Impact of Aluminium Ions on Adriamycin-type Ligands

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Potentiometric and spectroscopic measurements have shown that Al^{3+} ions form 1:1 complexes with adriamycin and its analogues. The major complex at pH > 5 is a tetrahedral species in which the metal ion is co-ordinated to the ligand *via* two anthracycline oxygens. Two OH^- groups complete the co-ordination sphere. Although the complexes are comparatively stable the formation of $[Al(OH)_4]^-$ at pH > 8.5 excludes adriamycin from the metal ion co-ordination sites. The unusual fluorometric behaviour of the drug molecule is also present in the complex formed at lower pH.

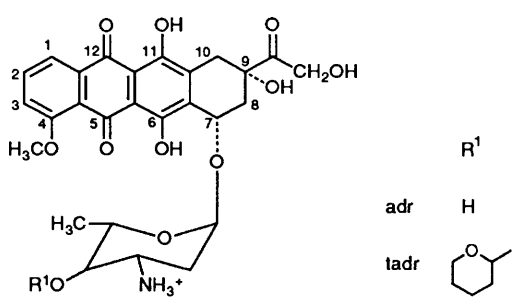
The formation of metal-ion complexes of the anthracycline derivatives could be considered as a way to influence the toxic properties of these drugs and several attempts have recently been reported.[1–12] The mechanisms of aluminium toxicity are poorly understood, although it has been established that it can bind to the specific metal-binding sites of the iron transport protein transferrin.[13–15] This suggests that aluminium may interfere with the uptake of iron and may induce some abnormalities in cells, including tumour cells. Although the effect of aluminium on cell proliferation may not be very large,[16] another representative of Group 13, gallium, has been shown to possess some cytotoxicity as a result of a severe decrease in iron uptake by the cells.[17–19] It has also been shown recently that iron ions may have a critical impact on the biological effects of anthracycline drugs including modification of their chemical structure.[20–24]

Both aluminium and gallium have been shown to bind rather effectively to phenolate as well as to quinone oxygens forming soluble complexes with interesting biological implications.[25–28] It is therefore likely that aluminium binding to anthracycline antibiotics may have some biological and chemical implications and that both Al^{3+} and drug may potentially interfere with each other in natural systems. These possibilities prompted us to perform a detailed study on the physicochemical behaviour of the aluminium–anthracycline antibiotic systems and the basic findings are presented here.

Experimental

Purified anthracycline antibiotics, adriamycin (adr) and 4′-*O*-(tetrahydropyranyl)adriamycin (tadr) were kindly provided by Laboratoire Roger Bellon and Rhone-Poulenc. Quinizarin-2-sulfonic acid (9,10-dihydro-1,4-dihydroxy-9,10-dioxoanthracene-2-sulfonic acid) (qnzs) and quinizarin (1,4-dihydroxyanthracene-9,10-dione) (qnz) were used as obtained from Sigma. The stock solutions of anthracyclines were prepared just before use to avoid ligand degradation caused by oxygen and light. The compound $AlCl_3 \cdot 6H_2O$ (Carlo Erba) was used as a metal-ion source. Stock aluminium(III) solutions contained 0.1% HCl to prevent significant hydrolysis.

Spectroscopic Measurements.—Absorption spectra were recorded on a Cary 219 spectrophotometer and circular dichroism (CD) spectra on a Jobin Yvon Mark V dichrograph. Results are expressed in terms of molar absorption coefficient ε



and molar CD coefficient $\Delta\varepsilon = \varepsilon_l' - \varepsilon_r$. The values of both coefficients are related to the total concentration of the ligand. Uncorrected fluorescence spectra were recorded at 20 °C on a Jobin Yvon JY3C spectrofluorometer.

The concentrations of ligands used in the spectroscopic measurements (adr and tadr) varied from 10^{-6} to 4×10^{-4} mol dm^{-3}. A broad range of the metal-to-ligand molar ratios was used. The spectroscopic results obtained for the two ligands are exactly the same so further mention of any ligand means the same data can be inferred for the other.

