The Complexation of Transition Series Metal Ions by Nalidixic Acid

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The following formation constants have been determined for nalidixic acid: proton, copper(II) complexation, magnesium(II) complexation, guanosine-5'-monophosphate–copper(II) complexation. Use of these data (together with the corresponding published constants for calcium(II), iron(II), manganese(II) and zinc(II)) supports the hypothesis that the drug acts at a site other than extracellular. Complex formation between nalidixic acid, metal ion and DNA (at guanosine residues) is suggested.

Introduction

Transition metals are present in extremely low concentrations *in vivo* and their ligand environment can be considerably altered when a therapeutically effective dose of a drug (*e.g.* an antibacterial agent) is administered. This change in the balance between the metal ion and the ligand may have a profound effect upon the activity of the drug against potentially susceptible bacteria. The correct balance may allow high bioavailability of the metal ion or ligand drug (*i.e.* engender antibacterial activity) whereas an incorrect balance may almost completely preclude the entry of a metal ion into the bacterial cell [1–5].

Antibacterial agents may exert their effects by diverse mechanisms [6]. For example, the activity of 8-hydroxyquinoline (oxine) against *Staph. aureus* (Gram-positive) and *M. tuberculosis* (acid-fast) requires the formation of a complex between the drug and either copper or iron [7, 8]. The metal ion in the complex must have some coordination positions which remain uncomplexed, since high ratios of oxine to metal result in diminished activity; a phenomenon called 'concentration quenching' by Albert [7]. The increased oil:water partition coefficient concomitant with complex formation, suggests that the site of action of the drug lies within the bacterial cell [7]; the requirement for the metal to possess surplus ligand binding sites and the observed enhancement of antibacterial activity in the presence of iron [9] indicate that the metal complex may interact specifically with a metal binding site within the cell.

Nalidixic acid I was first synthesized in 1962 [10] and is particularly active against Gram-negative bacteria *in vitro* and *in vivo* [11]. It has proved useful in the clinical management of urinary tract infections caused by *E. coli*, *Proteus* and *Klebsiella* species [12–14] and subsequently many analogues have been synthesized [15]: some of these (*e.g.* oxolinic acid II [16], pipemidic acid III [17], piromidic acid IV [18], cinoxacin V [19], tioxic acid VI [20], flumequine VII [21] and rosoxacin VIII [22]), have been clinically successful. During the last 20 years, the mechanism of action of such compounds has been extensively investigated and has recently been reviewed [23]. The bactericidal effect of nalidixic acid is diminished by increasing the concentration of the drug [24] and the characteristic biphasic dose response curve exhibited by nalidixic acid (and its congeners) bears a striking resemblance to that of oxine [25]. Moreover, it appears that the chelation of certain metal ions [26–31] between the carbonyl and carboxyl groups of these molecules and their binding to DNA [23, 32] (with some base specificity) may be essential prerequisites for their antibacterial activity.

However, it has been established [33] that there may be no direct correlation between the biological activity of a drug and the binding constant for the complex formed between the drug and a metal ion. The drug must be able to compete for trace metal ions in the presence of powerful endogenous ligands *in vivo* and there is an obvious need to determine which complexes between metal and drug may be formed under biological conditions. Since transition metals are present in low concentrations *in vivo*, conventional laboratory techniques are not sensitive enough to obtain the necessary data under biological conditions and computer simulation of the salient equilibria has become well established for this

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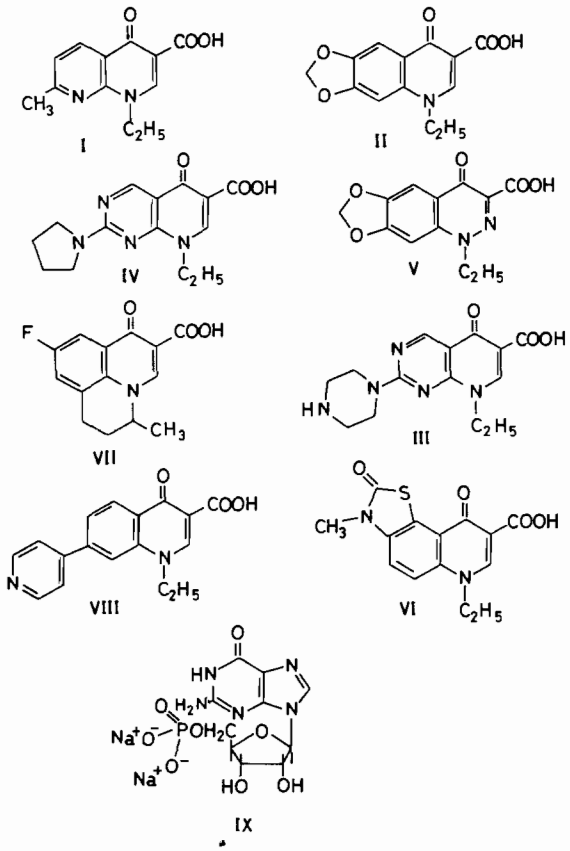

