick *et al.*[17]. All the gangliosides were stored in chloroform–methanol (1:1, v/v) at −20 °C under N_2. KCl, for the titration of surface potentials, was roasted at 500 °C for 5 h. Light petroleum, for the application of surface monolayers, was purified on alumina. All other reagents used in the purification of the lipids and in the studies described were A.R. grade, and were used without further purification. Water was doubly distilled and then redistilled from potassium permanganate. Glassware was cleaned successively in alcoholic potash, nitric acid, and permanganate distilled water.

Centrifugation of concurrently swollen mixtures

Solutions of mixed brain gangliosides *plus* lecithin, combined in proportions varying from 0 to 100 % of each substituent were dried together *in vacuo*, and shaken in 2 ml of an aqueous solution containing 9 mM KCl, and 1 mM histidine buffer, pH 6.5. Final total concentration of lipid equalled 1–2 mM. Mixtures with the three lowest percentages of ganglioside (0–20 %) were restudied at total concentrations of lipid equalling 10–25 mM in order to increase sensitivity. The resultant solutions and/or suspensions were equilibrated under N_2 at 22 °C for 24 h, and then centrifuged for 30 min in a Spinco preparative ultracentrifuge at 100000 × *g*, using a SW 39 head.

The concentration of phospholipid in the initial lipid swell, the supernatant and pellet were determined by determining phosphate[18]. Ganglioside concentrations were measured by the resorcinol reaction for *N*-acetylneuraminic acid[19].

Centrifugation of separately swollen mixtures

This was carried out in an identical manner to the above except that the two lipids were dried and suspended in separate tubes and then shaken with the aqueous phase before mixing in appropriate proportions and left as before for 24 h.

Monolayer surface potential studies

Monolayer surface potentials were studied in a scrupulously cleaned teflon trough (10 ml capacity, 10 cm^2 surface area) using an americium ionizing electrode, a Vibron electrometer 33 B-2, pH-calomel reference electrode, and a constant voltage source. Mixtures of monosialoganglioside, or disialoganglioside, or mixed mono-, di-, and trisialogangliosides, *plus* lecithin were dissolved in light petroleum (b.p. 40–60 °C)–ethanol (9:1, v/v), and 4 µl applied to the surface of solutions containing 1 mM KCl, 0.25 mM cacodylate buffer, 0.025 mM EDTA, pH 7.0. Confirmatory studies were per-

formed on phosphatidic acid–lecithin monolayers prepared by similar techniques and applied to the same buffer but at pH 6.2. Sufficient lipid was added to saturate the buffer surface, and studies were, therefore, conducted at collapse pressure. The surface potential of the monolayer was varied by the successive additions of aliquots of 2 M KCl beneath the surface up to a concentration of 100 mM.

Electron microscopy

The electron micrographs were all prepared by taking approximately 1 μmole of sample per ml in 0.5 % ammonium molybdate, and spraying onto carbon–colloidon grid, and air dried. Micrographs were taken on a Siemens Elmiskop 1A at an instrument magnification of 40000.

RESULTS AND DISCUSSION

Centrifugation of concurrently swollen mixtures

In Fig. 1 the ratios of the ganglioside in the supernatant and pellet to the total lipid in them are plotted against the proportion of ganglioside in the aqueous dispersion before centrifugation. In Fig. 2 the ratio of the total lipid remaining in the supernatant to the total lipid available in the initial dispersion as a percentage is plotted against the proportion of ganglioside to total lipid in the aqueous dispersion as a percentage.

The molar contents of ganglioside were calculated from an average molecular weight determined from the weight of vacuum desiccated material, the content of *N*-acetylneuraminic acid, and the substituent content of the extracted mixture of gangliosides determined by thin-layer chromatography. As shown in Fig. 1, the percentage of ganglioside in the pellet equalled the percentage of ganglioside in the initial swell at ratios of ganglioside–lecithin below 30 %. At these concentrations, more than