Fluorescence emission spectra for microvolume samples were recorded with a UV/VIS microspectrofluorometer prototype, developed in our laboratory.[29,30] Excitation was obtained by use of an argon-ion laser tuned at the 480 nm line. A × 100 objective and a luminous field diaphragm were used on the excitation path focusing the laser beam to a spot of about 4 μm in diameter. The fluorescence spectra were recorded in the region 500–750 nm on a 1024 diode-intensified optical multichannel analyser (Princeton Instruments). With this apparatus the screening effect due to reabsorption of fluorescence by the solution is very small and the fluorescence spectra of concentrated solutions can be recorded with high precision.

Potentiometric Studies.—Potentiometric titrations carried out for adr and qnzs ligands were performed with a Radiometer PHM 64 pH-meter and TTA 80 titration unit equipped with G20408 glass and K4040 calomel electrodes.

The concentration of the adr stock solutions was determined spectrophotometrically using $\varepsilon = 11\,500$ dm^3 mol^{-1} cm^{-1} at 480 nm, while the acid content was checked by the Gran method.[31] The ligand concentration was 5×10^{-4} mol dm^{-3},

Table 1 Macroscopic dissociation constants (pK_i) of adr and complex stability constants (log β) for the Al^{3+}–adr and –qnzs systems. Standard deviations are given in parentheses

(*a*) adr (H_2A^+)

pK_1	pK_2	$[NH_2]/[O^-]$	Conditions	Concentration/mol dm^{-3}
8.94[a]	9.95[a]	1.60[a]	0.1 mol dm^{-3} (KNO_3)	7×10^{-6}
8.15[b]	10.16[b]	6.27[b]	?	4×10^{-6}
9.10[c]	9.97[c]	2.45[c]	0.05 mol dm^{-3} (KCl)	10×10^{-6}
8.49(3)[d]	10.23(3)[d]		0.05 mol dm^{-3} (KNO_3)	5×10^{-4}

(*b*) Al^{3+}–adr[e]

Species	log β	Binding mode[f]
AlAH	16.70(10)	O, O^- (NH_3^+), $4H_2O$
$AlAH_{-1}$	7.45(4)	O, O^- (NH_3), $2OH^-$
$AlAH_{-2}$	−0.46(13)	O, O^- (NH_2), $2OH^-$

(*c*) qnzs (HA^-)

pK	9.30(3)

(*d*) Al^{3+}–qnzs[g]

AlA	9.04(9)	O, O^-, $4H_2O$
$AlAH_{-1}$	4.33(42)	
$AlAH_{-2}$	−1.47(8)	O, O^-, $2OH^-$
Al_2AH_{-1}	8.89(12)	
Al_2AH_{-3}	0.80(10)	O, O^-, $2OH^-$[h]

[a] Ref. 36. [b] Ref. 37. [c] Ref. 39. [d] This work. [e] For 104 experimental points. [f] The protonation state of the unbound amino group is given in parentheses. [g] For 103 experimental points. [h] Donor set for each metal ion.

and the ligand-to-metal molar ratios were 0.5, 1, 2 and 3:1. Titration curves at any ratio could be treated quantitatively only to pH ≈ 6–6.5 as above this a foam was formed, which might be accompanied by fine precipitation (it could not be detected visually because of the very intense colour of the solutions). Above pH 8.5–9 the foam disappeared and the pH was stable again. Within the safe pH ranges, constancy of the pH-meter reading could be achieved in a reasonable time (5–10 min), and the fit between experimental (30–40 points per titration) and calculated titration curves was fairly good. Duplicate titrations were performed with all samples of qnzs and with the samples of adr at 1:3 metal-to-ligand ratio and the reproducibility was always within 0.02 pH unit.

For the hydroxo complexes (OH^- is treated as −H in the calculations) of Al^{3+} the stability constants (log β) assumed were[32] −5.52 for $[Al(OH)]^{2+}$, −7.70 for $[Al_2(OH)_2]^{4+}$, −13.57 for $[Al_3(OH)_4]^{5+}$, −109.1 for $[Al_{13}(OH)_{32}]^{7+}$ and −23.46 for $[Al(OH)_4]^-$.

