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Interactions of Gangliosides with Phospholipids and Glycosphingolipids in Mixed Monolayers

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1. The interactions among five different gangliosides and three chemically related glycosphingolipids and their behaviour in mixed monolayers with six different phospholipids were investigated at the air/145mM-NaCl interface at pH5.6. 2. The mixed monolayers of any of the different gangliosides showed an immiscible behaviour at high surface pressures, with absence of interactions among them revealed by an ideal behaviour for mean molecular area and surface potential per molecule. 3. This behaviour was probably the consequence of steric hindrance and electrostatic repulsions between their polar head groups. 4. Di- and tri-sialogangliosides could be differentiated from neutral sphingolipids and monosialogangliosides on the basis of their interactions with phospholipids, which were correlated to the perpendicular electric field at the interface contributed by the carbohydrate residues. 5. The presence of the phosphocholine polar head group in phosphatidylcholine was important to establish interactions with di- and tri-sialogangliosides revealed by negative deviations from the ideal behaviour for mean molecular areas and mean surface potential per molecule. 6. The possible significance of these observations is discussed in relation to the participation of gangliosides in the organization of membranes and to their capability of inducing membrane fusion.

The complexity of the oligosaccharide chain of gangliosides and other glycosphingolipids and the presence of sialosyl residues in the polar head group are major factors conditioning their surface behaviour, their influence on the permeability of liposomes to glucose in the presence of neurotransmitters, or their capability to induce membrane fusion in chicken erythrocytes (Maggio *et al.*, 1977, 1978*a*,*b*).

Since the structure and function of a biological membrane is the result not only of the properties of single components but also of the interactions among them, we studied the behaviour of gangliosides in mixed monolayers with other glycosphingolipids as well as with natural or synthetic phospholipids. The results showed that the interactions occurring between glycosphingolipids and phospholipids produced changes of the molecular packing and surface potential that depended on the type of polar head group of the glycosphingolipid and the phospholipid.

Abbreviations used: Cer, ceramide (*N*-acylsphingoid); GlcCer, Glcβ1→1Cer; LacCer, Galβ1→4Glcβ1→1Cer; Gg_3Cer, GalNAcβ1→4Galβ1→4Glcβ1→1Cer; Gg_4Cer, Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1Cer; GM_3, NeuGcα2→3Galβ1→4Glcβ1→1Cer; GD_3, NeuAcα2→8NeuAcα2→3Galβ1→4Glcβ1→1Cer; GM_1, Galβ1→3Gal-NAcβ1→4Gal (3←2αNeuAc)β1→4Glcβ1→1Cer; GD_{1a}, NeuAcα2→3Galβ1→3GalNAcβ1→4Gal(3←2α-NeuAc)-β1→4Glcβ1→1Cer; GT_1, NeuAcα2→3Galβ1→3Gal - NAcβ1 → 4Gal(3 ← 2αNeuAc8 ← 2αNeuAc)β1→4Glcβ1→1Cer. Abbreviations are those recommended by IUPAC–IUB (cf. Maggio *et al.*, 1978*a*) for neutral glycosphingolipids and by Svennerholm (1963) for gangliosides.

Materials and Methods

The equipment used, the preparation of lipid monolayers and the source and purity of the glycosphingolipids were described previously (Maggio *et al.*, 1978*a*). Egg phosphatidylcholine and egg phosphatidylethanolamine were prepared and purified as described by Bangham *et al.* (1974); the oxidation index (A_{233}/A_{215}) as defined by Klein (1970) was below 0.08 and 0.12 for each phospholipid respectively. T.l.c. of the purified lipid preparations, used in amounts at least 10 times higher than those required for detection, showed no contaminant spots. Dipalmitoyl phosphatidylcholine (1,2-hexadecanoyl-*sn*-glycero-3-phosphocholine) and dipalmitoyl phosphatidylethanolamine (1,2-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine) were from Koch–Light Laboratories (Colnbrook, Bucks., U.K.). Bovine brain L-α-phosphatidylserine and L-α-phosphatidylinositol from soya bean (98% pure) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.)

