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Sylvie Blanc, Georges Jeminet, Anne-Marie Albrecht-Gary. Kinetic Aspect of the Complexing Ability of 4-Bromo-and N-methyl-Calcimycin for Calcium and Magnesium. 2026. <hal-05580170>

HAL Id: hal-05580170

<https://cnrs.hal.science/hal-05580170v1>

Preprint submitted on 3 Apr 2026

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**Kinetic Aspect of the Complexing Ability of
4-Bromo- and N-methyl- Calcimycin
for Calcium and Magnesium**

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Abstract. To understand why 4-bromo-calcimycin, a chemically modified analogue of calcimycin or A 23187, seemed to be a better biological transporting agent of calcium than calcimycin its complexing properties were studied in methanol. Using potentiometric and spectrophotometric methods protonation and calcium or magnesium complexation constants were determined. 4-Br-A 23187 formed two complexes a neutral two ligand one metal A_2M and a charged species AM^+ like calcimycin but three order of magnitude less stable than those of A 23187.

Introduction

Antibiotic A 23187 or calcimycin [1] (Figure 1) is used to follow the regulatory activities in cellular processes [2]. Many studies have been done to know the mechanism of cation transport of this ionophore [3,6] which shows high selectivity for divalent over monovalent cations [7] especially calcium. Chemical transformations of this compound have given some information between structure and function [8]. These experiences have proved that the substituents on the benzoxazole moiety of the antibiotic were very important [4,8-10] and it was verified by “ab initio” computation [11]. In this approach Debono *et al.* [12] have realised the synthesis of different bromo-derivates of calcimycin and 4-bromo-A 23187 (Figure 1) seems to possess enhanced transport discrimination between Ca^{2+} and Mg^{2+} compared with its parent. This compound has also lost the fluorescence of the benzoxazole chromophore and this change in spectral properties has been used in biochemical applications where ionophores are employed in conjunction with other fluorescent probes [13-27]. Accordingly, we decided to study the complexation of 4-bromo-calcimycin with calcium and magnesium in methanol. The thermodynamic constants will be compared with those of A 23187 [3,4] and an other analogue, the N-methyl-calcimycin [8].

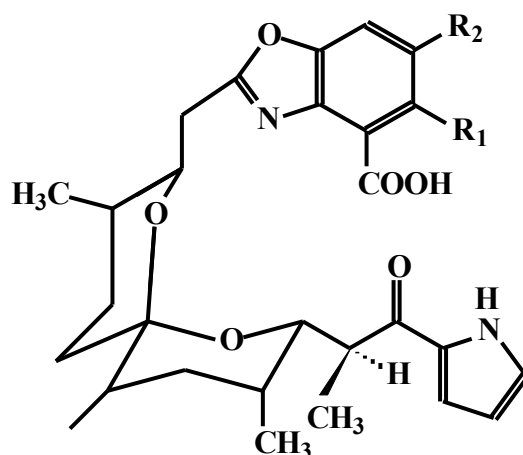


Figure 1: Chemical structures of a) A 23187 or calcimycin $R_1 = \text{NHMe}$ $R_2 = \text{H}$; b) 4-bromo-calcimycin $R_1 = \text{NHMe}$ $R_2 = \text{Br}$; c) N-methyl-calcimycin $R_1 = \text{NMe}_2$ $R_2 = \text{H}$

Results and Discussion

Thermodynamics and Spectrophotometry. Spectrophotometric titrations of 4-Br and N-methyl calcimycin with calcium and magnesium versus p[H] were carried out in order to calculate the binding constants of the complexes and to determine their corresponding electronic spectra (250-450 nm). The best fit of the spectrophotometric data was obtained by Letagrop-Spefo[28-32] program with a model including two protonated species, AH and AH_2^+ and two complexes a charged one AM^+ (one antibiotic : one cation) and a neutral one A_2M (two antibiotics : one cation). The the UV characteristics and stability constants values and of the various species are summarised in Figure 2 and in Table 1