Experiments were performed under argon at 25 °C with the ionic strength controlled at 0.05 mol dm^{-3} with KNO_3. To convert pH-meter readings into hydrogen-ion concentrations the electrode system was calibrated by the method of Irving *et al.*[33]

The concentration stability constants $\beta_{pqr} = [M_pA_qH_r]/[M]^p[A]^q[H]^r$ were calculated from the pH-metric titration curves by means of the PSEQUAD computer program.[34]

Results and Discussion

Potentiometric Study.—Proton complexes. Adriamycin and tetrahydropyranyl adriamycin (H_2A^+) contain two dissociable protons in the pH range studied (2–10): one on the ammonium group of the sugar moiety and another on the phenolic hydroxy group of 1,4-dihydroxyanthraquinone. The other phenolic OH of the anthracycline ring is very weakly acidic with a pK of about 13.7.[35] Because of the possible overlap between the dissociation of the sugar NH_3^+ group and the phenolic OH group the pH-metrically determined macroconstants listed in Table 1 are composite and cannot be directly assigned to the individual acidic groups. Most previously reported macroscopic dissociation constants were determined by spectrophotometric methods taking into account the overlapping dissociation microprocesses.[39,40] In these calculations the pK values and a parameter characteristic for the overlap between the isomer deprotonations (the ratio of the concentrations of the isomeric species HA and HA*, or the molar absorptivity of the monoprotonated ligand average forms) were refined simultaneously.

Previous experiences led to the conclusion[36] that because of the poorly conditioned system the least-squares evaluation method might result in rather large uncertainties in the calculated parameters. Besides the light and oxygen sensitivity of the ligand, this might be responsible for rather large differences in the reported constants (Table 1). It is also well known that at higher drug concentrations (above 100 μmol dm^{-3}) the self-association between the ligand species containing protonated phenolic OH groups can be significant.[37] This can affect the macroscopic dissociation constants determined pH-metrically at higher concentrations. Thus, the differences between the macroscopic dissociation constants determined in this work and those reported earlier can be understood. Our data are in reasonable agreement with those of Király and Martin[35] obtained also from potentiometric titrations for daunorubicin, a close derivative of adriamycin: $pK_1 = 8.61$ and $pK_2 = 10.0$. They also performed spectroscopic pH titrations in order to determine the microscopic dissociation constants of daunorubicin. Calculated results have shown that with a very good approximation pK_1 can be assigned to the deprotonation of the ligand ammonium group, pK_2 to the deprotonation of the phenolic group.

The pK values obtained by Frezard and Garnier-Suillerot[38] for tadr were 7.7 and 10.0, respectively.

Aluminium complexes. Since the co-ordination patterns for adr and tadr are exactly the same the potentiometric data are discussed for the system containing adr only since this has higher solubility at pH > 7.

The titration curves obtained for the Al^{3+}–adr system at different metal-to-ligand ratios (Fig. 1) clearly indicate that complex formation starts at pH > 3.5 and after a rather flat part there is a significant pH jump in each titration curve corresponding to the liberation of about three protons per metal ion, independent of the ligand excess. This result suggests very strongly the formation of 1:1 complexes only in the acidic pH region. Thus, the pH-metric titration curves were evaluated by

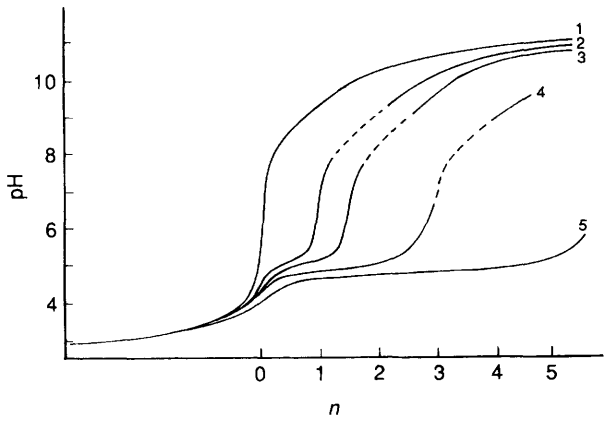


Fig. 1 Titration curves for Al^{3+}–adr solutions of metal-to-ligand ratios of 0:3 (1), 1:3 (2), 1.5:3 (3), 3:3 (4) and 6:3 (5); *n* corresponds to the number of base equivalents added per ligand. The samples contained an excess of strong acid; when it is neutralized $n = 0$

