STABILITY CONSTANTS FOR THE COMPLEXATION OF ALKALI AND ALKALINE-EARTH CATIONS BY *N*-ACETYL-NEURAMINIC ACID

Jean-Paul BEHR and Jean-Marie LEHN

*Institut de Chimie, Université Louis Pasteur, 4, rue Blaise Pascal, 67-Strasbourg, France**

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1. Introduction

N-Acetylneuraminic acid (NANA) is found in numerous glycoproteins and glycolipids. The carbohydrate components of these molecules, which may be either free or associated with membrane structures, take part in a variety of biological phenomena such as contact inhibition, antigenic and hormonal action, fibrinogin coagulation and receptor functions for neurotransmitters (see reviews in [1–4]). Removal of the NANA residues with neuraminidase affects markedly their properties (see review in [1–5]): thus plasma glycoproteins and gonadotropic hormones are rapidly eliminated from the circulation [6], and gangliosides loose their receptor properties [7].

On the other hand, the behaviour of most of these molecules is closely related to the presence of mono and divalent metal cations, especially of calcium ions. As a first step in a study of the interaction of gangliosides with various cationic species it was of interest to determine the association constants between metal cations and the most widely distributed component of these macromolecules bearing an anionic site: *N*-acetylneuraminic acid. Such data also bear on the role of gangliosides in cation transport phenomena [1]. In addition, much interest has been shown in recent years for the alkali and alkaline earth metal cation complexing properties of various natural [8, 9] and synthetic [10–12] substances.

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2. Materials and methods

2.1. *Measurements*

NANA from Sigma Co. (batch no. 30 C-0320) was used after drying under vacuum for 24 hr. The alkali salts of NANA have been prepared via neutralisation of the acid with the corresponding base followed by lyophilization. Stock solutions (0.1 M) of alkali salts have been prepared by dissolving the dried (4 hr heating with a Mecker burner, then 12 hr at 120°) salts in the required volume of dry methanol. Stock solutions (0.1 M) of the alkaline-earth salts were titrated with a solution of EDTA of known concentration. The measurements have been performed in a small polyethylene cell containing a magnetic stirring bar and placed in a double walled glass cell thermostated at 25 ± 0.1°, which was slowly swept by a flow of nitrogen gas saturated with the solvent.

The concentrations of the uncomplexed cations have been measured potentiometrically (Tacussel type TS60N pH meter) using cation specific electrodes and the corresponding concentrations have been obtained from calibration curves. The millivoltmeter was connected to a recorder in order to check the response time of the electrode and the stability of the potential during each measurement.

A calomel electrode (Tacussel C8) with a 0.1 M cesium chloride bridge was used as reference.

The concentrations of the alkali cations, Na^+ and K^+, have been determined, respectively, with a sodium selective glass electrode (Philips Type C15-Na [13]) and a monovalent cation glass electrode (Corning no. 476220) on 10^{-2} M solutions of the alkali salts of NANA. These electrodes were calibrated between

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10^{-4} and 10^{-2} M before and after each measure (slope 56.5 mV/p $[M^+]$). The concentrations of the alkaline-earth cations Mg^{2+} and Ca^{2+} have been determined with a divalent cation liquid membrane electrode (Orion No. 92-32) on solutions (5 ml; 10^{-2} M) of NANA potassium salt. The divalent ion was added in eight 100 µl fractions of stock solution. After each addition the solution was stirred until the potential stabilized (less than 2 min) and the measurement was performed after standing unstirred for 2–3 min. The pH, 7.3 ± 0.1, was monitored continuously using a semi-micro electrode (Ingold). Two series of measurements were performed for each cation. In order to eliminate the effect of K^+ and H^+ ions on the electrode potential, the electrode was calibrated before and after each measurement by adding the same increments of stock solution to 5 ml KCl solution (10^{-2} M) at pH 7.3. The least-squares slope was 26.6 mV/p $[M^{2+}]$ or 29.2 mV/p $[M^{2+}]$ with a mean deviation of about ± 0.1 mV. The liquid membrane electrode does not allow measurements to be performed in methanol.

2.2. *Calculations*

The pK of NANA being 2.6, the carboxyl group was assumed to be entirely ionized in our experimental conditions. The thermodynamic stability constant K_T for a 1/1 NANA cation complex is defined by:

$$K_T = \frac{(\text{NANA}, M^{(n-1)+})}{(\text{NANA}^-)\,(M^{n+})} = K_C \times \frac{f\,\text{NANA}, M}{f\,\text{NANA} \times f M}$$

where n equals 1 or 2 for mono or divalent cations, respectively, K_C is the concentration stability constant and f_i are activity coefficients. Since fNANA is unkown K_T cannot be calculated for a monovalent cation. Assuming that the activity coefficients of the singly charged species NANA, M^+ and $NANA^-$ are equal, K_T may be calculated for a divalent cation complex by a computer iterative fitting of the stability constant using scheme 1, where N_0, M_0, and M are, respectively, the total concentrations of NANA and of the cation and the total concentration of free cation. I is the ionic strength and (M) is the activity of free cation measured by the ion selective electrode. K_T was only calculated for the calcium complex in order to compare it with similar complexes (see discussion). For the others, we have calculated the concentration stability constants for a further comparison with gangliosides in the same conditions:

$$K_C = \frac{M_0 - M}{(N_0 - M_0 + M)M}$$

The error on K_C may be estimated to $\Delta(\log K_C) \sim 0.20$.