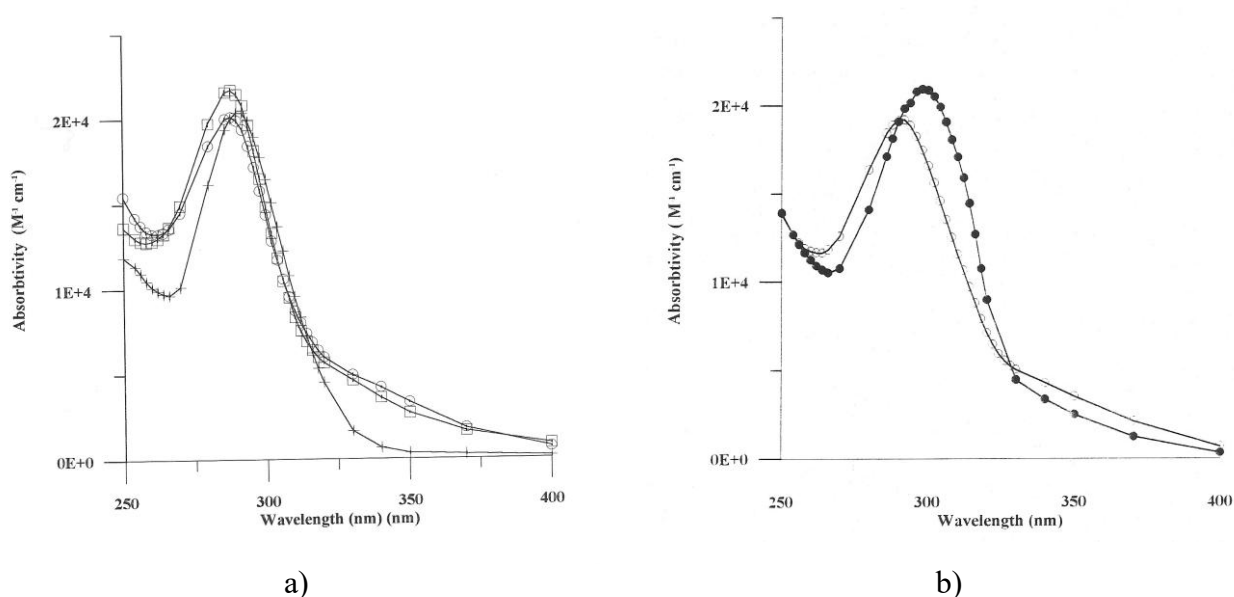


Figure 2 : Electronic spectra of
 a) di, mono and deprotonated species of 4-bromo-calcimycin : \bullet AH_2^+ , \square AH , \circ A^-
 b) calcium and magnesium monomeric 4-bromo-calcimycin complexes \circ AMg^+ , \bullet ACa^+

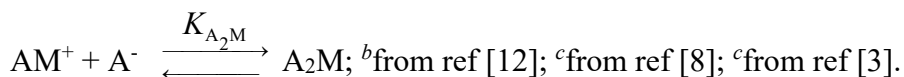
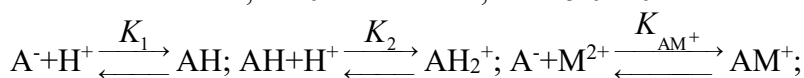
Solvent : methanol; T = 25. °C; I = 0.1.

It was not possible under our experimental conditions to calculate with accuracy the spectra of the A₂M complexes formed with 4-Br- or N-methyl-A 23187. These data are compared with the corresponding parameters obtained in the same conditions for calcimycin.[3,4].

Table 1 . Stability Constants of the Calcium and Magnesium Complexes and of their Corresponding Maximal Absorptivities.^a

	Species	Log K	λ_{\max} (/nm)	$\varepsilon \times 10^{-4}$ (/mol ⁻¹ dm ³ cm ⁻¹)
4-Br- A 23187	A ⁻	-	288	2.01
	AH	6.1 ± 0.4	288	2.17
	AH ₂ ⁺	2.9 ± 0.4	290	2.04
	ACa ⁺	3.0 ± 0.4	298	2.09
	A ₂ Ca	4.0 ± 0.5		
	AMg ⁺	3.1 ± 0.2	292	1.91
	A ₂ Mg	4.1 ± 0.5	305 ^b	3.2 ^b
N-methyl-A 23187	A ⁻	-	288	1.57
	AH	10.1 ± 0.1 (9.5 ± 0.2) ^c	288	1.97
	AH ₂ ⁺	4.1 ± 0.3 (3.8 ± 0.4) ^c	290	2.33
	ACa ⁺	(4.5 ± 0.3) ^c	302	1.96
			356	0.23
	A ₂ Ca	(4.0 ± 0.9) ^c	350	0.20
	AMg ⁺	4.2 ± 0.2 (4.5 ± 0.1) ^c	290	1.46
			348	0.25
	A ₂ Mg	5.1 ± 0.2 (5.0 ± 0.8) ^c	296	2.90
			360	0.7
A 23187 ^d	A ⁻	-	288	1.53
			350	0.38
	AH	10.4 ± 0.1	279	1.68
			380	0.76
	AH ₂ ⁺	3.3 ± 0.1	288	1.93
			380	0.78
	ACa ⁺	6.4 ± 0.1	302	1.77
			3371	0.74
	A ₂ Ca	7.7 ± 0.2	302	3.17
			370	1.56
	AMg ⁺	6.2 ± 0.1	296	1.57
			374	0.72
A ₂ Mg	7.6 ± 0.3	304	3.14	
		374	1.74	

^aSolvent methanol; I = 0.1 /mol dm⁻³; T = 25.0 ± 0.2°C.