Fig. 1. Structures of NAL, some of its congeners and of guanosine-5′-monophosphate. Key: I = Nalidixic acid, II = Oxolinic acid, III = Pipemidic acid, IV = Piromidic acid, V = Cinoxacin, VI = Tioxic acid, VII = Flumequine, VIII = Rosoxacin, and IX = Guanosine-5′-monophosphate.

type of investigation. This requires a knowledge of the formation constants for complexes between the metal ion and appropriate endogenous ligands (*e.g.* histidine) present and the corresponding parameter for the metal and the drug. Consequently, we have investigated complex formation between nalidixic acid and certain metal ions and, since mixed ligand complexes occur commonly in biological systems, ternary complexation with histidine was also studied. Moreover, in view of the suggested mode of action of nalidixic acid (*vide supra*), ternary complex formation between guanosine-5′-monophosphate (GMP) IX, metal ion and nalidixic acid was of particular significance. The chemical structures of these ligands are shown in Fig. 1.

Experimental

Materials

Analytical grade chemicals were used throughout. Nalidixic acid: Found: C, 61.9; H, 5.1; N, 12.1%; calculated for $C_{12}H_{12}N_2O_3$: C, 62.1; H, 5.2; N, 12.1%

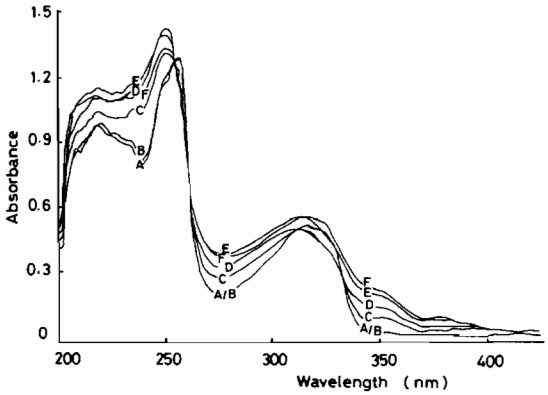

Fig. 2. Effect of iron(II) (7.5×10^{-5} mol dm^{-3}) on the ultraviolet absorption spectra of NAL (4.8×10^{-5} mol dm^{-3}) at pH = 2.4(B); 2.9(C); 3.2(D); 4.1(E) and 4.8(F); compared to nalidixic acid alone at pH = 2.4(A).

was supplied by Aldrich Ltd. Guanosine-5′-monophosphate was supplied by Sigma Ltd. Standard stock solutions of metal ions were prepared as their chloride salts except for ferrous ion which was prepared as its sulphate. The concentrations of metal ion and of mineral acid in these stock solutions were determined as described previously [1].

Methods

Ultraviolet absorption spectra were determined using an automatic spectrophotometer (Perkin-Elmer model 402). A typical series of spectra is shown in Fig. 2.

Computer methods were the same as those outlined previously [1]. Following our usual approach [1], potentiometric titrations were performed at 37 °C, I = 150 mmol dm^{-3} [NaCl] so that the formation constants obtained could be used directly in the computer simulation model of blood plasma. All solutions were prepared using distilled and degassed doubly deionised water and the dissociation constant of the medium under these conditions had been determined to be log $K_w = -13.31$. In the protonation studies, total ligand concentrations ranged between 5 and 15 mmol dm^{-3} while for binary and ternary metal ligand systems the absolute and relative concentrations were varied as much as possible within the limits of solubility. The concentration of GMP was kept below 15 mmol dm^{-3} in order to minimize 'stacking' of base molecules [34].

