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FT-IR and Raman Investigation of Cadmium Binding by DNA

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Abstract

Complexes of cadmium(II) with DNA in aqueous solution under physiological conditions are investigated by means of vibrational spectroscopy. It is shown that at low concentrations, Cd^{2+} binds primarily on PO_2^-, through a pure electrostatic interaction. This is followed by a cooperative coordination on both guanine and adenine** when large amounts of Cd(II) are present (3 eq. of Cd(II) for 1 eq. PO_2^-). Tests performed on DNA with Cd(II) concentrations met in real biological systems leave the DNA intact, indicating that the toxic effect of Cd(II) may not be directly related to its action on DNA. We demonstrate that FT-IR spectroscopy is useful in identifying the type of binding occurring on the PO_2^- sites of DNA, while Raman spectroscopy is remarkably well adapted for monitoring base complexation by Cd^{2+}.

Introduction

The toxic effects of cadmium and other divalent cations are presumed to be related to specific chemical and physical properties of the ion. Cadmium is recognized as a non-essential element that is toxic to many plants and animals [1]. So far a number of useful reviews on the chemistry of heavy metals with various biological molecules have been published, but few informations are available on the complex formation of purines, pyrimidines, nucleosides and nucleotides by Cd(II) (for a general review see refs. 1–7 and refs. cited therein). This is mostly due to the fact that no changes are observed with the usual analytical methods when one of these particular molecules is in the presence of Cd(II), except for large quantities of Cd(II). This is particularly true when macromolecules like DNA or proteins are under investigation.

While some macromolecules present specific coordination sites for metal ions and form complexes of high stability [8], others, by virtue of a large net negative charge, bind metal in a non-specific electrostatic manner. In addition, a cation's binding ability varies from one to another and is described by the well-known theory of soft and hard species (see general reviews already quoted). A theoretical framework has been set in this direction and the importance of the electrostatic effect has been stressed [8 and refs. cited therein].

Pioneering work on DNA–Cd(II) complexes has been done by means of UV spectroscopy [9] and the results were compared to those for other metals. For instance, it was reported that Cd^{2+} and Cu^{2+} both bind to the DNA bases, copper being more firmly bound than Cd^{2+}, and Cd^{2+} having a larger affinity for the bases than Zn^{2+}. Since then, more experimental work has been performed by electrochemistry or other methods and disagreement arises from this simple picture. Investigations carried out by polarography on the DNA–Cd^{2+} system [10] showed that at least two binding sites might exist, due to the fact that the Cd–DNA binding constant is equal to 25 M for native DNA and 67 M for thermally denatured DNA. This could be a clear evidence that Cd^{2+} is mostly linked to the bases and marginally to PO_2^-, because phosphate is mostly located on the surface of the molecule and is therefore equally accessible to Cd^{2+} whatever the secondary structure of DNA. But even so, just pure electrostatic interactions were suspected by invoking different PO_2^- affinities caused by changes in the bulk geometry of the macromolecule, leading to better reactivity towards Cd^{2+}. Additional work has been done on both tRNA [11, 12] and DNA [13] and unambiguously a pure electrostatic behavior was confirmed for low metal to DNA concentrations. Complexation on the DNA bases was therefore ruled out as long as the Cd^{2+}/DNA ratio remains below 0.5. This in agreement with the UV results showing that the DNA–Cd^{2+} melting curves for values below this ration (*viz.* 0.5) are identical to the curve for native DNA [9], hyperchromicity on the absorption coefficient being measured only for large metal concentrations. This latter fact (the hyperchromicity of the absorption spectra) might be related either to denaturation, to base coordination or to a com-

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**Abbreviations: Ade or A: adenine, Cyt or C: cytosine, Gua or G: guanine, Thy or T: thymine, R: ribose, NMP: nucleotide monophosphate.

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bination of both phenomena. For instance, denaturation could occur because the presence of large amounts of metal ions will increase the ionic strength, leading to an effect similar to salt denaturation.

More recently, a study mostly using pulse polarography [14] did not indicate any specific interaction on the bases. Additional evidence was also drawn from CD spectra [14], demonstrating that when the Cd^{2+}/DNA ratio is below the critical value of 3 (related to PO_2^- concentration), nothing happens. Beyond this limit, the results are explained in terms of a B to C conformation transition rather than to a pure Cd^{2+} coordination on the bases or another part of the macromolecule.