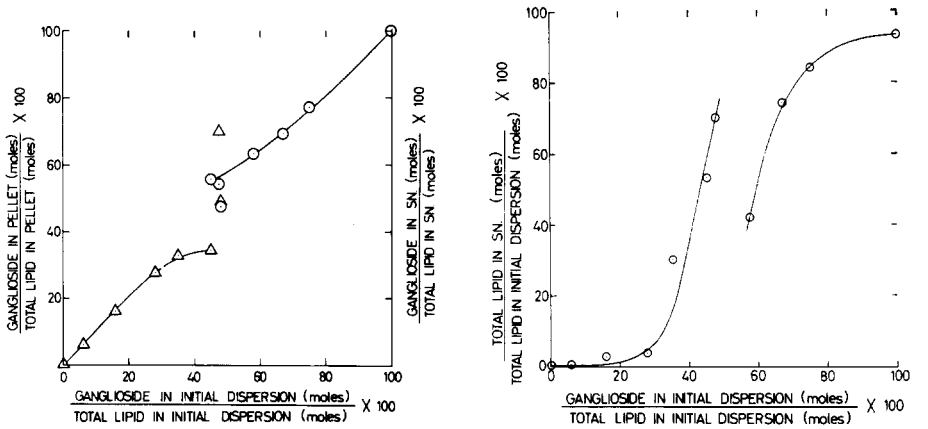

Fig. 1. The composition, expressed as a percentage of ganglioside, of the pellet (left-hand side) and supernatant (right-hand side) after centrifugation, are plotted against the initial lipid composition of the mixtures; the mixtures being made in organic solvent prior to dispersing in buffer.

Fig. 2. The percentages of the total lipid remaining in the supernatant after centrifugation are plotted as a function of the initial lipid composition of the mixtures; the mixture being made in organic solvent prior to dispersing in buffer.

95 % of the lipid sedimented as can be seen in Fig. 2 and the pellet was in the form of a smectic mesophase. By contrast, at the highest ganglioside–lecithin ratios (above 65 %), the percentage of ganglioside in the supernatant approximated the percentage of ganglioside in the initial swell. At these ratios, the bulk of the material remained in the supernatant, although as the relative proportion of lecithin was increased, a top to bottom lipid concentration gradient was present in the clear solution contained in the centrifuge tube. In the middle ratio zone, between 30 and 65 %, both a clear lipid-containing supernatant and a pellet were observed, and more variable relative concentrations were recorded. In this region it appears from Fig. 2 that increased amounts of the lipid is being micellized by the increasing concentration of gangliosides. At 45–48 % ganglioside–lecithin ratios, an unstable, homogeneous, clear viscous phase was observed, which resolved into pellet and clear supernatant on standing under N_2 at 22 °C for a total of 48 h.

Thus at low ganglioside–lecithin ratios, when the substituents are hydrated concurrently, ganglioside molecules are distributed in the lamellae of a combined ganglioside–lecithin smectic mesophase. The negative charge introduced widens lamellar spacing as can be seen when "myelins" of the ganglioside–lecithin mixture are viewed with crossed Nicol prisms and compared with those of pure lecithin. It is therefore, not surprising that, despite their water solubility, gangliosides in brain are essentially