For mixed films, solutions of the individual components were mixed before spreading the monolayers. All experiments were carried out on a subphase of 145mM-NaCl at pH5.6 at 20 ± 1°C. The behaviour of

the mixed monolayers was analysed by comparing the force–area and surface-potential–area curves with the theoretical isotherms for the corresponding films in which no interactions between molecules are assumed. The values for the ideally mixed monolayers were calculated by the additivity rule for molecular areas and surface potentials per molecule at different surface pressures as described previously (Maggio & Lucy, 1976; cf. Gaines, 1966; Shah, 1970). In some cases the miscibility between the molecules in mixed monolayers was studied by application of the surface-phase rule (cf. Gaines, 1966). In the cases in which the film molecules were miscible, the spontaneity of the interaction was analysed by calculation of the excess free energy of mixing by the method of Goodrich (1957) (Gaines *et al.*, 1964; Gaines, 1966).

Results

Glycosphingolipid–phospholipid interactions

The mean area per molecule in monolayers of the ganglioside GM_1 mixed with dipalmitoyl phosphatidylcholine, in different molar ratios, showed positive deviations from the additivity rule at all surface pressures (Fig. 1*a*). Besides changes in packing the molecules of these mixed films showed ion–dipole interactions revealed by positive deviations of the mean surface potential per molecule (Fig. 1*b*). A similar pattern was obtained for the mixtures of the monosialoganglioside GM_3 or the neutral sphingolipids Cer, GlcCer and Gg_4Cer with dipalmitoyl phosphatidylcholine. Conversely, mixed monolayers of the disialoganglioside GD_{1a} with this phospholipid exhibited interactions characterized by a closer packing and a decreased surface potential per molecule in relation to the ideal behaviour (Figs. 2*a* and 2*b*). Mixed films of the disialoganglioside GD_3 or the trisialoganglioside GT_1 with dipalmitoyl phos-

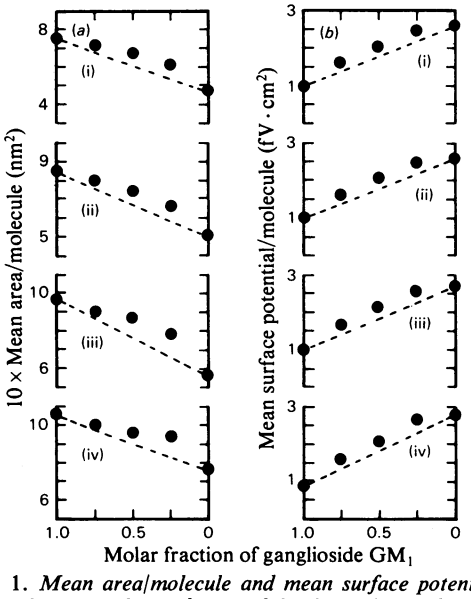

Fig. 1. *Mean area/molecule and mean surface potential/molecule in mixed monolayers of dipalmitoyl phosphatidylcholine and ganglioside GM_1*

The mean area/molecule (*a*) and the mean surface potential/molecule (*b*) were determined, as described in the text, for mixed monolayers of dipalmitoyl phosphatidylcholine and ganglioside GM_1 on subphases of 145mM-NaCl, approx. pH5.6. Determinations were made at different surface pressures: (i) 30mN/m; (ii) 20mN/m; (iii) 10mN/m; (iv) 5mN/m. The broken lines represent values calculated by the additivity rule.

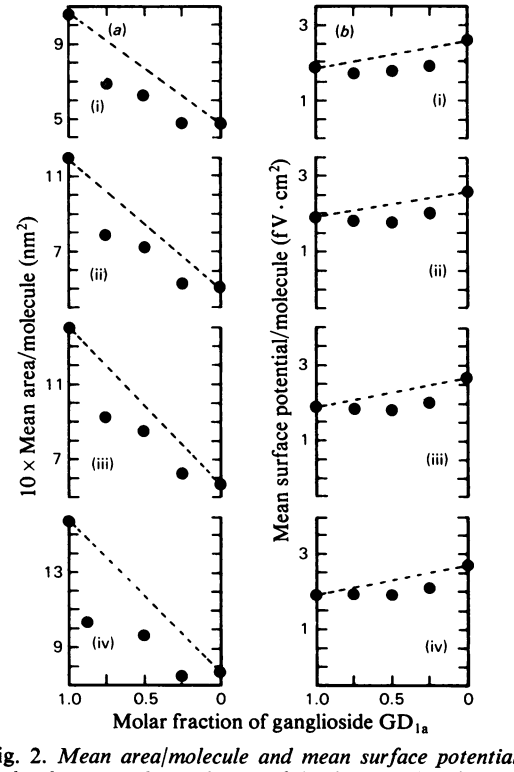

Fig. 2. *Mean area/molecule and mean surface potential/molecule in mixed monolayers of dipalmitoyl phosphatidylcholine and ganglioside GD_{1a}*

