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Stability Constants for, and Structural Investigation of, Divalent Metal Ion Complexes with Polyene Antibiotics

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Abstract

The stability constants for the formation of complexes between Ca(II), Mg(II), Cu(II), Zn(II) and Ni(II) with nystatin and amphotericin-B (polyene antibiotics) have been determined by both a potentiometric and a solubility method. The structures of the complexes have been investigated by NMR, ESR and CD spectroscopy. The transition metal stability constants are consistent with the Irving-Williams series. The structural results are discussed and related to the importance of such complexes in mode of action theories.

Introduction

In a previous paper we reported [1] the preparation and partial characterisation of complexes of the polyene antibiotics nystatin and amphotericin B with Ca(II), Mg(II), Cu(II), Ni(II) and Zn(II).

Stable solid complexes were formed with stoichiometry of 1:1 and/or 2:1 [1]. The interest in such complexes arises from their potential involvement in the mode-of-action of the polyenes, positive improvements in clinical efficacy through an increase in solubility and/or biological potency.

Following preparation of such complexes we now report an investigation of metal ion complexation by polyenes in solution.

Structural aspects in solution have been studied by ESR, CD and NMR spectroscopy. Thermodynamic aspects of complexation (determination of stability constants) have been investigated by potentiometry [2, 3, 4] and by a solubility method [2, 3, 4].

Experimental

Potentiometric Titrations

HCl (0.1 M) and NaOH (0.1 M) were of BDH AnalaR grade. Potassium hydrogenphthalate (0.1 M) was used to standardise the NaOH solution.

A glass electrode was soaked in 98% DMF/H_2O for 48 h and this 'pickled' electrode was used to measure the 'pH' of DMF/H_2O solutions with an E.I.L. 7050 pH meter. The pH meter was standardised, before each titration, with pH 7 buffer in water by a glass electrode. The response of the 'pickled' electrode to hydrogen ion activities was found to be reproducible and directly proportional to hydrogen ion activities.

The ionization constants of the polyenes were determined by the following procedure.

About 0.2 g of the polyene was accurately, weighed and dissolved in 10 ml of DMF. The resulting solution was made up to 50 ml with DMF. About 31 g of sodium perchlorate ($NaClO_4$), *i.e.* approximately 3 mol l^{-1}, were dissolved in 50 ml of deionised water. The two solutions were thoroughly mixed and 5 ml of standardised HCl (~0.1 M) was added. The final solution (105 ml) was stirred and placed into a water bath at 298 K. Nitrogen was slowly bubbled through the solution for 10 min and, during titration with standard NaOH. The pH was recorded for every 0.1 ml NaOH added.

The stability constants of the metal–polyene antibiotic complexes were measured as follows. About 0.3 g of nystatin was weighed accurately and dissolved in 10 ml of DMF. The resulting solution was made up to 50 ml with DMF. The appropriate metal salt was made up in 50 ml H_2O such that the molar ratio of metal:nystatin was 2:1, 31.35 g of $NaClO_4$ was added to this aqueous metal salt solution. The two solutions were mixed and 5 ml of 0.1 M HCl added. This solution (105 ml) was stirred well, placed in a waterbath at 298 K and, as before, titrated with ~0.1 M of NaOH solution. The pH after each 0.1 ml of NaOH addition was recorded.

Equilibrium Constant Calculations

All titrations were carried out in 3 M $NaClO_4$, reported equilibrium constants are 'Bronsted' constants in terms of activity of the $[H^+]$ ion and the concentration of other reactants.

The ionisation constants (pK_{a1} and pK_{a2}) of nystatin and amphotericin B were calculated from

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the acid-base titration results by the method of Rossotti [2, 6]. Stability constants for the metal complexes were calculated by the method originally due to Irving [6]. Linear plots of

$$\frac{1-\bar{n}}{2-\bar{n}[L]} \text{ against } \frac{\bar{n}}{(2-\bar{n})[L]^2}$$

where L = polyene, where constructed from which K_1 and K_2 were calculated by linear least-squares procedures.