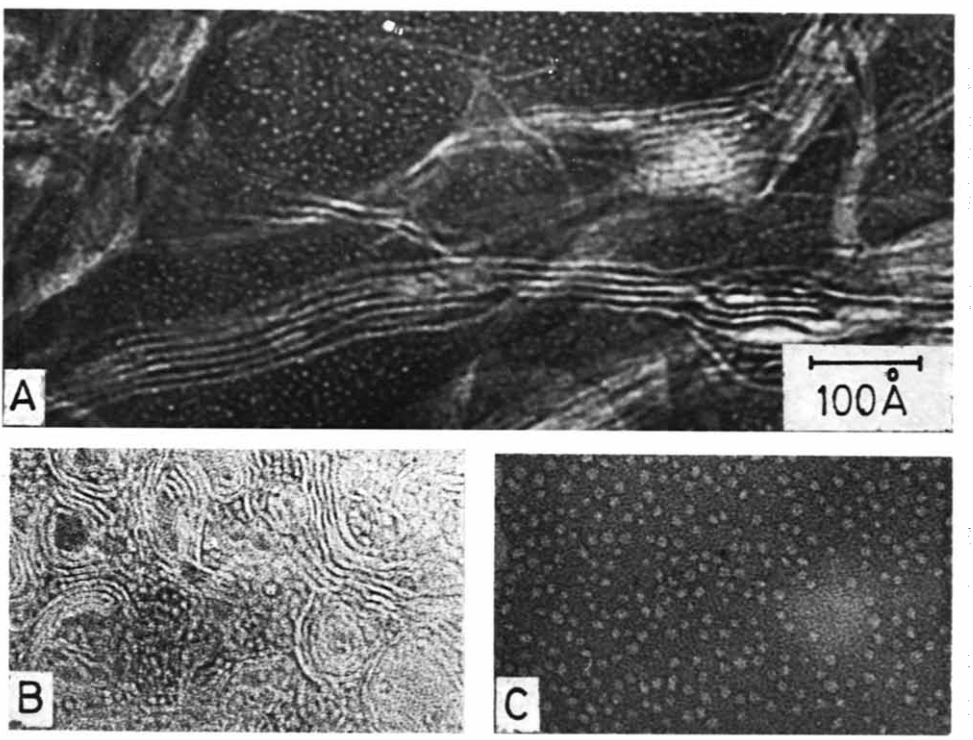

Fig. 3. Electron micrographs of (A) cylindrical micelles formed from 45–48 % ganglioside–phosphatidylcholine ratio, (B) smectic mesophase formed with low ganglioside ratios $< 30\%$, and (C) spherical micelles formed by high ganglioside concentration $> 80\%$.

all membrane bound. On the other hand at high ganglioside–lecithin ratios, the lipid-rich clear supernatant is composed of mixed ganglioside–lecithin micelles.

As the ratio is lowered an increasing percentage of the micellar material is sedimented suggesting an enlargement or alteration of the shape of the mixed micelles (right hand branch of curve in Fig. 2). The identity of the middle zone (ganglioside–lecithin ratios, 45–48 %) cannot be established until suitable X-ray diffraction studies are performed. It is possible, however, that this zone represents a transition between S_1 and G phases of the ganglioside–lecithin mixtures. The marked viscosity (and the strand-like appearance on preliminary ultramicroscopic examination) suggests the presence of a cylindrical micellar M_1 transition phase (Fig. 3A). Figs 3B and 3C show the appearance of the smectic mesophase at low ganglioside concentrations and micelles at high ganglioside concentrations prepared in the same way for comparison.

Centrifugation of separately swollen mixtures

While the foregoing results were obtained by centrifuging mixtures of ganglioside and lecithin swollen concurrently from mixed dried films, a major difference was observed if the ganglioside and lecithin were swollen in aqueous solutions separately and the solutions then combined. As can be seen in Fig. 4, which shows the ratio of the ganglioside in the supernatant to that in the initial dispersion as a function of the ratio of the ganglioside in the initial dispersion to total lipid in the initial dispersion, more than 80 % of the ganglioside remained in the supernatant at ganglioside–lecithin ratios of as little as 15 %. There was no measurable phospholipid in the supernatant at ganglioside–lecithin ratios up to 100 %. It thus appears that gangliosides can only penetrate the outer monolayers of the lamellae in 24 h and entry of ganglioside monomers into the inside of preformed lecithin "myelins" may well be retarded by the high energetic requirements for the movement of the bulky, hydroxylated and charged, hydrated head groups across the lipid bilayer. A similar hydration barrier has been reported in studies of the solubilization of methyl linoleate by cetomacrogol[20].

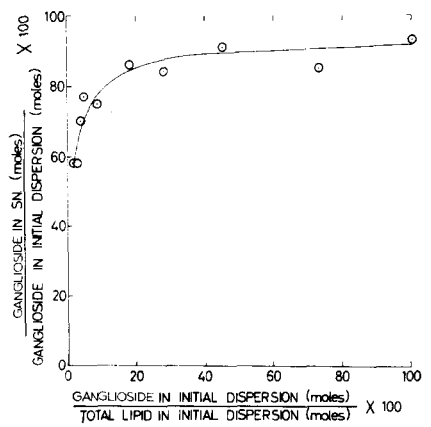

Fig. 4. The percentages of the total ganglioside remaining in the supernatant after centrifugation are plotted against the initial ganglioside concentration of the lipid mixture; the mixtures being made by adding aqueous dispersions of the two lipids.