The mean area/molecule (*a*) and mean surface potential/molecule (*b*) were determined, as described in the text, for mixed monolayers of dipalmitoyl phosphatidylcholine and ganglioside GD_{1a} on subphases of 145mM-NaCl, approx. pH5.6. Determinations were made at different surface pressures: (i) 30mN/m; (ii) 20mN/m; (iii) 10mN/m; (iv) 5mN/m. The broken lines represent values calculated by the additivity rule.

phatidylcholine also showed negative deviations of the mean area and mean surface potential per molecule. These results indicated that the polar head group of the glycosphingolipid was important for determining the interactions with phosphatidylcholine.

The type of polar head group in the phospholipid was also important in determining the type of interaction with different gangliosides, as shown in Fig. 3 for mixtures in molar ratios of 1:1. Similar results were obtained for glycosphingolipid/phospholipid molar ratios of 3:1 and 1:3 at all surface pressures. The type of interactions shown by GD_{1a}, GT_1 or GD_3 (results not shown) with dipalmitoyl phosphatidylcholine was different from that of the same gangliosides mixed with dipalmitoyl phosphatidylethanolamine. In this case the deviations were positive instead of negative both in mean area and mean surface potential per molecule. The behaviour of mixed films of gangliosides with egg phosphatidylcholine and egg phosphatidylethanolamine was similar to that found with the corresponding synthetic phospholipids. Positive deviations from the additivity rule for both parameters were also found for mixed films of glycosphingolipids with phosphatidylserine or phosphatidylinositol. Exceptions were the mixed films of ceramide with these phospholipids that showed negative deviations from the ideal behaviour.

The excess free energy of mixing for mixtures of glycosphingolipids and phospholipids in molar ratios of 1:1 are shown in Fig. 4. These values show only a particular trend of the interaction since the absolute values are subject to considerable error, owing to the experimental difficulty of extending the measurements at very low pressures and to the possibility of phase separations in regions of high compression (cf. Gershfeld, 1974). The values of excess free energy of mixing result from a difference between the areas under the isotherms of the experimental and ideal films up to a specified surface pressure and are thus independent of the particular values of area per molecule or any deviations from the ideal behaviour. Consequently, their positive or negative sign adds information on whether a particular interaction is energetically favoured compared with the mixture in which no intermolecular interactions are assumed (cf. Gaines, 1966). Most of the mixed monolayers of glycosphingolipids with phospholipids that showed positive deviations in mean area and mean surface potential per molecule showed positive excess free energy of mixing. On the other hand, negative excess free energy of mixing, indicating that the interactions

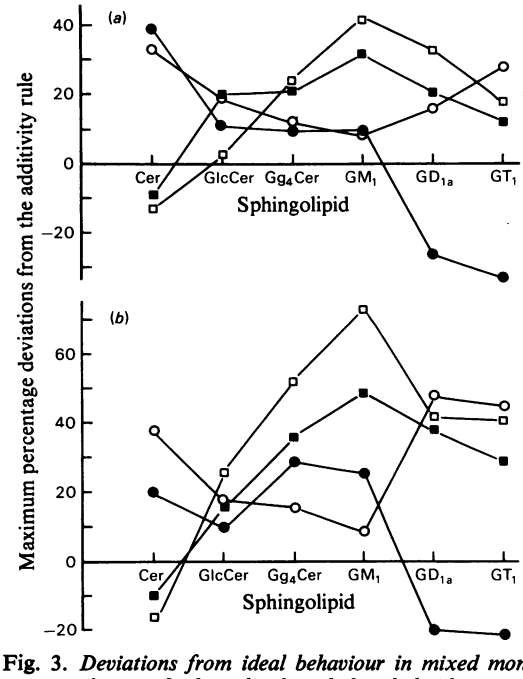

Fig. 3. *Deviations from ideal behaviour in mixed monolayers of sphingolipids and phospholipids*