Solubility Method

A small amount of nystatin (0.01 to 0.05 g) was dissolved in 1 ml of DMF in a polyethylene bottle. 3 mol l^{-1} $NaClO_4$ was dissolved in 99 ml deionised water and this was added to the DMF solution. About 0.2 to 0.5 g of metal–polyene antibiotic* complexes was added to the solution and the solution was shaken for about 7 h on a thermostatted waterbath (298 K). The undissolved metal salt was filtered off, the pH of the solution was recorded, the metal ion concentration of the solution was measured by EDTA titration and for Cu(II) by atomic absorption spectrophotometry. Equilibrium constants may then be calculated for complexation as described by Rossotti [2], details may be found in reference [5].

Electron Spin Resonance Spectroscopy

Instrumentation and methods were essentially as described in the preceding paper [1]. The low temperature spectra were interpreted as follows.

All low temperature spectra were axial or nearly so (Fig. 1). The parallel components of the spin-Hamiltonian parameters, $g_{/}$ ($=g_z$) and $A_{/}$ can be determined directly from the low temperature spectra with reasonable accuracy. Exact analysis of the high field portion of the spectrum is difficult especially if $g_x \neq g_y$ and $A_x \neq A_y$. When $g_x = g_y$ a reasonable estimate of $g_\perp$ is often given by the low field maximum of the spectrum, this is the value reported in this work.

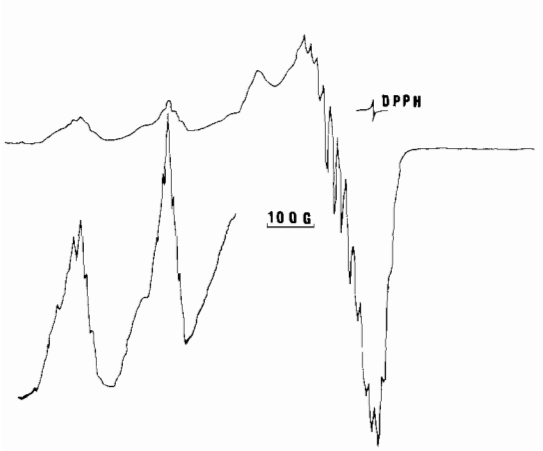


Fig. 1. ESR spectrum of Cu–amphotericin B complex.

*Prepared by the method described in the preceeding paper.

Nuclear Magnetic Resonance

Proton NMR spectra were recorded using a Perkin-Elmer R32, 90 MHz instrument.

0.02 g nystatin was used for each NMR study. Zinc and praseodymium nitrates were dried over silica gel before use. Nystatin was dissolved in 1 ml of deuterated DMF, TMS was used as an external standard in NMR experiments.

Zn(II) nitrate or praseodymium(III) nitrate was added in stoichiometric amounts to the polyene solution and the NMR spectra recorded. The stoichiometric ratio studied for the Zn(II):polyene antibiotic was up to 9:1 and for Pr(III):polyene was up to 2:1.

Circular Dichroism

Circular dichroism was measured between 900 and 450 nm at room temperature, with a JASCO J40 instrument.

A DMF solution of nystatin was mixed with aqueous solutions of Cu(II) to give a final 50% DMF/H_2O solution. The final concentration of nystatin was 1×10^{-2} M and of Cu(II) was 5×10^{-3} M. The mole ratio of Cu(II) concentration to nystatin concentration was therefore 1:2. Diluted HCl and NaOH was added dropwise to obtain different pH. The CD measurement was obtained for the 1:2 complex over a range of pH. The pH was measured with the 'pickled' electrode at room temperature.

Results and Discussion

The ionization constants of polyenes have been reported previously and this data, together with our results, are reported in Table I.

The results of our determinations of formation constant for metal-ion polyene complexes are summarized in Table II. Considering the quite different solvent systems used for the potentiometric (50% DMF/H_2O) and solubility methods (1% DMF/H_2O), there is reasonably good agreement between these measurements.