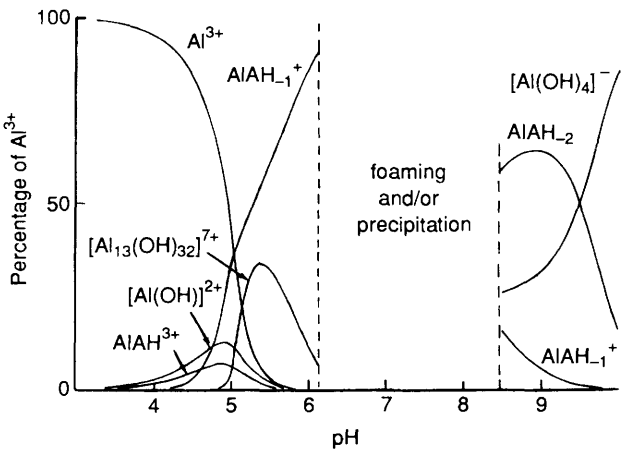


Fig. 2 Species distribution curves for the Al^{3+}–adr system. Total ligand concentration [adr] = 2×10^{-4} mol dm^{-3}, metal-to-ligand ratio 1:2

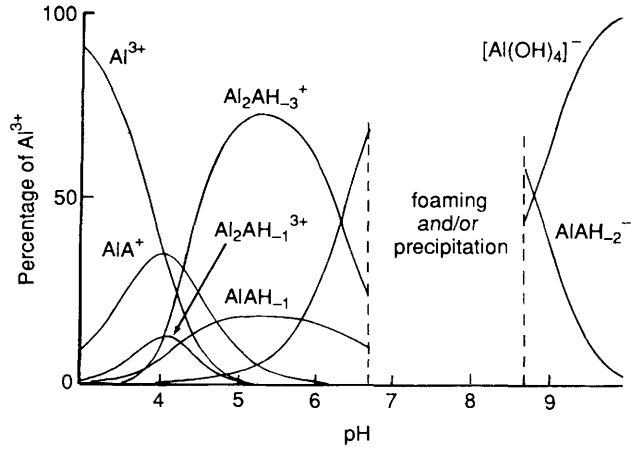


Fig. 3 Species distribution curves for the Al^{3+}–qnzs system. Total ligand concentration 2×10^{-4} mol dm^{-3}, metal-to-ligand ratio 1:3

the assumption of various 1:1 species, AlAH, AlA, $AlAH_{-1}$ and $AlAH_{-2}$. It is known that Al^{3+} ions co-ordinate preferentially oxygen donors, with negligible affinity for nitrogen donors, especially as monodentate ligands. Thus, the co-ordination site for this metal ion is centred at the anthracycline ring involving two oxygen donors. The sugar amino group is most likely excluded completely from co-ordination (see below).