Maximum percentage deviations (negative or positive) in mean molecular area (*a*) and mean surface potential/molecule (*b*) for mixed monolayers relative to the corresponding parameters for ideal films. The values represent deviations obtained above 30mN/m for mixed films, in molar ratios of 1:1, of the sphingolipid indicated with: ●, dipalmitoyl phosphatidylcholine; ○, dipalmitoyl phosphatidylethanolamine; ■, phosphatidylinositol; □, phosphatidylserine. Disialoganglioside GD_3 showed a behaviour similar to that of type GD_{1a}.

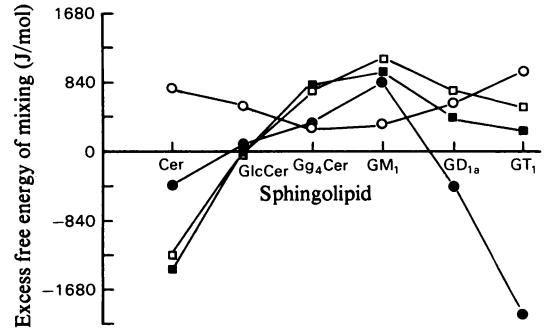

Fig. 4. *Excess free energy of mixing for mixed monolayers of sphingolipids and phospholipids*

The excess free energy of mixing values were obtained by calculation of the area under the pressure–area isotherm up to a surface pressure of 30mN/m by the Simpson's rule. The values are given for mixed films, in molar ratios of 1:1, of the sphingolipids indicated with: ●, dipalmitoyl phosphatidylcholine; ○, dipalmitoyl phosphatidylethanolamine; ■, phosphatidylinositol; □, phosphatidylserine.

were more favoured than in the ideal state, were found for the mixtures that exhibited negative deviations from the ideal behaviour in the mean area and mean surface potential per molecule.

Glycosphingolipid–glycosphingolipid interactions

The maximum positive or negative percentage deviations from the ideal behaviour in mean areas and mean surface potential per molecule for mixtures of these sphingolipids are shown in Table 1. Negative deviations in area and surface potential per molecule were found for mixtures of mono-, di- and trisialogangliosides with neutral sphingolipids. Mixtures of neutral sphingolipids followed the additivity rule for mean molecular area and showed ion–dipole interactions resulting in positive deviations from the ideal behaviour for the mean surface potential per molecule. The excess free energy of mixing indicated that most of these interactions were more favoured than in the ideal situation although some of the values are too small to have any significance.

Mixed monolayers of different gangliosides followed the additivity rule of mean area and mean surface potential per molecule, indicating that no intermolecular interactions occurred between different ganglioside species. This was consistent with the observations that each molecular class behaved independently and that the mixtures collapsed within

Table 1. *Excess free energy of mixing and deviations from ideal behaviour in mixed monolayers of gangliosides and neutral sphingolipids*

Maximum percentage deviations (negative or positive) in mean molecular area and mean surface potential/molecule for mixed monolayers relative to the corresponding parameters for ideal films are shown. The values represent deviations obtained above 30mN/m for the mixed films indicated, in molar ratios of 1:1. Similar results were obtained with other molar ratios (3:1 and 1:3) and at lower surface pressures. The excess free energy of mixing was calculated as described in Fig. 4.

	Percentage deviations from the additivity rule						Excess free energy of mixing (J/mol)		
	Mean area/molecule			Surface potential/molecule					
	Cer	GlcCer	Gg_4Cer	Cer	GlcCer	Gg_4Cer	Cer	GlcCer	Gg_4Cer
GM_1	−10	−30	−20	−8	−29	−20	−420	−12.6	+252
GD_{1a}	−18	−21	−10	−8	−24	−8	−1554	−1134	−378
GT_1	−9	−26	−11	−12	−16	−8	−1386	−840	−504
Cer	—	0	0	—	+22	+18	—	−29.4	−987
GlcCer	—	—	0	—	—	+22	—	—	−63