The variation in the magnitude of the equilibrium constants is in the expected, Irving-Williams order, Ni(II) < Cu(II) > Zn(II), for the transition metals. As would also be predicted, all these complexes are much more stable than those of either calcium or magnesium. Comparison of the equilibrium constants with literature values [7] is difficult, due to the lack of other data for non-aqueous systems of the kind here studied. The substantial values measured for the equilibrium constants in DMF/H_2O mixtures do strongly suggest that metal-ion co-ordination by

TABLE I. pK_a Values for Nystatin and Amphotericin B.

	Concentration	Mean pK_1	Mean pK_2	
Nystatin	*ca.* 1×10^{-3}	4.117(±0.286)	8.222(±0.399)	this work
	ca. 2×10^{-3}	4.122(±0.235)	8.200(±0.346)	
	ca. 3×10^{-3}	4.195(±0.267)	8.238(±0.383)	
	Av.	4.14	8.22	
Amphotericin B	*ca.* 1×10^{-3}	3.712(±0.123)	8.658(±0.519)	this work
	ca. 2×10^{-3}	3.679(±0.258)	8.382(±0.547)	
	Av.	3.70	8.52	

pK_a values reported for nystatin in methanol/H_2O are pK_1, 2.64; pK_2, 9.73 and for amphotericin B, pK_1, 2.98; pK_2, 9.57.

TABLE II. Summary of log K_1[a], log K_2 and log β_2 of the Metal–polyene Antibiotics Determined by pH Titration and Solubility Method[b].

Metal complex	Method	log K_1 (298, I ~ 3)$^+$	log β_2
Copper–nystatin	pH titration	5.39	10.24
	solubility	5.88	11.04
Copper–amphotericin B	pH titration	5.83	11.34
	solubility	6.09	11.95
Zinc–nystatin	pH titration	3.64	7.07
	solubility	4.48	7.70
Zinc–amphotericin B	pH titration	4.78	8.72
	solubility	5.17	9.63
Nickel–nystatin	pH titration	5.33	8.51
	solubility	4.41	8.43
Nickel–amphotericin B	pH titration	4.90	9.05
	solubility	4.33	9.14
Magnesium–nystatin	solubility	2.87	4.45
Calcium–nystatin	solubility	3.54	6.93

[a]No standard deviation exceeded 5% of log K_1. [b]The solution used for pH-titration was 50% DMF/H_2O. The solution used in solubility measurements was 1% DMF/H_2O.

polyenes should not be ignored when their mode-of-action is discussed. The results have been modelled (see experimental section for details) in terms of two macroscopic metal–ligand binding constants. These give an accurate idea of the overall stability of the complexes. The problems inherent in the study of this system preclude the kind of detailed analysis of potentiometric data now possible by computational methods. However, the values are consistent with the kind of co-ordination indicated by our spectroscopic studies; notably N_2O_2 binding of copper(II) is suggested by the ESR results.

Solutions containing amphotericin B and Cu(II) gave rise to two characteristic ESR spectra. An approximately 1:1 mixture of amphotericin B and Cu(II) (in 50% DMF/ethanol) clearly shows the presence of two species (Fig. 1). Species (**I**) accounting for ~25% of the Cu(II) is characterised by $g_{//} = 2.260$, $A_{//} = 190 \times 10^{-4}$ cm^{-1}. No evidence for nitrogen hyperfine coupling could be observed for this complex. Species (**II**) is characterised by $g_{//} = 2.210$ and $A_{//} = 202 \times 10^{-4}$ cm^{-1}. The low field component of $g_{//}$ for this complex clearly show nitrogen hyperfine coupling, there are five lines in approximately the 1:2:3:2:1 ratio expected for two equivalent nitrogens (I = 1) bound to a single copper [8].

Solutions containing only an individual specie could be prepared in the following way, a 10:1 amphotericin B to Cu(II) solution showed only the presence of species (**I**), on neutralisation (with a quantity of NaOH equivalent to the concentration of amphotericin B) only species (**II**) was observed. ESR parameters for both species from various spectra are summarised in Table III.

The ESR spectra of Cu(II)–nystatin complexes show only one species, $g_{//} = 2.245$ and $A_{//} = 178 \times 10^{-4}$ cm^{-1}. No hyperfine coupling was observed.