The errors are estimated to 2 nm for the wavelengths and to 3σ for the stability constants and the extinction coefficients.

The first protonation constant of 4-Br-A 23187 is attributed to the carboxylic function as for calcimycin. We observed a decrease of 10^4 of this constant for 4-Br-A 23187 in comparison with calcimycin (Table 1). This comportment can be related to the electronic properties of the bromine borne by the benzoxazole moiety of the calcimycin. An electron negative substituent at position 4 reduces the electron density (or partial negative charge) on the carboxylate oxygen atoms, decreasing their hydrogen-bond acceptor capabilities. The second protonation constant concerns the secondary amine and is very similar to that of calcimycin (Table 1).

The coordination properties of 4-bromo- and N-methyl-calcimycin with calcium and magnesium are very similar (Table 1); 4-bromo-A 23187 forms complexes that are one order of magnitude less stable than the corresponding N-methyl-calcimycin species, the latter are themselves three order of magnitude less stable than those of calcimycin.[12]. This result can be related first to the different basic properties of the binding sites. We namely observe the same order for the complex stability constants and the first pK values of the ionophores. More basic is the carboxylic function more stable are the complexes. To illustrate the competition between protons, Ca^{2+} or Mg^{2+} for the three studied antibiotics in methanol we present in Figure 3 the formation curves which were calculated using the Haltafal program [33] and the previous data under various acidic and a ionophore/calcium ratio corresponding to physiological environment. We observed that half of 4-Br-A 23187 is not complexed by the calcium in basic medium which is a consequence of its low stability constants. However, we noticed for all considered antibiotics that the charged complex ACa^+ is the predominant species in solution. It is already formed at $p[\text{H}] = 4$ for 4-Br-A 23187 and only at $p[\text{H}]$ higher than 5 for the two others. This prove that in biological systems we have to take this species into account as it is present at a high level.

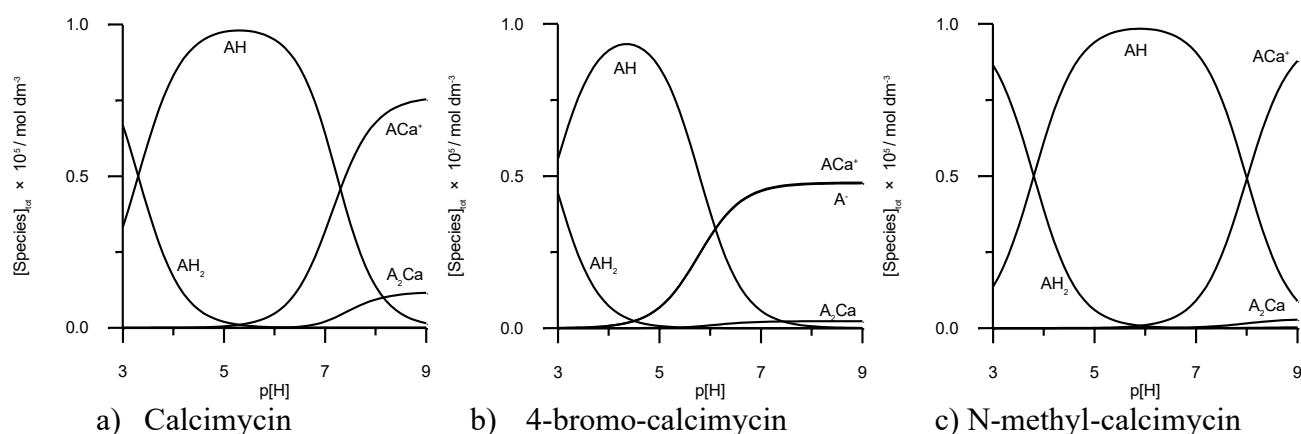


Figure 3 : Distribution curves of calcium complexes formed with the three studied ionophores under various acidic conditions.

$[\text{A}]_{\text{tot}} = 10^{-5} / \text{mol dm}^{-3}$; $[\text{Ca}^{2+}]_{\text{tot}} = 10^{-3} / \text{mol dm}^{-3}$;

Solvent : methanol; $T = 25 \text{ }^\circ\text{C}$; $I = 0.1$.