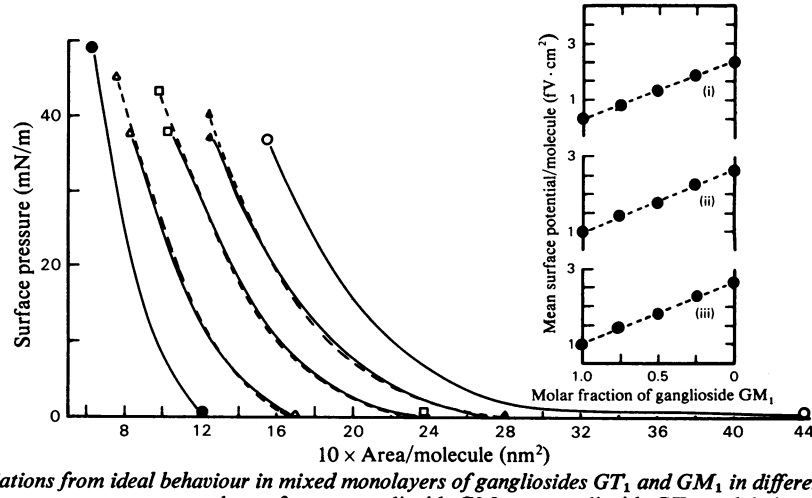

Fig. 5. *Deviations from ideal behaviour in mixed monolayers of gangliosides GT_1 and GM_1 in different molar ratios*

The surface pressure–area curves are shown for: ●, ganglioside GM_1; ○, ganglioside GT_1, and their respective mixtures in molar ratios of 3:1 (△), 1:1 (□) and 1:3 (▲). The inset shows the mean surface potential/molecule for mixed films in different molar ratios at: (i) 30mN/m; (ii) 20mN/m; (iii) 10mN/m. The broken lines represent values for ideal films.

± 1 mN/m of the collapse pressure of the film with the lowest collapse pressure; according to the surface-phase rule (cf. Gaines, 1966) this is indicative of molecular immiscibility at high pressures. This behaviour is illustrated in Fig. 5 where the isotherms of the single gangliosides GT_1 and GM_1 and of their mixtures in different molar ratios are given. Mixed films of the gangliosides GM_1 and GM_3, of GM_1 and GD_{1a}, of GD_{1a} and GD_3 and of GD_{1a} and GT_1 behaved also as immiscible monolayers.

Discussion

The absence of interactions and the immiscibility in surface films at high pressures of the different ganglioside species is probably the result of steric hindrance and electrostatic repulsions between their polar head groups. This was concluded because mono-, di- and tri-sialogangliosides showed spontaneous interactions with the neutral glycosphingolipids, as shown by a decrease in molecular packing and a decrease in surface potential (Fig. 5 and Table 1), and also with phospholipids that were negatively charged at the pH studied, although the interactions with the latter were not energetically favoured (Figs. 3 and 4). Electrostatic repulsions and steric effects in the polar head groups are responsible for increasing the distance between glycosphingolipid molecules in monolayers of single lipids (Maggio *et al.*, 1978*a*), and this resulted in the energy of the intermolecular interaction becoming lower as the number of carbohydrate and sialosyl residues increased (Fig. 6). These observations are consistent with the fact that gangliosides do not spontaneously form bilayers unless some kind of stabilization is provided by adding bilayer-forming lipids (Hill & Lester, 1972). For an amphipathic molecule there are well-defined requirements involving thermodynamic, geometric and packing constraints so that it can be spontaneously assembled into a bilayer structure (Israelachvily *et al.*, 1976, 1977), which gangliosides probably do not fulfil because of their negatively charged bulky polar head group. These results suggest that it is unlikely that there are regions consisting of pure or mixed gangliosides closely packed in a biological lipid bilayer unless effects such as ganglioside–protein interactions, binding of bivalent cations or other ligands, or ternary lipid complexes are also involved. Ganglioside–lipid interactions in a membrane in the absence of the above effects would probably occur with neutral glycolipids or phospholipids. Behaviour indicating changes in packing and ion–dipole interactions in the polar head groups was observed in mixed monolayers of glycosphingolipids and phospholipids.