If formation of *O,O'* co-ordinated bis and tris complexes also took place at lower pH (*i.e.* before the pH jump), the pH jump should occur at different $OH^-:Al^{3+}$ ratios depending on the metal-to-ligand ratio. The best fit between the experimental and calculated titration curves was obtained assuming the complexes listed in Table 1. Species such as $Al(AH)_2$ and $Al(AH)_3$ or their deprotonated forms were also included in the calculations but all were rejected by the computer program when the titration curves were treated together in the calculations. The concentration distribution curves of the species found using potentiometry are shown as a function of pH in Fig. 2. It can be seen that besides some hydroxo complexes, mainly $[Al(OH)]^{2+}$ and $[Al_{13}(OH)_{32}]^{7+}$, only three 1:1 species AlAH, $AlAH_{-1}$ and $AlAH_{-2}$ are formed with the adriamycin drug. The AlAH complex is a minor species at pH < 5 while $AlAH_{-2}$ is dominant at pH > 8.5. The major species formed is the $AlAH_{-1}$ complex which dominates above pH 5. The latter two species, $AlAH_{-1}$ and $AlAH_{-2}$ should be written more accurately as $AlAH(OH)_2^+$ and $AlA(OH)_2$, respectively. Thus, during the formation of the major complex $[AlAH(OH)_2]^+$ from $[AlAH]^{3+}$ two protons are lost in very overlapping processes. This co-operative proton release occurs most likely from two bound water molecules. [The spectroscopic data (see below) exclude the possibility of proton removal from the second phenolic OH group in the pH range studied.] The simultaneous deprotonation of two water molecules suggests that during this process the structure changes from octahedral $[AlAH]^{3+}$ to more stable tetrahedral[41,42] $[AlAH(OH)_2]^+$. At pH > 7 deprotonation of the non-co-ordinating sugar NH_3^+ group leads to formation of $AlA(OH)_2$ (Table 1).

In order to support the results discussed above potentiometric titrations were also performed for the Al^{3+}–quinizarin-2-sulfonic acid system. The latter ligand possesses a very similar donor set to the anthracycline ring of adriamycin. Quinizarin-2-sulfonate (HA),[9] contains one dissociable proton with $pK = 9.30$ corresponding to a phenolic OH group. It is a slightly more acidic group than that of adr.

The co-ordination pattern of Al^{3+} with qnzs is basically similar to that found for adr, although the participation of both anthraquinone donor sites in metal-ion binding is more favoured (Table 1, Figs. 2 and 3). This may result in the formation of a dimeric complex Al_2AH_{-1}, or by the linkage of more monomeric units form a chain polymer with the general stoichiometry $(AlAH_{-1})_n$. The formation of the dimeric complex with quinizarin derivatives[43,44] is well established for Fe^{3+}, while formation of a similar long-chain polymer was observed between various dihydroxyquinoid ligands and Cu^{2+} in the solid state.[45] It is interesting that there is also a co-operative deprotonation for the AlA species of qnzs (which corresponds to the complex AlAH of adr, *cf.* the different deprotonation schemes of the free ligands), and also of the dimeric Al_2AH_{-1} complex. Thus, the structure variation (O_h to T_d) suggested above seems to be a reliable assumption. This co-operative release of two protons in closely overlapping processes leads to the formation of complexes $AlAH_{-2}$ and Al_2AH_{-3}, respectively. A co-operative effect has also been observed in the hydrolysis of Al^{3+} ions.[41]

The co-ordination pattern as well as the similar stabilities of the respective complexes of adr and qnzs indicate that the main binding sites of Al^{3+} with anthracycline drugs are the oxygens of the anthraquinone ring. The higher tendency of qnzs to co-ordinate metal ions simultaneously at both binding sites may result from the fact that this ligand is much smaller and the lack of a sugar moiety avoids steric hindrance which could prevent co-ordination of a second metal ion.

Spectroscopic Study.—Anthracyclines such as adr or its derivative tadr exhibit very characteristic and intense absorp-

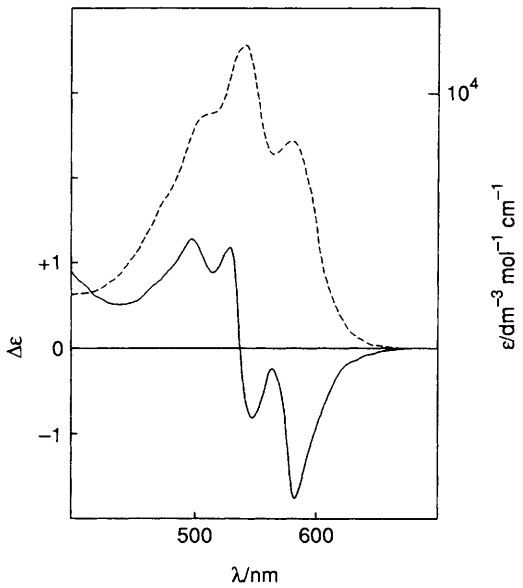


Fig. 4 Visible absorption (— — —) and CD (——) spectra of Al^{3+}–adr complex **1**. [adr] = 10^{-4} mol dm^{-3}, [Al^{3+}] = 5 × 10^{-4} mol dm^{-3}, pH 5; ε and Δε are calculated for total concentration of ligand

