THE BINDING OF DIVALENT CATIONS BY PURIFIED GANGLIOSIDES

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1. Introduction

The gangliosides are glycosphingolipids containing sialic acids. They are mainly found in certain membrane fractions of the central nervous systems [1]; but they may also be detected in the visceral system where they are implicated in intercellular interactions and seem to inhibit the proliferation of malignant cells ([2] and references therein). Numerous data suggest that they may play an important role in the nervous system, especially via the carboxyl groups of the sialic acids [1, see discussion]. These carboxylates contribute to the membrane electric charge and interact with various cationic species [3]. Divalent metal cations, especially calcium, play a fundamental role in the release of biogenic amines and in secretory processes in general [4]. It seemed thus of interest to study the stability constants and the nature of the complexes formed between such cations and ganglioside aggregates, which could be considered as simplified membrane models. Cation association with sialic(*N*-acetylneuraminic)acid itself has been studied previously [5].

2. Materials and methods

A Sigma grade (Type III; 500 mg) bovine brain ganglioside mixture was chromatographed in two equal portions of 250 mg over a column of Silicagel H (200 × 15 mm) using as eluant a mixture of $CHCl_3$/MeOH/H_2O/NH_4OH, 60/35/7/1. The flow (8–10 ml/hr) was adjusted via nitrogen gas pressure. The 3 ml fractions were analyzed by thin-layer chromatography on silica using the same eluant and the Svennerholm [6] reagent as developer. The eluates were combined into four ganglioside fractions and one initial phospholipid fraction (about 10% of the total). Each fraction was evaporated to dryness and chromatographed again on a similar column. The purified gangliosides obtained from the different fractions were deionized on Amberlite MB3 and lyophilized, yielding 90 mg G_{GNT1} (monosialo-), 110 mg G_{GNT2a} (disialo-), 55 mg G_{GNT3a} (trisialoganglioside) (nomenclature and identification following ref. [1] and references cited therein). Two other gangliosides were obtained in quantities too small to allow physical measurements to be performed.

The association constants with Mg^{2+} and Ca^{2+} cations were determined using a divalent cation liquid membrane electrode on 5×10^{-3} M ganglioside solutions, neutralized with KOH according to the procedure described previously [5]. Two or three measurements were performed for each cation/ganglioside pair; after each run the cation free ganglioside could be recovered by passing the material twice over an Amberlite MB 3 column.

Association constants and stoichiometries were extracted from Scatchard plots [7] using $(M_0-M)/M$ (ordinate) and (M_0-M) (abscissa); M_0 and M are respectively the total concentration of divalent cation and the concentration of free cation. The plots are either straight lines or straight segments linked by an arc (variable stoichiometry). The stoichiometries were confirmed by the maximum value of M_0-M = ganglioside/stoichiometry for a given straight line segment.

The carbon-13 NMR spectra of sialic acid and of its Ca^{2+} complex have been measured on a Varian XL-100-15 spectrometer equipped with a Fourier Transform accessory, using 0.2 M aqueous solutions at pH 7 in 12 mm o.d. microcells (Wilmad Co.).

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Table 1

Stability constants log$K$ ($\ell \times M^{-1}$) and number of monomeric units m per complexation site for ganglioside–divalent cation association.

Cation	n	G_{GNT1}			G_{GNT2a}			G_{GNT3a}		
		M_0	m	log$K$	M_0	m	log$K$	M_0	m	log$K$
Ca^{2+}	4.5	–	–	–	–	–	–	$< 2.5 \times 10^{-3}$	1.5	4.7
	4	–	–	–	$< 2 \times 10^{-3}$	2	4.8	–	–	–
	3	$< 2 \times 10^{-3}$	3	4.2	–	–	–	$> 5 \times 10^{-3}$	1	3.6
	2	$> 2 \times 10^{-3}$	2	3.2	$> 5 \times 10^{-3}$	1	3.2	–	–	–
Mg^{2+}	4.5	–	–	–	–	–	–	–	1.5	3.6
	4	–	–	–	–	2	3.8	–	–	–
	3	–	3	3.9	–	–	–	–	–	–

The experimental conditions and the symbols are given in sect. 2 and 3.

3. Results and discussion

The critical micellar concentration of the gangliosides in water is about 0.2 g/ℓ. Under our experimental conditions, the gangliosides exist in the form of micelles containing about one hundred monomeric units [8]. Analysis of the data by a Scatchard treatment [7] leads to the number m of monomeric units per complexation site and to the stability constant K for 1/1 complexation of this site (which contains n sialic acid residues) with the divalent cation. The results are listed in the table. Measurements have been performed at various total cation concentrations M_0.