It is also interesting to remark that 4-Br-A 23187 begins to form the neutral complex at $p[H] = 5.5$, the calcimycin at $p[H] = 6.5$ and the N-methyl calcimycin at $p[H] = 7.5$. If we consider that A_2Ca is the transporting species, the bromo derivative should be more efficient than the calcimycin between $p[H] 5.5$ and 6.5 . Hence the A 23187 antibiotic forms under basic conditions with calcium the neutral complex at highest concentration ($0.12 \times 10^{-5} / \text{mol dm}^{-3}$) compared with N-methyl ($0.10 \times 10^{-5} / \text{mol dm}^{-3}$) and 4-Br-A 23187 ($0.5 \times 10^{-5} / \text{mol dm}^{-3}$).

The second reason of the decrease in the calcium or magnesium complexes stability between calcimycin and the two others ionophore is related to the ligand conformation. It was proved for A 23187 that the presence of an hydrogen bond between the acid carboxylic function and the nitrogen of the benzoxazole ring (Figure 4) is necessary to obtain the optimally coordinating configuration. We have previously observed that the N-methyl-calcimycin lacks this hydrogen bond and forms much less stable complexes. [34,35] An atom of brome at position 4 on the calcimycin obliged the secondary amine group to rotate because of its important volume ($r = 1.96 \text{ \AA}$) and hindered also the formation of this hydrogen bond. The lost of this hydrogen bond induces a less favourable coordination geometry around the cations and is a factor leading to a decrease of the complex stability as observed between the calcimycin and the two other antibiotics.

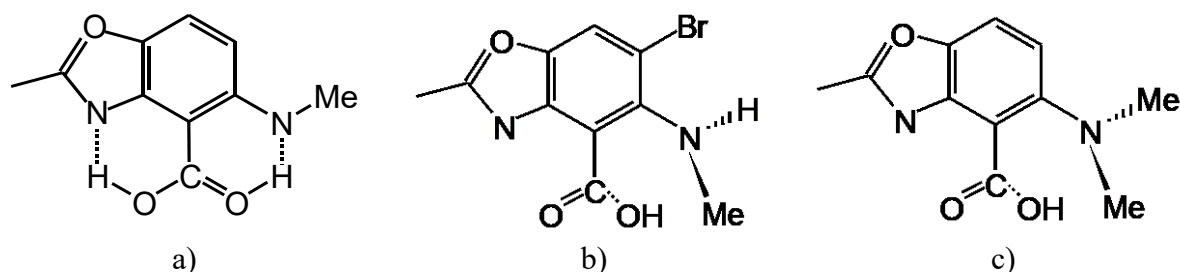


Figure 4 : Hydrogen bonds in monoprotonated species of

a) calcimycin; b) 4-bromo-calcimycin; c) N-methyl-calcimycin.

If we consider the following successive complex formation equilibrium, thermodynamic cooperativity, as defined by Perlmutter-Hayman [36], should be regarded as positive when $K_{A_2M}/K_{AM^+} > 0.25$, negative when $K_{A_2M}/K_{AM^+} < 0.25$, and statistical when $K_{A_2M}/K_{AM^+} = 0.25$.



In our case, the ratios of K_{A_2Ca}/K_{ACa^+} are equal to 20, 10 and 0.3 and those of K_{A_2Mg}/K_{AMg^+} are calculated to be 25; 10 and 1.3 for calcimycin, 4-Br and N-methyl-calcimycin respectively. These values are higher than 0.25 for the three studied ionophores and illustrate a thermodynamic positive cooperativity formation of the neutral species either for the calcium or for the magnesium complexes. This indicates that the driving force for the formation of the A_2M complex is maintained for the three ligands. Hence, structural studies done on calcimycin have indicated that two interligand intramolecular hydrogen bonds contributed to enhanced stability of the A_2Ca complex.[34, 35] We suppose that this interligand hydrogen bond could be maintained in the 4-Br- and N-methyl-calcimycin complexes and then explain the thermodynamic positive cooperativity of the neutral complex formed from the charged one. The decrease in the volume of the substituents from calcimycin to 4-Br and N-methyl-calcimycin induced a reduction of the steric hindrance between the two ligands, favoured the interligand hydrogen bond and lead to a strong enhancement of the positive cooperative effect in the formation of the neutral complexes between calcimycin and the 4-Br and N-methyl-calcimycin.

Formation and Dissociation Kinetics

The reactions of alkaline earth cations with complexing ligands are generally quite rapid and require the use of rapid mixing techniques. The following overall reaction for the formation of the neutral calcium and magnesium complexes with N-methyl-calcimycin was studied by a stopped flow device.