Results and Discussion

Formation Constants

Binary formation constant data for nalidixic acid (NAL) with copper(II) and magnesium(II) are reported in Table I. The insolubility of nalidixic acid (at $-\log[H^+] < 6.0$) and of its complexes with

TABLE I. Formation Constant Data for Nalidixic Acid at 37 °C. I = 150 mmol dm^{-3} [NaCl].
$\beta_{pp'qr} = [M_qL_pL'_{p'}H_r]/[M]^q[L]^p[L']^{p'}[H]^r$.

Stoichiometry				Log β values	Sum of squares in Residuals	MINIQUAD R Factor	No. of data points	No. of titrations
p	p'	q	r					
Protonation								
1	0	0	1	5.943(0.003)[a]	9.7×10^{-8}	0.006	180	7
Copper								
1	0	1	0	6.00 (0.008)[a]	3.8×10^{-8}	0.005	149	7
2	0	1	0	11.54 (0.06)[b]	1.0×10^{-7}	0.003	154	10
1	1	1	0	15.43 (0.04)[c]	4.3×10^{-7}	0.007	173	8
1	1	1	1	19.58 (0.09)[c]				
Magnesium(II)								
1	0	1	0	3.05 (0.02)[a]				
2	0	1	0	5.95 (0.03)[a]	6.7×10^{-8}	0.007	257	9
1	0	1	−1	−4.65 (0.03)[a]				

L' = Histidinate; L = Nalidixate.
[a]Binary data. [b]From mixed ligand studies with ethylenediamine. [c]From mixed ligand studies with L-histidinate.

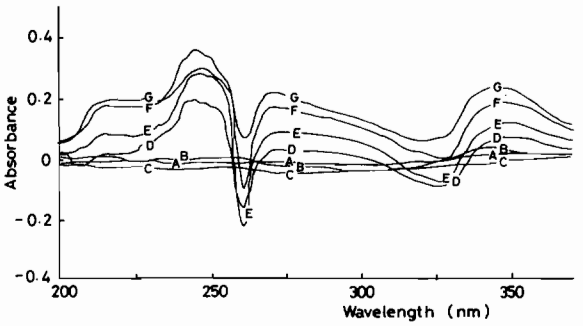

Fig. 3. Difference ultraviolet absorption spectra comparing the effect of iron(II) (7.5×10^{-5} mol dm^{-3}) *versus* reference solution of NAL (4.8×10^{-5} mol dm^{-3}) at pH = 2.4(A) and 4.9(B). Curves represent iron(II):NAL solutions at pH = 2.4(C); 2.9(D); 3.2(E); 4.1(F) and 4.8(G).

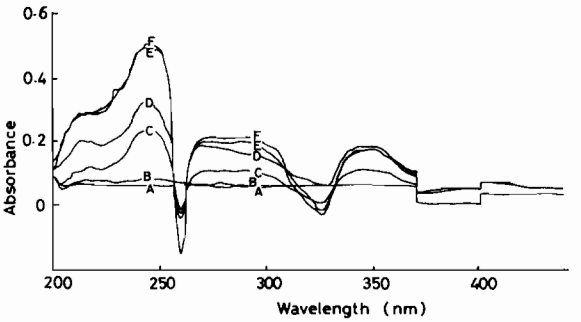

Fig. 4. Difference ultraviolet absorption spectra comparing the effects of iron(II) and iron(III) (total concentration of metal ions = 7.5×10^{-5} mol dm^{-3}) at comparable pH values *versus* reference solution of NAL (4.8×10^{-5} mol dm^{-3}) at pH = 2.4(A). Curves represent iron(II):NAL solutions at pH = 2.3(B), 2.6(C) and 3.8(D) and iron(III):NaL solutions at pH = 2.3(E) and 2.6(F).