Recent papers have also investigated model systems or DNA–metal complexes by means of FT-IR [15–19] or Raman spectroscopy [20, 21] with various degrees of success. Thus, we intend to simultaneously apply these two techniques to the Cd^{2+}/DNA adducts in order to ascertain the pure electrostatic model and/or to elucidate the role of the bases in Cd^{2+} binding. If the B to C transition is really taking place, Raman spectroscopy should be a good probe to investigate this conformation change. In addition, we will propose a semi-quantitative approach by using marker bands in order to measure the relative extent of complexation.

Experimental

Calf thymus DNA (sodium salt) was purchased from Sigma Chemical Co. and used without further purification. All the salts were reagent grade and used as supplied.

The DNA solutions were prepared by dissolving a few mg of the compounds in a 0.15 M NaCl stock solution in doubly distilled water, adjusted to pH *ca.* 7. The pH of the stock solution was adjusted either with NaOH 1 N or HCl 1 N. Typically, the concentration of DNA was *ca.* 7% *w/w* (or 0.14 M PO_2^-) for IR measurements and about 5% (or 0.1 M PO_2^-) for Raman studies. In both cases, the samples were prepared by adding a given volume of salty solution to DNA. A gentle hand stirring with a tiny glass rod was applied until a good sample homogeneity was obtained. The samples were then kept overnight in the refrigerator and occasional stirring was reapplied. The solutions containing DNA plus metal were prepared by using a 0.4 M stock $CdCl_2$ solution in 0.15 M NaCl adjusted to pH *ca.* 6–6.2 (beyond this value, $Cd(OH)_2$ precipitation occurs). The Cd^{2+} quantity was calculated and the desired volume added to DNA already dissolved in an equivalent volume of 0.15 M NaCl. The mixing procedure is the same as given above for dissolution of pure DNA. Upon addition of large Cd quantities, the DNA solution becomes like a gel. For this reason, pH measurements of the complex are difficult and the pH of the solution was assumed to be near 7 since the concentrated DNA solution should act like a buffer. The pH value was checked before and after solution preparation when possible.

The spectra of these solutions were recorded by transferring the sample into a capillary tube for Raman measurements or by squeezing a few μl of the solution between two AgCl windows for FT-IR studies. In the Raman experiments, a 0.01 M $NaNO_3$ internal standard was added to the 0.15 M NaCl stock solution. Due to strong anion absorption, no such internal standard was used in FT-IR. However, we checked the reproducibility of our FT-IR measurements by monitoring the absolute intensity of the H_2O bending mode at *ca.* 1640 cm^{-1} for data taken from different sets of scans on the same solution. According to this, our experimental method is believed to be accurate within 10%. The spectra of solutions at *ca.* 7% *w/w* DNA/water (0.15 M NaCl) have been recorded at pH *ca.* 7 (6.5) with and without Cd^{2+}. The water subtraction was performed with a 0.15 M NaCl aqueous solution used as reference at pH 7. A good subtraction was characterized by a flat baseline around 2200 cm^{-1}, caused by the cancellation of the water combination mode (1600 + 600 cm^{-1}) [22]. This criterion is a rough estimate, but removes the water contribution in a satisfactory way, although some distortions might appear. We did not run the experiments in D_2O because the hydrogen bond network is not exactly the same as in H_2O. For the Raman spectra the procedure for water subtraction is straightforward because it is removed by scaling the two spectra with the common 1047 cm^{-1} band of NO_3^-.

The FT-IR spectra were recorded on a Digilab FTS 50 spectrometer using a 2 cm^{-1} resolution and 256 scans, corresponding to a recording time of 15 min. The water rotation bands were removed by smoothing the raw data with a Savitzky-Golay procedure.

The Raman spectra were recorded on a SPEX Triplemate spectrograph coupled to an EG & G Princeton Applied Research detector, model 1422, and were excited with 250 mW of the 488.8 nm laser line (Spectra-Physics CW Ar^+ laser, model 2016) in order to minimize the fluorescence of the sample. The sealed capillary was mounted on a thermally regulated sample holder and maintained at a temperature of 10 °C. The spectra were recorded with a 2 cm^{-1} slit width during 15 or 30 min. The fluorescence background was routinely subtracted with an appropriate *n*th order polynomial.

UV results are derived from experiments similar to those reported in ref. 9. Besides DNA, we recorded the absorption spectra for 5′-NMP (nucleotide monophosphate) under identical conditions with a metal to NMP ratio ranging from 1 to 3 when possible.