To reduce the reaction rate, the initial concentration ratio of the reactants was chosen according to the stoichiometry of the reaction (2 ligands / 1 cation). If the reaction is first order with respect to the ionophore and the metal, the spectrophotometric detection of the reaction kinetics yields the following equation:

$$\frac{A - A_0}{A_\infty - A} = 2 \times [A^-]_0 \times k \times t \quad [4]$$

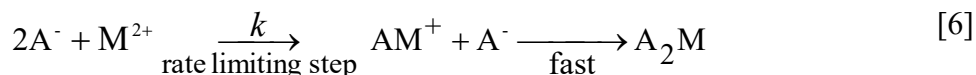
With: k second order rate constant ($/\text{mol dm}^{-3} \text{ s}^{-1}$); A_0 , A and A_∞ , the initial, instantaneous and final optical density respectively and $[A^-]_0$: the total initial concentration of ionophore.

We observed for calcium and magnesium N-methyl-calcimycin complexes a single rate limiting step with no more loss of spectrophotometric amplitude than that expected from the dead time of the stopped-flow technique used. The kinetic data fitted equation [3] with an excellent statistical confidence. These results agree with the following rate law:

$$v = k \times [A^-] \times [M^{2+}] \quad [5]$$

with $[A^-]$ and $[M^{2+}]$ instantaneous concentration of total ionophore and cation.

Our experimental observations, which agree with our previous results obtained for calcimycin suggest that the rate limiting step of the overall formation of the neutral A_2M N-methyl-calcimycin complexes is the formation of the charged ligand:



The variation of the second order rate constants with proton concentrations is presented in for N-methyl-calcimycin magnesium complex. Because more experimental kinetic data are available for the magnesium species than for the calcium complex, due to slower formation kinetics of the magnesium complexes, we have chosen to show the variations of the rate constants k with proton concentrations for the magnesium complex(Figure 5).

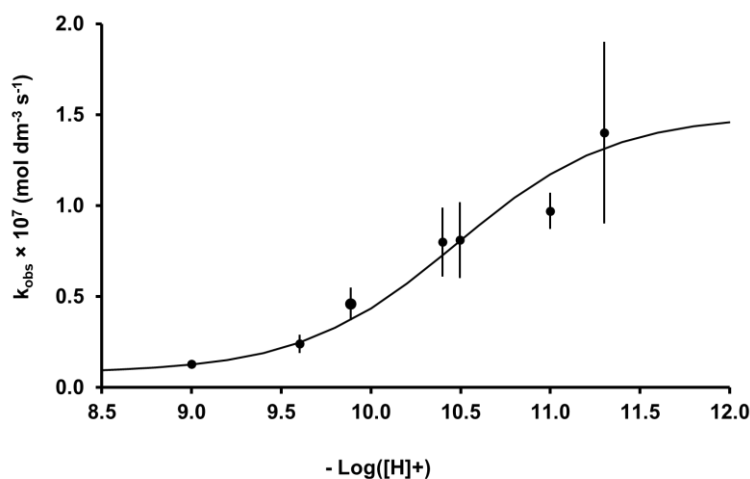


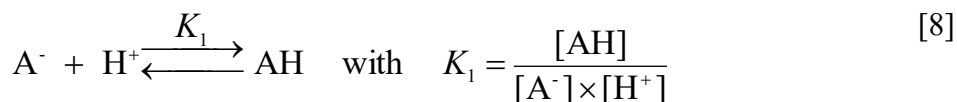
Figure 5 : Experimental first order rate constants for the decomplexation of N-methyl-AMg⁺ as a function of p[H]. Solvent : methanol; $T = (25.0 \pm 0.1) ^\circ\text{C}$; $I = 0.1$.

The sigmoidal shape of the curve $k = f(-\text{Log} [H^+])$ suggests two competing reactions via the deprotonated species A^- and via the monoprotonated AH of the free ionophore. The following mathematical expression [7] was fitted by a statistical non-linear square's method for the different values of k measured at different concentrations of protons:

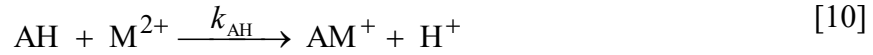
$$k = \frac{a + b[H^+]}{1 + c[H^+]} \quad [7]$$

If we write the chemical equations involved in the proposed mechanism, we have to consider:

- the acid-base equilibrium of the free N_methyl-calcimycin:



- and the two rate-limiting steps of complexation:



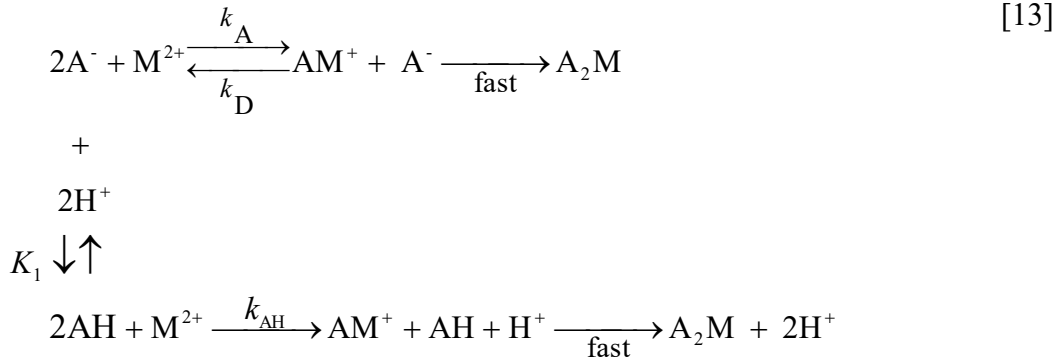
The corresponding rate law can be written:

$$v = k \times [A^-]_{\text{tot}} \times [M^{2+}] = k_{AH} \times [AH] \times [M^{2+}] + k_A \times [A^-] \times [M^{2+}] \quad [11]$$

The expression of the second order rate constant k with $[H^+]$ is:

$$k = \frac{k_A + k_{AH} \times K_1 \times [H^+]}{1 + K_1 \times [H^+]} \quad [12]$$

The overall mechanism is presented here:



The thermodynamic K_1 value related to the protonation of the free antibiotic cezomycin and the kinetic parameters k_A and k_{AH} related to the formation of the charged complex AMg^+ , respectively via the deprotonated species of N-methyl-calcimycin A^- and the monoprotinated AH , were determined from the kinetic measurements. They are for $k_A = (1.5 \pm 0.1) \times 10^7 / \text{mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$; $k_{AH} = (8 \pm 2) \times 10^5 / \text{mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$.

The stability of the A_2M complex is so low that this complex is never the predominant species in solution. For this reason, we did not attempt to study dissociation kinetics of the dimeric neutral complex. We studied the decomplexation of the monomeric complexes by hydrogen ions. This is a fast reaction which required the use of rapid mixing techniques and was studied by a stopped flow device to follow the reaction:



However, the dissociation of ACa^+ was too fast to be studied by this method. The decomplexation reaction of the charged N-methyl-calcimycin magnesium AMg^+ complex under acidic conditions followed first order kinetics with respect to the complex, the hydrogen ion concentration being kept constant during the reaction either by using a sufficient excess or by buffering the reaction medium. The observed rate constants are independent of hydrogen-ion concentrations over a large range, from $10^{-2} / \text{mol dm}^{-3}$ to $10^{-6} / \text{mol dm}^{-3}$. The observed rate law is then:

$$v = k_{\text{obs}} \times [\text{AM}^+] \text{ with } k_{\text{obs}}: \text{observed first order rate constant (s}^{-1}\text{)} \quad [15]$$

The mechanism of decomplexation in this case is simple, a direct dissociation of the complex caused by the change of $[\text{H}^+]$. The average value of k_{obs} gives $k_{\text{D}} = 64 \pm 4$ ($/\text{s}^{-1}$). The direct decomplexation pathway (k_{D}) occurs in the absence of protons and is related to the thermodynamic stability of the complexes under our experimental conditions and the rate law becomes:



The striking feature here compared with the calcimycin is the hydrogen ion independence of the decomplexation rate. The natural ionophore has been shown to have an important hydrogen-ion assisted pathway. The absence of the secondary amine hydrogen, which is now replaced by a methyl group has then a profound effect on the behaviour of A 23187. The direct dissociation of N-methyl- Mg^+ has a rate constant $k_{\text{D}} = 64 \pm 4$ (s^{-1}) as compared to the $k_{\text{D}} = 0.3$ (s^{-1}) for the natural ionophore. The monomeric magnesium complex of N-methyl-calcimycin dissociates more readily than the natural ionophore AMg^+ complex. As in all the previous studies a marked difference in the kinetics of calcium and the magnesium complexes was observed. The magnesium complex was much slower than the calcium complex, which could not be followed with the stopped-flow techniques.

The sum of our experiments gives us a clear picture of the complexation kinetics of N-methyl-calcimycin with calcium and magnesium, in methanol. The overall rates of complex formation and dissociation are governed by the monomeric charged complex.