The resultant contributions of the dipole moment of the glucose, galactose and the sialosyl residue, attached to the galactose proximal to the ceramide moiety, of the polar head group of glycosphingolipids and monosialogangliosides were in the opposite direction to the overall dipole moment of the whole molecule (Maggio *et al.*, 1978*a*). By contrast, the contributions of the second and third sialosyl residues were of a similar value to that of the first sialosyl residue but in the same direction as that of the overall molecular dipole. Therefore a distinction between neutral glycosphingolipids and monosialogangliosides on one side and di- and trisialogangliosides on the other could be made on the basis of the perpendicular electric field at the interface contributed by the neutral carbohydrates and sialosyl residues. A differentiation between the two groups of glycosphingolipids that can be correlated to the electrical properties of their polar head groups is also possible from the results of the present work (Figs. 3 and 4). Considering the deviations from the additivity rule for mean areas, mean surface potential per molecule or the spontaneity of the interaction in mixed monolayers with any of the phospholipids a definite change in the type of the interaction takes place for di- and tri-sialogangliosides compared with monosialogangliosides and neutral glycosphingolipids; in Figs. 3 and 4 this is shown by a change in either the upward or downward tendency of the curves or in the positive or negative sign of the deviation. For phosphatidylcholine (natural or synthetic) the interactions with di- and tri-sialogangliosides occurred with negative deviations and were thermodynamically favoured, unlike those found for neutral glycolipids and monosialogangliosides or with other phospholipids. If the spontaneity of the interaction is considered, di- and tri-sialogangliosides, in preference to neutral glycosphingolipids or monosialogangliosides, should be associated to phosphatidylcholine in a complex lipid interface in the absence of other constraints.

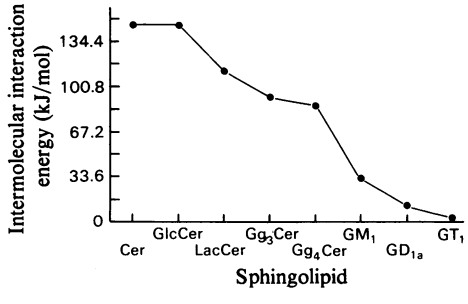

Fig. 6. *Intermolecular interaction energy of sphingolipids*
The interaction energy was calculated at 30 mN/m by the method of Salem (1962), taking the number of interacting methylene pairs $n = 18$ for each of the sphingolipids indicated.

Negative deviations in mean areas and mean surface potential per molecule together with negative excess free energy of mixing occur when the gangliosides GD_{1a}, GD_3 and GT_1 are in mixed films with phosphatidylcholine, but not with other phospholipids, showing that the dipolar properties of the phosphocholine group are also important for establishing this behaviour. The glycosphingolipid–phospholipid interactions probably resulted from polar-head-group interactions largely independent of the hydrophobic chains present in the phospholipid or of the phase of the monolayer of the pure components. The liquid-expanded di- or tri-sialogangliosides showed the same type of interactions with the liquid-condensed dipalmitoyl phosphatidylcholine containing saturated chains as with the liquid-expanded egg phosphatidylcholine having unsaturated fatty acyl residues. Conversely, the behaviour was different with liquid-condensed or -expanded natural or synthetic phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol. On the other hand, the more liquid-condensed neutral glycosphingolipids showed the same type of behaviour as the more liquid-expanded gangliosides with the synthetic or natural phosphatidylethanolamine.

The interactions of di- and tri-sialogangliosides with phosphatidylcholine and phosphatidylethanolamine resembled those exhibited by lipids that induce membrane fusion (Maggio & Lucy, 1976), and on this basis it was possible to anticipate that gangliosides GD_{1a}, GD_3 and GT_1, but not GM_1, GM_3 or the neutral glycosphingolipids, could induce fusion of chicken erythrocytes (Maggio *et al.*, 1978*b*) in an experimental system similar to that described by Ahkong *et al.* (1973). These findings and the results of the present work indicate that the correlation between the interactions of fusogenic lipids with phosphatidylcholine, involving changes in surface potential, and their ability to induce membrane fusion (Maggio & Lucy, 1975, 1976, 1977; Maggio *et al.*, 1976) is maintained for di- and tri-sialogangliosides.

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