Monolayer surface potential studies

The surface potential measurements are depicted in Figs 5A and 5B, in which $\Delta(\Delta V)$ is plotted against the log of the salt concentration. Results are shown for the titration of mono- and disialoganglioside with estimated surface charge densities of 4.8 and 5.1 %, respectively. The charge density was calculated as moles of charged moiety (*N*-acetylneuraminic acid) per mole of lecithin in the surface, expressed as a per cent. Therefore, the disialoganglioside monolayer studied (approx. 5 % charge density) contains approximately half the number of moles of ganglioside as the monosialoganglioside monolayer (also approx. 5 %).

The points in Fig. 5 represent the means, and the bars the ranges of three determinations performed on separate monolayers. $\Delta(\Delta V)$ was calculated from a base value of 0 at 10^{-1} M. The stippled areas represent values for surface potential predicted by the Gouy–Chapman relation taking values of the area per molecule of lecithin as 55–65 Å^2 (ref. 21) to calculate A, the area per surface charge, at values of c from 1 to 100 mM. Approximate values of A are necessary as the cross-sectional area of the lecithin molecule has not been measured definitely, and as there is a small correction due to the different cross-sectional area of the ganglioside molecule. Values of V_0 for water above -390 mV were accepted, and ΔV for monolayers of lecithin was found to be 450 mV.

For both monosialoganglioside and disialoganglioside, the curve obtained closely approximated that predicted by the Gouy–Chapman equation. At the ionic strengths examined, the maximum slope predicted equalled 51 mV per decade, whereas that observed equalled 52 mV.

The deviations from the predicted curve over two decades of salt equalled 5 %, the measured values giving a greater $\Delta(\Delta V)$ than the theoretical even assuming the lower value of the area per molecule. As was pointed out in the introduction the effect of the head group orientation would change the $\Delta(\Delta V)$ on both the mono- and disialoganglioside by a very small amount, so we cannot say from these results what

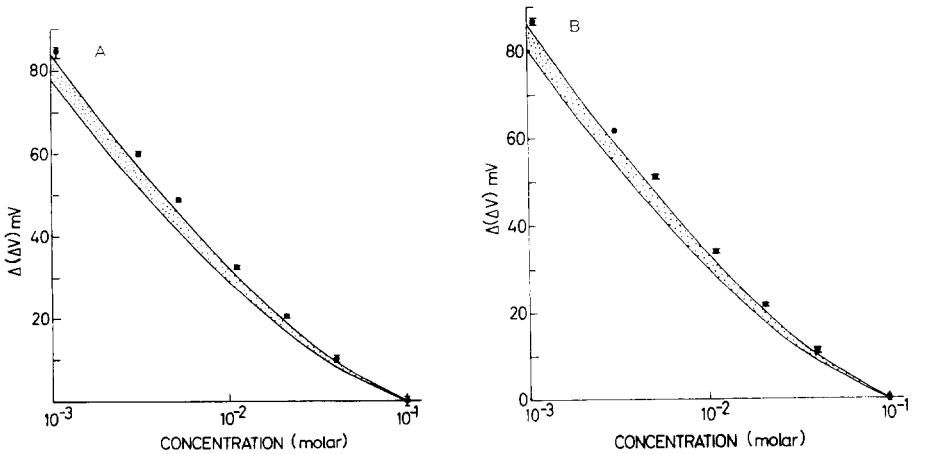

Fig. 5. The surface potential, $\Delta(\Delta V)$, of monolayers (A) 4.79% monosialoganglioside (measured as *N*-acetylneuraminic acid) in phosphatidylcholine and (B) 5.13% disialoganglioside charge (measured as *N*-acetylneuraminic acid) in phosphatidylcholine, as a function of KCl concentration. The bars represent the range of three determinations and the stippled area the range of theoretical values (see text).

orientation occurs, although energetically in the case of the disialoganglioside the free energy is reduced if there are two planes of charge, the reduction is small approx. 5 ergs/cm^2 or kT per charged group. If no other effect predominates the thermal motion would ensure a continuous distribution of charges in the region up to the maximum possible displacement of the charge from the interface.