Infrared measurements have also been done with a relative metal–biomolecule concentration equal to 1, but at a larger final concentration (*ca.* 5–7% *w/w*) under physiological conditions and pH.

Results and Discussion

FT-IR spectra of pure DNA and Cd^{2+}/DNA in a 3:1 ratio are shown in Figs. 1 and 2. The Cd^{2+}/

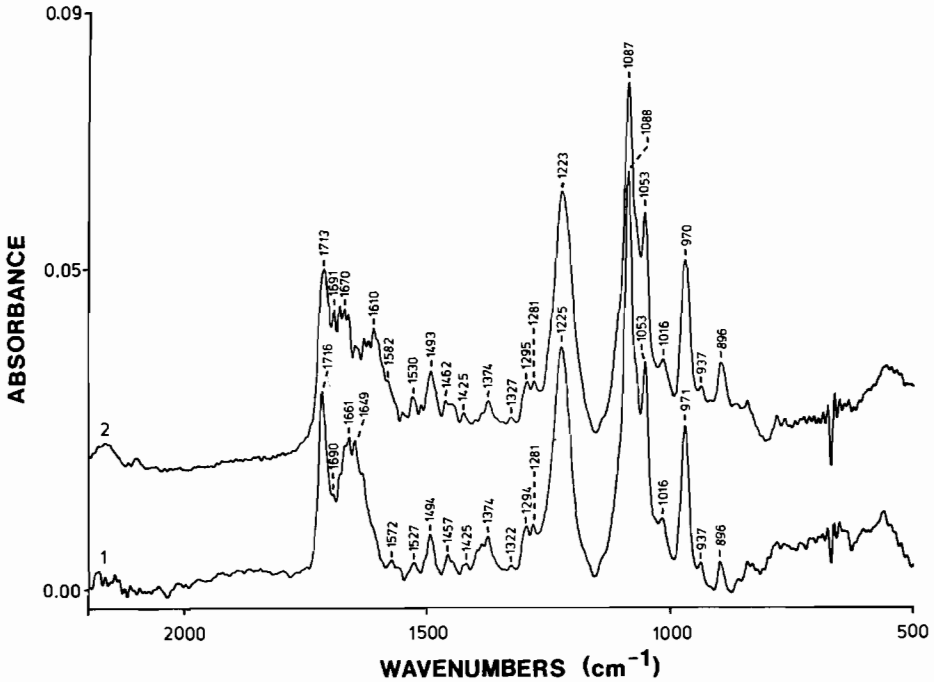

Fig. 1. FT-IR spectra of DNA (curve 1) and DNA + Cd^{2+} at a metal/DNA ratio equal to 3 (curve 2) at pH *ca.* 6–7 in the 2200–500 cm^{-1} region.

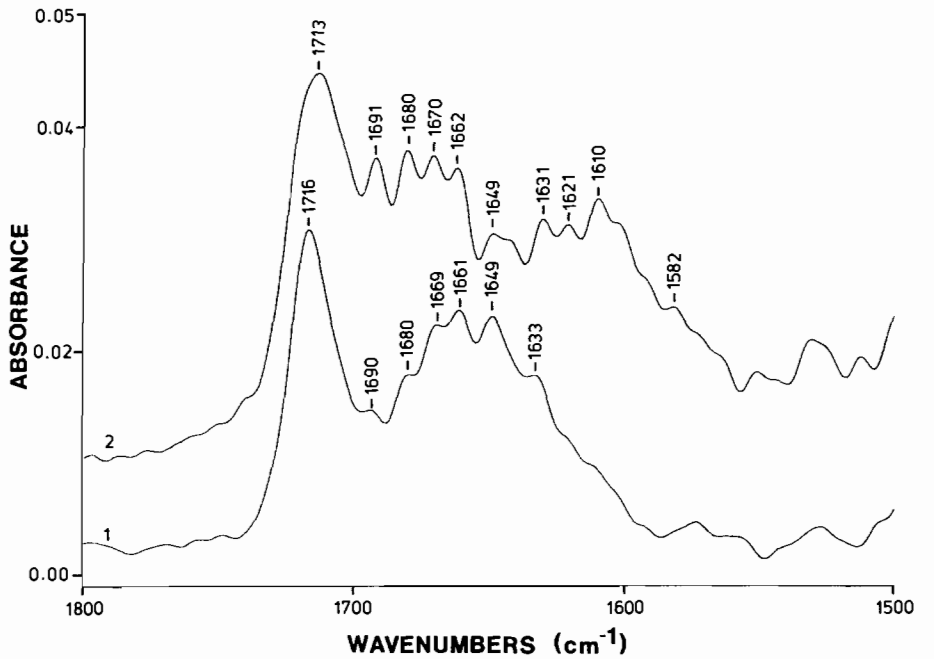

Fig. 2. FT-IR spectra of DNA (curve 1) and DNA + Cd^{2+} at a metal/DNA ratio equal to 3 (curve 2) at pH *ca.* 6–7 in the 1800–1500 cm^{-1} region.