3. Results and discussion

The results are given in table 1.

Table 1
Stability constants log K_C for NANA, cation association.

Cation	Na^+		K^+		Mg^{2+}	Ca^{2+}
Solvent	H_2O	MeOH	H_2O	MeOH	H_2O	H_2O
log K_C ($1 \times M^{-1}$)	< 0.1	2.2	< 0.1	2.4	1.5	1.9

The experimental conditions are described in sect. 2. The ionic strength was 0.01 for Na^+, K^+ and 0.02–0.04 for Mg^{2+}, Ca^{2+}; the pH = 7.3 ± 0.1.

3.1. *Monovalent Na^+ and K^+ cations*

The NANA anion does not complex Na^+ and K^+ cations in water. As expected a notable complexation is found in anhydrous methanol. However no appreciable K^+/Na^+ selectivity is observed. When the acid itself is used instead of the anion no complexation is detectable; this agrees with the very low stability constants (0–0.1 lM^{-1}) found for the complexation of alkali cations by neutral sugars [14]. The complexes formed by NANA anion are thus due almost entirely to the ionic interaction of the cations with the carboxylate group, with the possibility that one or two hydroxyl groups of the remainder of the molecule may replace water molecules of the hydration shell.

3.2. *Divalent Mg^{2+} and Ca^{2+} cations*

The analysis of the experimental data assuming various stoichiometries indicates that the results are compatible with a 1:1 stoichiometry when the total cation concentration is of the same order or greater than the concentration of the ligand. The same stoichiometry has been found for acyclic polyhydroxyacids [15, 16] and for uronic acids [17].

The stability constants for Mg^{2+} and Ca^{2+} complexation by the NANA anion are much higher than for Na^+ and K^+ and a selectivity $Ca^{2+}/Mg^{2+} \sim 2.5$ is found. Thus Ca^{2+} will displace Mg^{2+} in competition conditions.

Scheme 1

$$K_T = \frac{M_0 - (M)/f_M}{N_0 - M_0 + (M)/f_M} \times \frac{1}{(M)} \rightarrow M = \frac{-K_T f_M (N_0 - M_0) - 1 + ((K_T f_M (N_0 - M_0) + 1)^2 + 4 K_T f_M M_0)^{1/2}}{2 K_T f_M}$$

$$\uparrow \qquad\qquad \downarrow$$

$$\log f_M = -2.04 \left(\frac{(I)^{1/2}}{1+(I)^{1/2}} - 0.3\, I\right) \leftarrow I = N_0 + M_0 + 2\, M$$

The complexation of Ca^{2+} by galacturonic acid anion is $\log K_T = 1.8$ in terms of activities [17]. Assuming that the activity coefficients of singly charged species are equal and applying the iterative fitting of the stability constant one finds $\log K_T = 2.3$ for the (NANA, Ca^{2+}) complex. Thus this complex is more stable than the complex (Ca^{2+}, galacturonic acid). The crystal structure of calcium galacturonate [18] shows that the cation is nonacoordinated and is located in the center of a trigonal prism having three water molecules and three hydroxyl oxygens at the corners and three carboxylate oxygens in the center of each face.

Molecular models show that the terminal hydroxyl groups of the glycerol residue present in NANA could replace one water molecule in the complex which would then be more stable. A similar effect has been proposed for explaining the higher stability constant found for polymers of galacturonic acid with respect to the monomer [18]. At very low total divalent cation concentration the association constant increases continuously. Taking into account the change in ionic strength by iterative fitting of the stability constants with a computer or assuming the formation of complexes of 1:2 and 1:3 stoichiometries still leads to variable stability constants. It is possible that higher order aggregates are formed.

Calcium ion is fixed passively by membranes of various origins and phospholipids do not seem to contribute appreciably to this fixation [19, 20]. For human erythrocyte membranes there is a correlation between the calcium fixation ability and the NANA content [21]. In addition an inhibition of ca. 30% is found in the presence of an equivalent quantity of Mg^{2+} [21].

Assuming a competition between these two ions for a same site containing NANA this amount of inhibition corresponds to a Ca^{2+}/Mg^{2+} selectivity of the same order as the selectivity found here for the free anion. Since NANA is preferentially localized on the surface of the membranes [22], on the inner one as well as on the outer one [21, 23], it may function as a primary receptor for cationic species. These could be calcium ions but also neurotransmitters which contain a quaternary ammonium group. Studies in this direction using gangliosides as model systems are presently underway.

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