Comparable results were obtained with monolayers of equal charge density composed of mixed (mono-, di- and tri-) gangliosides–lecithin, and of phosphatidic acid–lecithin studied by similar techniques.

These results establish that gangliosides are held in insoluble monolayers when mixed with lecithin at low ganglioside–lecithin ratios and confer a charge on the monolayer concordant with the number of charged groups added and not the number of molecules.

CONCLUSION

A detailed discussion of the pathogenesis of Tay–Sachs disease in the light of these findings has been published elsewhere[22]. The role of the ganglioside in normal brain is still unclear. It seems most likely that all or most of the ganglioside will be membrane bound, and present in quantities that will not disrupt the structure overall. But this does not mean that at local sites the concentrations could not be large enough to permit disruption of the lipid part of the membrane, with concurrent changes in the membrane properties. On the other hand even at low concentration the number of sugar moieties on the surface of the membrane would be very large, with a consequent change in the water structure and hence properties of the membrane surface. Further experiments with this system are in progress to try and elucidate the biological role of the gangliosides.

APPENDIX

In order to ascertain if there is a measurable difference in surface potential or significant difference in the free energy between the two possible extreme configurations of the disialoganglioside (that is all the charges coplanar or half the charges each in two planes separated by a distance of approximately 15 Å) it is necessary to solve the Poisson–Boltzmann equation for two overlapping double layer systems giving the electrostatic potential at the air–water interface in terms of the charge on each plane and their separation, and from this deriving the electrical part of the free energy.

The system of one positively and one negatively charged plane has been analyzed by Hanai *et al.*[23], but their method is not directly applicable, as it uses the fact that there is a position between the two planes of charge where the potential is zero, a valid assumption only if the charges are of an opposite sign on each plane. A similar treatment to the one here is given by Haydon[24]. Fig. 6 represents the model used here, and taking the Poisson–Boltzmann equation and notation from Verwey and Overbeck[25] we have

$$\frac{d^2y}{d\xi^2} = \frac{-4\pi ve\rho}{\varepsilon kT\kappa^2} = \sinh y \qquad \text{(A1)}$$

where $y = ve\psi/\boldsymbol{k}T$, $\xi = \kappa x$ and $\kappa^2 = 8\pi ne^2/\varepsilon \boldsymbol{k}T$. ψ is the electrostatic potential; ρ the charge density in solution at the point x; e the charge on the electron; $\boldsymbol{k}$ Boltzmann's constant; T the absolute temperature; n the number of ions (of valency v), per cm^3 away from the double layer; and ε is the dielectric constant of the medium in the double layer region, assumed constant for this analysis.

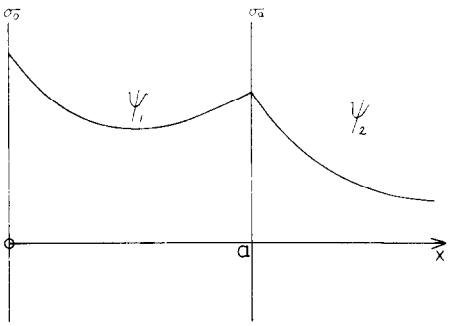

Fig. 6. The electrostatic model used for the quantitative analysis of the effect of two charged planes σ_0 and σ_0 separated by a distance a. The hydrocarbon–water interface being at the plane $x = 0$ and having surface charge density σ_0 and the second charge plane at $x = a$ having surface charge density σ_a.

The first integration of Eqn A1 gives:

$$\left(\frac{dy}{d\xi}\right)^2 = 2\cosh y + C_1 \tag{A2}$$

where C_1 is the constant of integration and equals -2 for $x>a$.