metal ions complicated the potentiometric titrations but it was possible to determine that NAL has a single protonation constant (pK_a 5.94) in the pH range 2–9. This is in good agreement with values reported [27, 29–31] previously and corresponds to ionization of the carboxylic acid group at position 3. However, the value is somewhat higher than is normal for carboxylic acids and this may be attributed to intramolecular H-bonding between the hydroxyl function and the carbonyl group at C4 of the molecule. This phenomenon is clearly revealed by X-ray crystallographic data [35] and is reflected in the infrared spectrum of NAL which exhibits a broad band at *ca.* 2000–3000 cm^{-1} (due to hydroxyl stretching) and another at *ca.* 1600 cm^{-1} (corresponding to the carbonyl stretching of the vinylogous amide). The formation constants for complexes of NAL with copper(II) and magnesium(II) ions also agree well with those reported [30, 31] previously, particularly with regard to the different temperatures and ionic strengths at which the various measurements were performed.

It is apparent from Table I that magnesium(II) forms both hydrolysed and bis species with NAL in addition to the normal mono complex. Precipitation precluded investigation of species other than the mono complex of copper(II) and the drug and the infrared spectrum of the precipitate [36] showed absence of the H-bonding displayed by NAL itself. Formation of this mono complex produced changes in the ultraviolet absorption spectrum of NAL similar to those observed during protonation except that the absorption maximum at 320 nm was insensitive to changes in pH. These observations are consistent with involvement of the carboxyl

TABLE II. Ternary Formation Constant Data for Copper(II), Nalidixate and 5'-GMP at 37 °C. I = 150 mmol dm^{-3} [NaCl]. $\beta_{pp'qr} = [M_qL_pL'_{p'}H_r]/[M]^q[L]^p[L']^{p'}[H]^r$

p	p'	q	r	Log $\beta_{pp'qr}$ values	Sum of squares in Residuals	MINIQUAD R. Factor	No. of data points	No. of titrations
1	1	1	1	20.93(0.04)	2.5×10^{-6}	0.015	117	4

group in the complexation of the metal ion [see also 23, 29–31].

Histidine was used as a competing ligand [37] in an attempt to measure the formation constants for complexes of copper(II) and NAL other than the mono species. This was not successful but some evidence for the formation of ternary complexes between copper(II), NAL and histidine was obtained. Replacement of histidine with 1,2-diaminoethane (ethylenediamine) afforded a log β value of 11.54 for a bis copper(II) complex.

NAL forms powerful complexes with iron(II) and iron(III) [26–28, 31] and these ions have a pronounced effect upon the ultraviolet absorption spectrum of the drug (Figs. 2–4). Potentiometric titrations using these metal ions were not affected by precipitation (*cf.* those with copper(II) – *vide supra*) but the measurement of formation constants for complexes of drug and metal were restricted by the ability of iron(III) to chelate NAL at pH values below 1 and the apparently irreversible nature of the complexation between iron(II) and the drug. However, during the latter titrations a yellow colour developed and the solution did not become colourless again on subsequent acidification. Investigation of this phenomenon by ultraviolet absorption spectroscopy indicated that formation of the yellow colouration corresponded closely to that of the iron(III):NAL complex and suggested that the corresponding iron(II) complex had been oxidised by traces of oxygen present in the solutions as the titrations progressed. This possibility appears to have been recognized also by Vincent *et al.* [31] who took appropriate precautions when determining formation constants for iron(II)–NAL using fluorescence spectroscopy. Consequently, this value, together with that for zinc(II)–NAL [31] and those for calcium(II)–NAL [30] and manganese(II)–NAL [30], were appropriately revised to account for the non-physiological conditions of their measurement. The resultant values were used in the blood plasma simulation model together with the corresponding values for copper(II)–NAL and magnesium(II)–NAL reported here (Table I).

Binary formation constant data for GMP have been published previously [1] and the results for the copper(II)–GMP system indicate that polynuclear species (which often occur when metal ions interact with nucleosides [38, 39]) are formed. These results bear some resemblance to those of Berger and Eichhorn [40] who reported that adenosine-5'-monophosphate formed a 2:2 complex. A variety of other polynuclear species were tested and a log β_{323} value of 41.9 appeared to be the most satisfactory although it did not produce a significant improvement in the computed statistics. Ternary formation constant data for GMP–copper(II)–NAL are reported in Table II. There is a reduced tendency to form polynuclear species in ternary systems because the polynuclear chain may be interrupted by the second ligand (*cf.* the effects of adenosine on the formation of polynuclear hydrolysed copper(II) species [41]). Evidence for the formation of polynuclear species in the GMP–copper(II)–NAL system was not obtained and it is best characterized by the formation of a ternary complex.