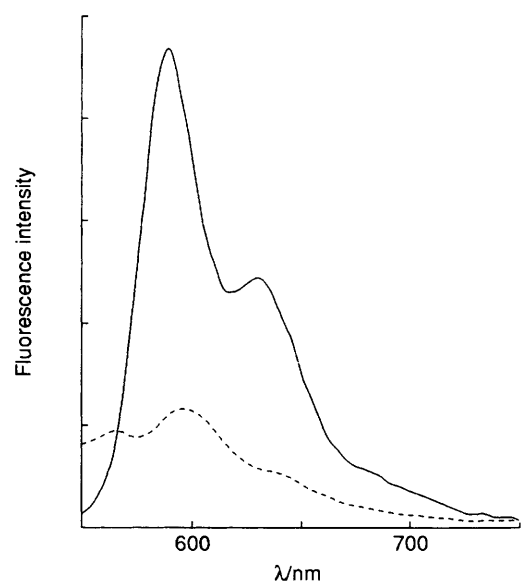


Fig. 5 Uncorrected fluorescence spectrum of complex **1** (Al^{3+}–adr) (——) and metal-free adr (— — —) at pH 5. [Al^{3+}] = 5 × 10^{-6} and [adr] = 1 × 10^{-6} mol dm^{-3}

tion spectra centred around 480 nm (Fig. 4). This set of transitions is sensitive only to changes of the phenolic OH groups, such as deprotonation or metal-ion co-ordination,[1,2,4,6,8] and only very slight variations of these spectra are observed in metal-free solutions at pH < 9. In addition the CD spectra of the aromatic ring unit as well as the fluorescence spectra are characteristic for different protonation states of the aromatic ring chromophore. Thus, absorption, CD and fluorescence spectra can be used as reliable tools to follow the metal-ion binding ability of the anthracycline ligands studied.[1–4,6–8]

The species distribution curves calculated for the Al^{3+}–adr system at the total ligand concentrations used in the spectral studies and at different metal-to-ligand ratios indicated that at any molar ratio from 1:1 till 1:3 there is a considerable concentration of the unbound ligand, *e.g.* in the 1:1 solutions at pH ~6.0 (highest pH available before precipitation) only about 50% of the metal is bound to the drug while the remainder is involved in the oligomeric hydroxo-species. Thus, for all samples measured the spectra obtained will always consist of two components, often overlapping, derived from metal-free and bound forms of the ligand. This causes series problems in quantitative treatment of the spectroscopic data.

The complexation of Al^{3+} to anthracycline (adr or tadr) strongly modifies the ligand absorption, CD and fluorescence spectra, all transitions being shifted towards lower energies. Let us consider the spectroscopic characteristics of complex **1** (AlAH), which is formed at pH around 4–5. The absorption spectrum of the 1:1 Al:adr solution is only slightly different from that of the free drug since only 20% of the ligand is co-ordinated to Al^{3+}. However, the appearance of a band at around 570 nm attests to the formation of a minor species (complex **1**). The modification of the absorption and CD spectra at pH around 5, *i.e.* the concentration of **1**, can be considerably increased by adding an excess of Al^{3+}. A molar ratio of metal to adr of 5:1 is required effectively to co-ordinate all the ligand. The absorption and CD spectra of **1** obtained under these conditions are shown in Fig. 4. One very surprising result obtained for this species is its very strong fluorescence. Usually the fluorescence of anthracycline is quenched through complexation with metal ions[4] and as we shall see below this is the case for complex **2** ($AlAH_{-1}$). Fig. 5 shows the fluorescence spectra of **1** obtained by excitation at 536 nm. For comparison, the fluorescence spectrum of free adr recorded under the same conditions is also shown.