TABLE III. ESR Parameters[a] for Cu(II)/Polyene Complexes.

		$g_{\parallel}$	$A_{\parallel} \times 10^4$ cm^{-1}	g	
Cu:Nystatin	1:10	2.245	178	2.057	
	1:10	2.245	189	–	+NaOH
	1:10	2.242	178	2.066	
	1:10	2.266	180	2.056	50% DMF/ETOH
	1:10	2.243	178	2.058	neutralised
Cu:Amphotericin B	1:1	2.210	202	–	main species ~75%
	1:1	2.260	190	–	minor species
	1:10	2.266	193	~2.06	–
	1:10	2.210	202	~2.07	+NaOH

[a]All results for frozen glasses 50% DMF/EtOH 77 K.

In the amphotericin B system the presence of nitrogen hyperfine coupling in the spectrum of species (II) shows it to be a bischelated approximately square coplanar complex of amphotericin B and Cu(II). ESR parameters ($A_{\parallel}$ and $g_{\parallel}$) are typical of CuN_2O_2 chromophore [9, 10, 11]. The groups involved are very similar to the well known ethanolamine complexes of Cu(II) [12, 13], the pK_a of the co-ordinated hydroxyl in such complexes have been the subject of some controversy [12, 13].

Species (I) is more difficult to assign, values of $g_{\parallel}$ and $A_{\parallel}$ are into the region expected for a O_4 chromophore [9] and nitrogen hyperfine coupling could not be observed. Further, this appears to be the dominant form in slightly acidic solutions. This complex is suggested to be a bischelated specie formed through the carboxylate and neighbouring hydroxyl grouping.

The Cu(II)–nystatin complex gave values of $g_{\parallel}$ and $A_{\parallel}$ close to the N_2O_2 chromophore [9] although no nitrogen hyperfine coupling was observed. It is suggested that the Cu(II)–nystatin is a bischelated square planar complex formed through the amino nitrogen and the neighbouring hydroxyl grouping. The greater chemical purity of amphotericin B may well account for the better resolution of the ESR spectra of its complexes as compared to those of nystatin. Nystatin is well known to be heterogeneous [14].

The CD spectra obtained from pH titrations of the Cu(II)–(nystatin)$_2$ complex fall into three distinct groups. In acid pH, the absorption maximum at 660 nm shows a negative Cotton effect. Both absorption maxima in neutral pH show positive signs and in alkaline solution, the spectrum gives one negative and one positive absorption maximum, typical spectra are illustrated in Fig. 2. The results are similar to those previously reported for copper(II)/nystatin solutions [15].

Some important assignments for the NMR spectrum of nystatin are tabulated in Table IV. The NMR spectrum of the nystatin gives four doublets in the

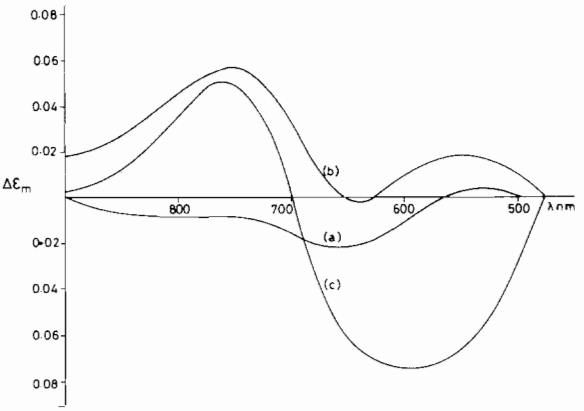
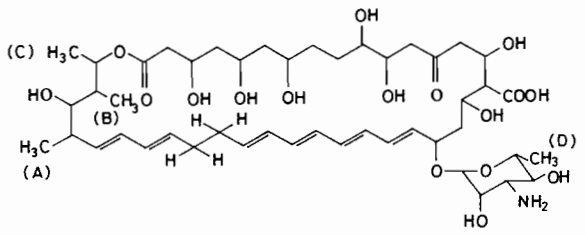


Fig. 2. CD spectra of Cu(II)–(nystatin)$_2$ in 50% DMF/H_2O at pH (a) 4.76; (b) 6.99; (c) 9.16.