Blood Plasma Simulation

Administration of a normal therapeutic dose (1 g) of NAL to humans produces concentrations of the drug of $\approx 10^{-4}$ mol dm^{-3} in plasma [42, 43] and this typical value has been used in the computer simulation (Table III). It is apparent that over 90% of the drug is present in plasma in the anionic form (in agreement with the results of Vincent *et al.* [31]) if interactions with calcium(II) and magnesium(II) are neglected. However, when these biologically important metal ions are used in the model, it is apparent that approximately half of the total amount of NAL in plasma is bound by them and this result is accentuated when oxolinic acid (OXO) is considered (Table IV). Metals other than calcium(II) and magnesium(II) do not appear to have a significant effect upon the distribution of either NAL or OXO amongst low molecular weight species in blood plasma. Moreover, when the concentration of drug in plasma is 10^{-4} mol dm^{-3}, the model indicates that both drugs have only a marginal effect on low molecular weight species (*i.e.* log PMI values $<$ 0.1 in Table V). With a higher concentration (10^{-3} mol dm^{-3}) of NAL or OXO there are significant effects on equilibria involving iron(II), manganese(II) and magnesium(II).

The results of this computer simulation study suggest that NAL is mainly non-complexed in plasma and so probably acts intracellularly; the animal

TABLE III. The Computed Formation of Nalidixic Acid (NAL) Metal Ion Complexes in Blood Plasma (NAL concentration = 10^{-4} mol dm^{-3}). The formation constants used, except for Ca, Mn, Fe and Zn, have been determined in this study.

Species	% Total NAL	% LMW Metal	log β values used
(a) Modelled omitting alkaline earth metal ion complexes			
NAL^-	93.6	–	–
NALH°	3.4	–	5.94
$Zn(NAL)^+$	–	0.3	3.7[a]
$Mn(NAL)^+$		14.5	3.5[b]
$Fe(II)(NAL)^+$	–	26.5	4.3[a]
$Fe(II)(NAL)_2^0$	–	4.1	7.5[a]
$Cu(II)(NAL)_2^0$	–	0.0	6.0
Cu(II)(NAL)(HIS)°	–	3.7	15.43
Cu(II)(NAL)(HIS)(H)	–	0.0	19.58
(b) Including alkaline earth metal ion interactions			
NAL^-	47.4	–	–
NALH°	1.7	–	5.94
$Mg(NAL)^+$	24.7	3.5	3.0
$Mg(NAL)_2$	2.1	0.1	5.95
Mg(NAL)(OH)	13.8	1.9	−4.65
$Ca(NAL)^+$	8.5	0.6	2.2[b]
$Zn(NAL)^+$	–	0.1	3.7[a]
$Mn(NAL)^+$		7.7	3.5[b]
$Fe(II)(NAL)^+$	–	16.1	4.3[a]
$Fe(II)(NAL)_2^0$	–	1.2	7.5[a]
$Cu(NAL)^+$	–	0.0	6.0
Cu(NAL)(HIS)°	–	1.8	15.43

[a]Estimated from Vincent *et al.* [31]. [b]Estimated from Timmers and Sternglanz [30].

TABLE IV. The Computed Distribution of Low-molecular-weight Complexes of NAL and OXO with Metal Ions in Blood Plasma (concentration of drug = 10^{-4} mol dm^{-3}).

Species	% Total Drug	% LMW Metal	log β values used
NAL^-	57.0	–	–
NALH	3.1	–	6.13
$Mg(NAL)^+$	29.7	4.2	3.0[b]
$Ca(NAL)^+$	10.2	0.7	2.2[b]
$Mn(NAL)^+$	–	3.8	3.1[b]
$Zn(NAL)^+$	–	0.2	3.8[b]
$Cu(NAL)^+$	–	–	5.6[b]
$Fe(II)(NAL)^+$	–	7.9	3.86[a]
$Fe(II)(NAL)_2^0$	–	0.5	6.86[a]
OXO^-	37.8	–	–
OXOH	12.3	–	6.91

(continued overleaf)

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