In conclusion the 4-bromo-calcimycin shows any thermodynamic calcium selectivity and its magnesium and calcium complexes are much less stable than those of their corresponding natural calcimycin complexes in methanol. Our results prove one more that the hydrogen bond between the carboxylic acid function and its substituent in α position is very important to form very stable complexes in the calcimycin family. The transport properties observed for 4-Br-A 2138 [37] has to be related to kinetics parameters and are not due thermodynamic properties. Previous studies have also pointed out the effects of $\text{p}[\text{H}^+]$ conditions on cation transport by ionophores A 23187 and ionomycin in biological systems. [38]

The stability constant can be written in terms of kinetic parameters as:

$$K = \frac{k_{\text{f}}}{k_{\text{D}}} \quad [17]$$

where k_{f} is the formation rate constant with the anionic form of the ionophore.

The value of k_f is the corresponding value for the natural ionophore was previously determined as $4.6 \times 10^6 / \text{mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$. As these values of k_f are similar the loss of complex stability for N-methyl-AMg⁺ is due principally to the increased rate of direct decomplexation of the modified ionophore.

Conclusion

Our kinetic studies of calcium and magnesium complexes have permitted elucidation of the rate complexation process in homogeneous medium. These steps are associated with the charged AM⁺ complex. Formation studies have illustrated that the anionic A⁻ species reacts with magnesium and calcium which correlate well with the rapid exchange of inner sphere solvent molecules for these cations. Evidence was also found for the formation of AM⁺ from the protonated species. This formation pathway was times slower for AMg⁺ and 200 slower for ACa⁺ with respect to the anionic formation pathway. In all cases our results in methanol indicated that the kinetic behaviour of calcium complexes is faster than that of magnesium. Thus kinetically, there is a distinction between these alkaline earths. The decomplexation of the monomeric N-methyl-A 23187 magnesium and calcium complexes is much more rapid than for the natural ionophores complexes. The decomplexation was independent of hydrogen ion. Again, as at equilibrium the kinetics of the slightly modified N-methyl-A 23187 point out the importance of the secondary aromatic amine hydrogen to the behaviour of A 23187.

The formation rate constants measured depends on hydrogen ion concentration as formation take place with A⁻ or AH as the reactive species. Thus, formation of the carrier species will depend on the interfacial hydrogen ion concentration and the antibiotic used.

The increased rate of decomplexation should be a contributing factor to the unloading rate.

Our work indicates that formation of highly stable complexes depends on the planarity of the moiety. Kinetically absence of this increases the dissociation rate of AM⁺ and makes the rate independent of H⁺.

Cooperative formation of the dimeric species with respect to the monocharged complex for all studied antibiotics. However, the species present under physiological conditions is the AM⁺ cation. The substituent play an important role for the acid base properties of the ionophore. Decreasing the protonation of the carboxylic function will have various consequences. The complexes could be formed at slower p[H] and could explain why the efficacy of the ionophores differs with acid-base conditions.

Experimental

Materials

4-bromo-calcimycin and N-methyl-calcimycin were synthesized according to previously published procedures.[8,12] Anhydrous methanol, Merck, Waterfree, containing less than 0.01 % of water was chosen as solvent. Our experiments were always carried out at $25.0 \pm 1^\circ\text{C}$ and at constant ionic

strength 0.1 using tetrabutylammoniumtrifluoromethane sulfonate (TBATFMS, Fluka, purum). We used perchloric acid (Merck, for analysis 70 %) and tetrabutylammonium hydroxide (Merck, 25 % in methanol). Calcium cation was introduced as perchlorate ($\text{Ca} , \text{ClO}_4, 2, 4\text{H}_2\text{O}$, Fluka, purum) which was previously dried in a vacuum oven at 60°C , and commercial magnesium trifluoromethane sulfonate ($\text{Mg}(\text{CF}_3\text{SO}_3)_2$, Fluka, purum) was used for magnesium cation. The solutions were continuously swept with solvent saturated argon to exclude atmospheric carbon dioxide without evaporation of the solvent. Hydrogen-ion concentrations were measured with a combined glass electrode (Ingold, High Alkalinity). The Ag/AgCl reference electrode was filled with $0.05 \text{ /mol dm}^{-3}$ tetramethylammonium chloride (Fluka, p.a.) in pure methanol. Potential differences were given by a Tacussel Isis 20,000 millivoltmeter. Solutions with known hydrogen-ion concentrations in methanol [39-42] were used to verify the linearity of the glass electrode.