TABLE IV. Some Values Derived from the NMR Spectrum of Nystatin.

(ppm)	Assignment	
1.14	$CH_3-CH(COH)(C=C)$	$(A)^+$
1.12	$CH_3-CH(C-OR)(COH)$	(B)
1.29	$CH_3-CH(OR)(CR)$	(C)
1.36	$CH_3-CH(OR)(COH)$	(D)
1.75	$RCH_2-C=C$ RCH_2-COH RNH_2	
2.35	$RCH_2C=O$	
2.59	$R'R''CHCOOH$	
3.50	$RCHNH_2$	
6.15 6.45	$H-C=C-C=C$	

region $\delta = 1$ ppm to 1.5 ppm. These doublets represent the 4–CH_3 groups of the nystatin molecule. These are denoted by **(A)**, **(B)**, **(C)** and **(D)** in the structure below.



Nystatin Molecule

Each doublet represents each $-CH_3$ group with different α and β substituents. The $-CH_3$ group in the mycosamine ring is denoted by **(D)** and is at $\delta = 1.36$ ppm. This $-CH_3$ **(D)** doublet is useful in evaluating the lanthanide induced shift on the addition of praseodymium.

The NMR spectra as praseodymium was titrated into nystatin shows that the $-CH_3$ doublet **(D)** shifts upfield. The lanthanide induced shift (LIS) was plotted against the [Pr]/[nystatin] ratio (Fig. 3). The results suggest that one molecule of lanthanide shift reagent (LSR) forms a stable complex with two nystatin molecules. At low concentration of LSR, a linear concentration dependence of LIS is observed. As the concentration of LSR is increased, the deviation from linearity also increases, this maybe due to the limited solubility of the LSR.

The $-CHNH_2$ group can be assigned at $\delta = 3.50$ which is merged into the –OH resonance peak in the spectrum of nystatin. The spectrum of 9:1 Zn(II)–nystatin complex indicates a triplet at $\delta =$ 3.50 ppm which can be distinguished from the other peaks. This peak is also clearly seen from the spectrum of the 1:1 Pr(III)–nystatin complex but in that of the 2:1 complex, this peak was merged with another –OH resonance at $\delta = 2.90$. A shoulder at $\delta = 2.00$ ppm is assigned to the RNH_2 group which is shielded from the electrons of the metal ion, and

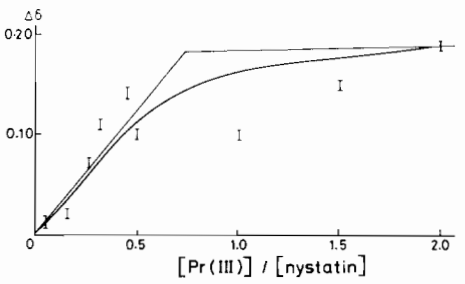


Fig. 3. Plot of lanthanide induced shift *vs.* [Pr]/[nystatin] ratio.

is only present in the spectra of the metal–nystatin complexes. It is therefore concluded that both Zn(II) and Pr(III) co-ordinate to the hydroxy amino group of nystatin.

The –CHCOOH group was assigned at $\delta = 2.59$ ppm. It was found that the doublet was moved upfield when the concentration of praseodymium was twice the concentration of nystatin. This group shifted upfield and was at higher field than the $-CH_2C{=}O$ peak, which in turn was at higher field than the –CHCOOH peak, which in turn was at higher field than the –CHCOOH peak in the nystatin spectrum. This behaviour suggested that the Pr(III) reacts with the carboxylic group if the amino group is occupied.

The existence of complexes of polyene antibiotics with metal ions demonstrated in the previous paper and here described further by determination of their stability constants and by solution phase studies indicates the need to consider the role of such complexes in mode of action theories. However the solvent medium used in these studies does not mimic the lipoid matrix within which some such complexation may take place in biological cells. The complexes, with their small increase in solubility in water over nystatin itself may allow improved levels of bioactivity to be attained in biological fluids.

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