DNA spectra with 1:1 and 2:1 ratios are almost identical to the one of the native DNA. The main bands come from the PO_2^- group and the ribose moiety in the 900–1300 cm^{-1} region and also from the bases ν(C=N), ν(C=O), ν(C=C) at 1600–1700 cm^{-1}. Additional features are present elsewhere, but the weakness of the signal does not allow a clear interpretation. Extensive IR studies have been done on DNA and were reviewed in the literature [23–26]. This technique is not very selective for investigations of the base coordination due to strong overlap of the respective contribution from each residue. Therefore, we performed studies on model compounds such as nucleoside monophosphates (NMP) in order to have a better opinion of what we may expect to see on DNA with this particular metal.

From the well known X-ray structure of the Cd–5′-NMP [6], base coordination and PO_3^- interaction are well established. It is interesting to note that, generally speaking, Cd–N bond lengths (or with any other kind of heteroatom), are long compared to nucleotide complexes with other metals (*i.e.* for Cd–NMP, r = 2.3–2.4 Å and for M–NMP, r = 1.9–2.2 Å [6]). This point bears some importance in the prediction of the perturbation of the purine and pyrimidine modes by Cd(II). The Cd–X bond being long, one can expect smaller perturbations of the ring vibrations compared to other metals.

We will attempt to rationalize general studies done at neutral pH when 5′-NMPs are mixed stoichiometrically with Cd(II). Upon metal addition a precipitation occurs that can be more or less important depending whether the NMP adduct is a purine or a pyrimidine. There is an equilibrium between a precipitate and another species remaining in solution for the pyrimidines and only a precipitate for the purines.

For 5′-GMP, the crystal structure is known [27], but nobody has ever been successful in crystallizing 5′-AMP. The literature only reports an adenine–cadmium complex, where the N3 and the N9 positions are concerned [28], this being of little use for our purposes. Due to the low solubility of 5′-GMP and 5′-AMP upon complexation, the solutes are readily washed out from the aqueous phase and a white solid can be isolated. Therefore, we assume that their structures are identical to the ones determined by X-ray diffraction. Thus, the involvement of both sites (bases and PO_3^-) is taken for granted, meaning that at neutral pH, the probability of fixing Cd^{2+} to both sites is equal. (The affinity of Cd^{2+} for PO_4^- is believed to be large at this pH, because the mixing of an HPO_4^- salt to Cd^{2+} is followed by a total precipitation.)

For 5′-CMP, the situation is identical and the Cd^{2+} is both linked to the N_3 ring atom and to the phosphate moiety [29, 30]. For 5′-TMP, the structure is not known, but the base–Cd–PO_3^- interaction is general for most metal–NMP compounds [6]. For pyrimidines, the complexes are more soluble since the original compounds are themselves more soluble, therefore we are in the presence of both a precipitate and a complex left in solution. The IR spectra of the solution and precipitate indicate that Cd is mostly bound on the N_3 position of Cyt and C_4=O_4 of Thy. PO_3^- is also involved. Between the precipitate and the solution there is no significant difference, meaning that the base–Cd–PO_3^- interaction is retained in solution, adding proof to early statements made in this direction [31]. However, due to the DNA structure, we do not expect to be able to complex these two sites since they are not directly accessible as they are involved in H bonding. The precipitate obtained from these compounds is readily put back in solution by using a 1:1 Cd^{2+}/glutathione molar ratio (glutathione is a thiol containing tripeptide), meaning that it is easy to destroy the complex.

Now, we would like to resume our discussion of DNA. We will treat the problem in two parts: (1) the phosphate and ribose part and (2) the base entity.