Spectrophotometric Measurements

The method employed in our study is based on the classical potentiometric method of hydrogen ion competition to determine stability constants [4,10]. Experiments were started with the antibiotic , respectively $10^{-4} \text{ /mol dm}^{-3}$ and $2 \times 10^{-4} \text{ /mol dm}^{-3}$ for for bromo and N-methyl- calcimycin, in the presence or absence of an alkaline earth cation ($[\text{M}^{2+}]_{\text{tot}}/[\text{A}]_{\text{tot}} = 1, 30, 80 \text{ or } 300$) in an acid medium ($10^{-3} \text{ /mol dm}^{-3} < [\text{HClO}_4]_0 < 10^{-1} \text{ /mol dm}^{-3}$). 40 ml of solution, placed in a jacketed cell, were titrated with tetrabutylammonium methoxide (Merck, 25 % in methanol) over the $\text{p}[\text{H}^+]$ range 1 to 12. These solutions were purged for 5 to 10 minutes beforehand with solvent saturated argon. A sample of 0.5 ml was then taken using a precision piston micropipette (Micropettor) and absorption spectra , 250-450 nm, were recorded on an Uvikon 860 (Kontron) spectrophotometer equipped with 0.2 cm quartz cells (Hellma). A typical set of spectra is given in Figure 6.

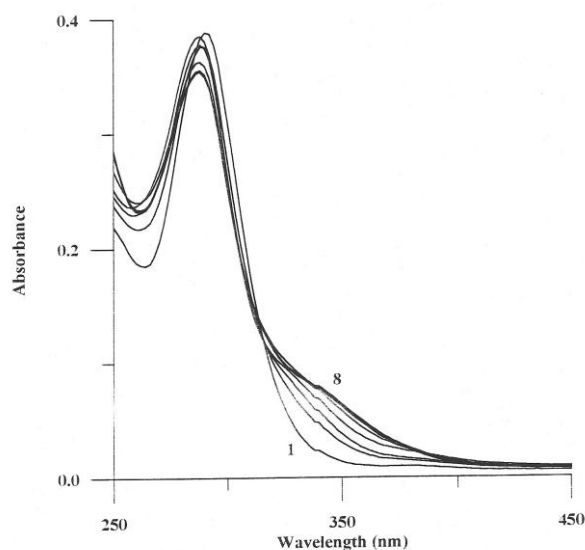


Figure 6: Evolution of absorption spectra of magnesium 4-bromo-calcimycin complexes at different concentrations of protons.

$[4\text{-bromo-calcimycin}]_0 = 10^{-4} \text{ /mol dm}^{-3}$; $[\text{Mg}^{2+}]_0 = 8 \times 10^{-4} \text{ /mol dm}^{-3}$; $[\text{HClO}_4]_0 = 8 \times 10^{-2} \text{ /mol dm}^{-3}$; Base: [tetrabutylammonium methoxide] = $0.64 \text{ /mol dm}^{-3}$. Spectra 1-8: $\text{p}[\text{H}^+] = 2.06, 3.06, 3.63, 4.57, 7.16, 8.33, 9.33, 10.01$ respectively.

The spectrophotometric data were analyzed with the Letagrop-Spefo[28-32] program which adjust the absorbivities and the stability constants of the species formed at equilibrium. It uses the Newton-Raphson algorithm to solve mass balance equations and a pit-mapping method to minimize the errors and determine the best parameter values.

Kinetic Measurements

Complex formation and dissociation with N-methyl-calcimycin are fast reactions and were studied by a stopped flow method (Durrum Gibson spectrophotometer). The spectrophotometric data were stored using a Datalab DL 905 transient recorder, and an on-line Apple II microcomputer was programmed to treat first order or pseudo-first order kinetics[43]. From our thermodynamic studies and with the help of the Haltafal program[44] it was possible to calculate experimental conditions providing complete formation and dissociation reactions for the kinetic measurements.

We studied the dissociation kinetics of the the AM^+ complex of N-methyl-calcimycin with calcium and magnesium. These complexes were totally formed with respectively a calcium or magnesium and N-methyl-calcimycin concentration of $10^{-3} \text{ /mol dm}^{-3}$ and $2 \times 10^{-5} \text{ /mol dm}^{-3}$ in basic conditions (hydrogen ion concentration less than $10^{-9} \text{ /mol dm}^{-3}$). Complete dissociation of the complexes was observed for hydrogen ion concentrations higher than $10^{-4} \text{ /mol dm}^{-3}$ and perchloric acid solutions were used. The values of the pseudo-first order rate constants k_{obs} determined in these experimental conditions are presented in

We have studied the formation kinetics of the neutral A_2M magnesium and calcium N-methyl-calcimycin complexes at various proton concentrations. Because the reactions are too fast for the stopped-flow technique, we were unable to adopt pseudo-first order conditions. A 2:1, ionophore:cation, concentration ratio was chosen with $[\text{N-methyl-calcimycin}]_0 = 8 \times 10^{-5} \text{ /mol dm}^{-3}$. The range of hydrogen ion concentrations studied was from 10^{-12} to $10^{-9} \text{ /mol dm}^{-3}$; a tris buffer was used in these conditions, $\text{pK} = 10.05 \pm 0.06$, [4].

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