3.1. *Complexation of calcium cations*

In the case of calcium ions, each ganglioside presents two different stoichiometries in the explored range M_0 (10^{-3} to 10^{-2} M). The values of M_0 above or below which a given stoichiometry is observed, are listed in the table. It is worth noting

i) that the changes in stoichiometry occur when all available sites are saturated with Ca^{2+};

ii) that, as the cation concentration increases, the micelles tend always to complex as much calcium as possible, first via a high global stability constant (at low M_0) and then by changing stoichiometry to a lower sialic acid/Ca^{2+} ratio;

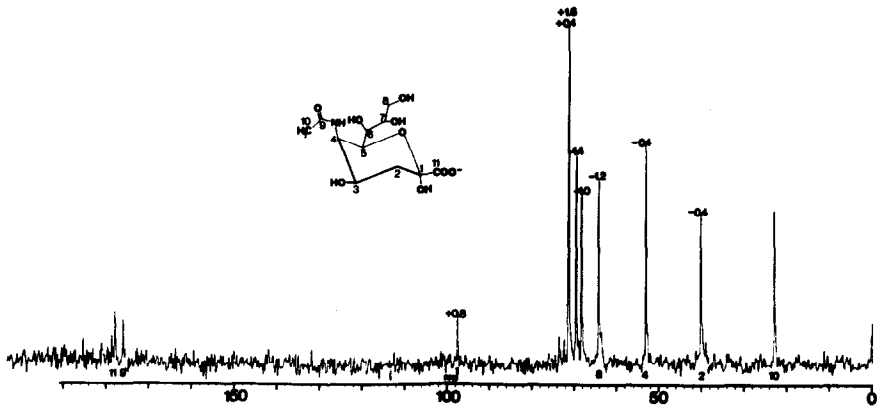

Fig. 1. Carbon-13 FT-NMR spectrum of sialic (*N*-acetylneuraminic) acid in water. The numbers below a given peak give the assignment; the numbers above the peaks give the change in chemical shift (ppm) upon calcium complexation. The reference signal is external TMPS.

iii) that the complexation ability (log K)/n per sialic acid residue decreases as n increases but is the same for mono- and disialogangliosides (c.a. 1.6); it is however appreciably lower for the trisialoganglioside. This may arise from the special structure of G_{GNT3a} (two directly end-to-end linked sialic residues) which may not allow sufficient proximity of the three carboxyl groups. In addition, one may expect a decrease in the electrostatic contribution of a third carboxyl group to the complex stability.

3.2. *Complexation of magnesium cations*

Gangliosides are known to have much less affinity for magnesium cations than for calcium ones in solvent mixtures like $CHCl_3$/MeOH/H_2O [9]. In our experimental conditions only *one* stoichiometry is observed for Mg^{2+} complexation, and it corresponds in each case to the highest stoichiometry found with Ca^{2+}. The much larger charge–charge interactions of a carboxylate group with the small Mg^{2+} cation as compared to Ca^{2+} favours high values of n, while on the other hand repulsion between the negatively charged and voluminous carboxylate groups may explain the lower *stability* constants for Mg^{2+} as well as the increasing Ca^{2+}/Mg^{2+} *selectivity* with increasing number of sialic acid residues per complexation site.

Direct comparison of the present results with our previous data on Ca^{2+} and Mg^{2+} complexation by sialic acid itself [5] is difficult since the stoichiometries are different and the stability gain per carboxylate group diminishes with the number n of such groups. Indeed the log K values for sialic acid (Mg^{2+}: 1.5; Ca^{2+}: 1.9 $\ell \times M^{-1}$) are higher than the (log K)/n values found here but show the same $Ca^{2+} > M^{2+}$ selectivity.

3.3. *Carbon-13 NMR study of sialic acid and of its Ca^{2+} complex*

Attempts to study the carbon-13 NMR spectra of the gangliosides were discontinued because of linewidth problems. The noise-decoupled ^{13}C spectrum of sialic acid itself is presented in the figure, together with the chemical shift changes brought about by the addition of excess $CaCl_2$.

The partial assignment of the peaks is based on the undecoupled spectrum and on chemical shift considerations. On calcium complexation all carbons linked to a hydroxyl group are appreciably shifted especially those of the glycerol side chain (signals between 60 and 80 ppm). Since the ^{13}C chemical shifts are very sensitive to conformational changes, it appears very likely that this side chain takes part in the complexation process (in agreement with our previous discussion [5]), probably by wrapping around the cations as much as possible.

3.4. *Effect of additives*

It has been suggested that gangliosides and especially their sialic acid residues, play a role in the receptor properties of membranes towards acetylcholine [11], serotonin [12–15], noradrenaline and other biogenic amines [12]. However, we could not detect any release of calcium cations when adding up to 10^{-2} M acetylcholine or noradrenaline to an aqueous solution of ganglioside (5×10^{-3} M) and Ca^{2+} (2×10^{-3} M). It is possible that a higher concentration of additives may be required if the corresponding stability constants are very weak. It has also been suggested that the gangliosides may play a role in the activation of acetylcholinesterase [16], among other factors because of their aggregation–disaggregation behaviour. The changes in stoichiometry found for calcium may be due to a change in micellar conformation or to a disaggregation process. Such conformational changes in the presence of Ca^{2+} have been observed for several phospholipids ([17] and references therein).

Changing the calcium concentration probably affects the structure of the membrane models. Similar effects are not observed in the case of magnesium cations, which however are known to inhibit calcium dependent secretory processes [18–21].

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