(1) Phosphate and Ribose

First, we will compute the ratio that involves the symmetric PO_2^- stretch coupled with the C5′–O5′ ribose stretch at *ca.* 1088 cm^{-1} and the antisymmetric stretching vibration located at *ca.* 1225 cm^{-1}, as it will give a good picture of the perturbation experienced by the phosphate. Usually, the frequency position of the antisymmetric stretch at *ca.* 1225 cm^{-1} is more sensitive to the geometry of the molecule and may be shifted by −5 to +20 cm^{-1} depending on which form of DNA is present [23]. As far as the intensity is concerned, different conformations do induce changes but they remain small and mostly concern the ν_s mode [23]. The result of the calculated intensity ratio, namely $I_{1225\,cm^{-1}}/I_{1088\,cm^{-1}}$ is plotted in Fig. 3.

The curve has been drawn from four spectra obtained at various Cd/DNA ratios ranging from 1 to 4 and at pH *ca.* 6–7. By using the Nakamoto COO^- formalism [32], this data shows that there is a significant interaction between PO_2^- and Cd^{2+}. This is accomplished by treating the O–P–O moiety in terms of localized and delocalized double bonds. With this approach, two kinds of adducts will be considered: one type of complex is formed when one or more Cd^{2+} cations are strongly bound to one or two oxygens of the PO_2^- moiety (type I), and the other when a single Cd^{2+} ion forms a more or less defined asymmetric electrostatic interaction with PO_2^- (type II involves resonance between O=P–$O^-\cdots Cd^{2+}$ and $O\cdots P\cdots O^-\cdots Cd^{2+}$).

The type I complex is an extreme case seldom met in this kind of chemistry, except when protonation occurs or when hard cations like Ca^{2+} or

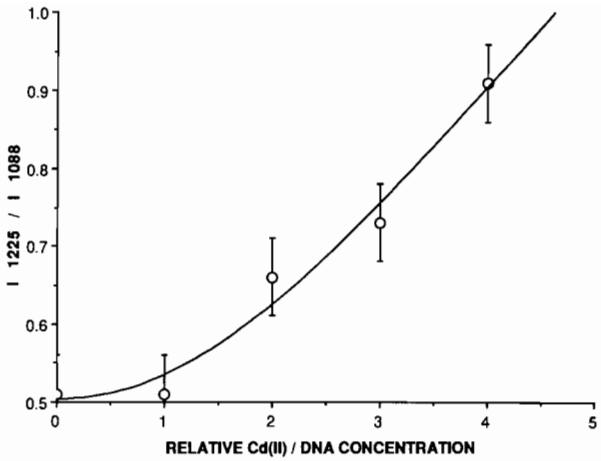

Fig. 3. Ratio of the DNA band intensities at 1225 and 1088 cm^{-1} as a function of Cd^{2+} concentration.

or Mg^{2+} are involved. For the type II complex, we expect some typical spectral changes: for instance, the ν_{as} should increase in frequency and in intensity as the double bond character is reinforced. (However this behavior may be hidden by a change in the conformation of the biopolymer.) The ν_s mode will remain untouched or slightly perturbed in frequency, but should decrease in intensity. It would be realistic to think that, when we are in the presence of Cd^{2+} bound more to one PO bond than to the other, this asymmetry will induce an intensity drop at ν_s at the expense of an increase at ν_{as}. However, because the double bond is not really localized, no drastic frequency shift is expected. This seems to be true according to our intensity calculations. From previous results taken from the literature [1, 2, 9–14], the behavior of the complex is partly predicted. It is obvious that as long as the Cd^{2+} concentration is kept low, the complex is not formed. The dominant species are purely ionic, and they are spectroscopically silent. This fact can be anticipated from the reported value of the bulk equilibrium constant which suggests that the complex is not formed for low metal concentrations. From IR studies made on DNA mixed with Hg^{2+} and Ag^{+} [16, 17], it has been shown that no modification occurs on PO_2^- as the group is not directly involved in complexation, but the likelihood of the presence of a loose electrostatic link as it is known to exist on NMP–metal adducts was not mentioned [6, 18]. Therefore, from the plot of Fig. 3, a semi-quantitative approach can be derived from the intensity ratio. Under our experimental conditions, the complexation starts to take place on PO_2^- only when the concentration of Cd(II) is at least larger than one equivalent PO_2^-. From Fig. 3, it is seen that for a Cd/DNA ratio between 1 and 4, the intensity ratio is roughly proportional to the Cd concentration. The fact that we do not see a plateau in Fig. 3, means that no saturation occurs. This may seem surprising, but is possibly due to the fact that when more Cd^{2+} is added, denaturation may occur and thus more sites become available. This picture is in agreement with UV measurements [9] and explains the increase of the association constant upon denaturation [10].