In the region $x<a$ the second integration gives

$$\xi = \int_{y(0)}^{y(a)} \frac{dy}{\sqrt{2\cosh y + C_1}} \tag{A3}$$

From the conservation of charge we get

$$\sigma_0 + \sigma_a = -\int_0^\infty \rho\, dx = \frac{\varepsilon \boldsymbol{k}T\kappa^2}{4\pi ve} \cdot \frac{1}{\kappa} \cdot \int_0^\infty \frac{d^2y}{d\xi^2}\, d\xi \tag{A4}$$

or

$$\frac{4\pi ve}{\varepsilon \boldsymbol{k}T\kappa}(\sigma_0 + \sigma_a) = -\left(\frac{dy_1}{d\xi}\right)_0 + \left(\frac{dy_1}{d\xi}\right)_a - \left(\frac{dy_2}{d\xi}\right)_a \tag{A5}$$

If we now make the condition that $y<1$, that is $\psi<25$ mV everywhere Eqn A2 becomes

$$\left(\frac{dy_1}{d\xi}\right)^2 = -y_1^2 + C_1 + 2 \tag{A6}$$

and Eqn A3

$$\xi = \int_{y(0)}^{y(a)} \frac{dy_1}{\sqrt{y_1^2 + C_1 + 2}} \tag{A7}$$

On integration this yields:

$$\xi = \ln \frac{y_1(a) + \sqrt{y_1^2(a) + C_1 + 2}}{y_1(o) + \sqrt{y_1^2(o) + C_1 + 2}}$$

$$= \ln \frac{y_1(a) + \left(\frac{dy_1}{d\xi}\right)_a}{y_1(o) + \left(\frac{dy_1}{d\xi}\right)_0}$$

from Eqn A6.

$$y_1(o) + \left(\frac{dy_1}{d\xi}\right)_0 = e^{-\kappa a} y_1(a) + \left(\frac{dy_1}{d\kappa}\right)_a \tag{A8}$$

From Eqn 2 the value of $y_2(a)$ can be found from

$$\left(\frac{dy_2}{d\xi}\right)_a = - y_2(a) \tag{A9}$$

We now obtain an expression for the potential at $x = o$ from Eqns A8, A9 and A5 using the fact that $y_1(a) = y_2(a)$,

$$y_1(o) = \frac{4\pi ve}{\varepsilon kT\kappa}(\sigma_0 + \sigma_a) + (e^{-\kappa a} - 1)\left(\frac{dy_1}{d\xi}\right)_a - \left(\frac{dy_2}{d\xi}\right)_a$$

As $a \to \infty$, $y_2(o) \to (4\pi ve/\varepsilon kT\kappa)\cdot\sigma_0$. The value of $y_1(o)$ in terms of the charge densities on the planes of charge and the distance apart can be derived as,

$$y_1(o) = \frac{4\pi ve}{\varepsilon kT\kappa}(\sigma_0 + e^{-\kappa a}\sigma_a)$$

$$= C(\sigma_0 + e^{-\kappa a}\sigma_a) \tag{A10}$$

The total free energy F of charging the two double layers is given by

$$F = -\int_0^{\psi_0} \sigma(o) d\psi_0{}^1 - \int_0^{\psi_a} \sigma(a) d\psi_a{}^1 \tag{A11}$$

From Eqn A10

$$dy_0{}^1 = \frac{4\pi ve}{\varepsilon kT\kappa}[d\sigma_0 + e^{-\kappa a} d\sigma_a]$$

Thus

$$F = -\frac{4\pi}{\varepsilon\kappa}\int_0^{\sigma_0} \sigma(o) d\sigma(o) + e^{-\kappa a}\int_0^{\sigma_a} \sigma(o) d\sigma(a) - \int_0^{\psi_a} \sigma(a) d\psi a^1$$

$$= -\frac{4\pi}{\varepsilon\kappa}\frac{\sigma_0{}^2}{2} + e^{-\kappa a}\sigma_0\sigma_a - \int_0^{\psi_a} \sigma(a) d\psi a^1$$

as $a \to 0$

$$F = -\frac{4\pi}{\varepsilon\kappa} \cdot \frac{(\sigma_0 + \sigma_a)^2}{2}$$

$$\int_0^{\psi_a} \sigma(a)\,\mathrm{d}\psi a^1 = -\frac{{\sigma_b}^2}{2}$$

So the free energy is given by

$$F = -\frac{4\pi}{\varepsilon\kappa}\left[\frac{(\sigma_a + \sigma_b)^2}{2} - (1 - e^{-\kappa a})\sigma_a\sigma_b\right] \tag{A12}$$

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