The spectroscopic characteristics of complex **2** were more difficult to determine because of the presence of uncomplexed ligand even after the addition of an excess of Al^{3+}. At pH > 6.5 most of the metal excess was precipitated as $Al(OH)_3$ and these samples were not quantitatively evaluated. However, using potentiometric and spectrofluorometric data it was possible to determine the concentration of free ligand present in the precipitation-free samples. For this purpose the fluorescence spectra of aqueous solutions (pH 6.2) of Al^{3+}–tadr at molar ratios 1:1, 1:2 and 1:3 were recorded together with the absorption and CD spectra. The drug concentration was constant and equal to 200 μmol dm^{-3}. At this pH metal-ion complexation to tadr (or adr) gives rise to a quenching of the drug fluorescence.[4] Thus, the fluorescence observed can be assigned to the free drug present in solution. In order to determine the free-ligand concentration present in the Al^{3+}–drug solutions a calibration curve was drawn from measurements of the fluorescence of tadr solutions at different concentrations. The intensities of the fluorescence spectra of the metal-free ligand at concentrations 50, 100 and 200 μmol dm^{-3} were found to be 1050, 1650 and 2500, respectively. These values were used to evaluate the amount of free and bound ligand in solutions containing 200 μmol dm^{-3} drug and with metal-to-ligand molar ratios of 1:1, 1:2 and 1:3. The fluorescence intensities of these solutions were found to be 1600, 1650 and 2000, respectively. Taking into account that the fluorescence of the complexed ligand is 0, the amount of bound ligand was evaluated to be 108 (54), 103 (51) and 73 μmol dm^{-3} (36%), respectively. On the other hand, according to potentiometric data (Table 1), the amount of bound ligand was found to be 112 (56), 94 (47) and 66 μmol dm^{-3} (33%), respectively. Thus, the evaluations of the amounts of bound and metal-free ligand in the solutions studied by two independent techniques are in very good agreement. This indicates that the complex distribution shown in Fig. 2 is reliable.

The absorption and the CD spectra of an equimolar solution at pH 6 are shown in Fig. 6. The contribution of the metal-free ligand has been subtracted from the total spectroscopic signal and the absorption and CD spectra corresponding to complex **2** have thus been determined.

Rapid acidification of an equimolar Al^{3+}–adr solution by addition of HCl to pH < 3 yielded complex **1** which after some

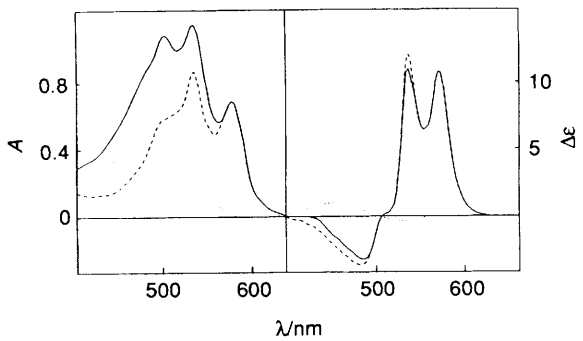


Fig. 6 Visible absorption (left) and CD (right) spectra of complex **2** (Al^{3+}-adr). [adr] = 2×10^{-4} and [Al^{3+}] = 2×10^{-4} mol dm^{-3} at pH 6.2. Contribution of metal-free adr (92 μmol dm^{-3}, ····) has been subtracted from the spectrum obtained for the solution at pH 6.2 (——) yielding the pure spectrum of complex **2** (– – – –). See text for details

minutes underwent dissociation. This suggests that the protonation of **2** (**2** + $2H^+ \rightarrow$ **1**) and the structure variation from T_d (**1**) to O_h (**2**) (*i.e.* binding of two water molecules) is very fast compared to the dissociation of ligand from the metal coordination sphere.

An increase in pH to >7 led to a decrease in intensity in the spectrum of the complex because of precipitation of metal hydroxide as well as the formation of more stable $[Al(OH)_4]^-$. It should be also mentioned that the spectral intensity may depend strongly on time within the first 24 h because of the complicated equilibria in which oligomeric species are involved.[41,46]

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