The use of the ν_{as}/ν_s ratio may be questionable since it can also represent a measurement of a conformation change of the macromolecule and not only the degree of complexation on PO_2^-. However, we do not think it can be confounded with conformation transition or denaturation even if similar changes might appear in the presence of a Z form. To demonstrate this latter point, we have recorded the spectra of DNA in a 4 M NaCl solution in the presence and absence of Cd(II). In both cases, the ratio was equal to 0.52. In these particular situations, the base pairing in DNA was destroyed as the characteristic peak at *ca.* 1710–20 cm^{-1} had disappeared [23]. No change was observed in the two spectra for the two PO_2^- modes, meaning that the link between Cd(II) and PO_2^- did not exist in this situation and had previously been mostly electrostatic. The lack of bonding is attributed to shielding of the PO_2^- moiety by the salt. Similar experiments have been done on basic (pH $>$ 12) and acidic (pH $<$ 3.5) DNA solutions in 0.15 M NaCl solution, leading to a ratio equal to 0.61. This result proves that the degree of ionization of PO_2^- plays an important role in the computation of this variable. Finally from the literature we have computed the ratio for the A and Z forms of DNA [23]. They are 0.54 and 0.64 respectively. (The data were collected from films cast on IR transparent windows.) From that, we can deduce that if the Z form of DNA is present, our early calculations will not represent a pure image of the degree of PO_2^- bonding, but will also include some information on the DNA geometry. The difference spectrum of ([DNA + Cd]–DNA) scaled with the 971 cm^{-1} band, indicates that there is a significant positive peak located at 1206 cm^{-1} that may correspond to the Z form (not shown). Usually this band is shifted from 1224 to *ca.* 1215 cm^{-1} and accompanied by an intensity increase when a B to Z transition takes place. Other features will also be modified especially in the 1700 cm^{-1} region, where the 1716 cm^{-1} should be shifted in frequency to *ca.* 1690 cm^{-1}, but this region cannot be used as it is also similarly perturbed by base coordination. However, if this correlates in part with the data in Fig. 1, a certain number of the other characteristic bands of the Z form are absent: specifically, the $\nu_s PO_2^-$ is not shifted upward from 1088 to 1090 cm^{-1} and no strong intensity increase is seen at *ca.* 1016 cm^{-1}. This latter band is one of the best marker peaks. More subtle features well described in ref. 23 are also absent or undetec-

table. Thus if the Z form is present it will be below our detection range. This particular point will be rediscussed in the next section. At this step the modifications not attributable to direct or electrostatic interaction on PO_2^- are suspected to be due to base coordination or simply to denaturation.

(2) Bases

For the remaining part of the molecule three potential sites are available to complexation according to general reviews [1 and refs. cited therein] and quantum mechanical calculations [33]. The first will be Gua N_7–O_6, then Ade N_7 and finally Thy O_2. The probability of complexing Cyt at N_3 is low, because the bond energy between G–C is about −19 kcal/mol [34]. This site is not exposed and its cohesion energy is large compared to −5 kcal/mol for the A–T pair hydrogen bond network or to −7.5 kcal/mol for the denaturation energy. From this theoretical approach, a few points bear some importance. First, if coordination takes place on N_7 of Gua, the H bond network of the G–C pair is reinforced by a new charge distribution. Upon complexation of the N_7 of Ade, the H bond force field of the A–T pair remains almost unchanged. The formation of a chelate involving both the N_7 and NH_2 position of adenine is thermodynamically unfavorable. The quantum mechanical calculations [33] have been performed with Zn^{2+} as metal and the presence of PO_2^- has not been taken into account, therefore some caution should be applied when using these predictions.

It must be kept in mind that IR lacks specificity as the changes observed upon complexation might be similar to the ones observed upon DNA unfolding, and interpretation of IR spectra is not clear cut, because there is a strong overlap of the absorption of the bases (see Table 1). Therefore, a correlation cannot easily be found, if we limit the entire study to specific regions and to this technique. Other groups have already noted this fact for the case of DNA mixed with Hg^{2+} and Ag^+ systems [16, 17]. For instance, they came to the conclusion that Hg^{2+} was bound mostly on thymine and adenine, but they also quoted that no conclusive argument could totally rule out the possibility that guanine would also be a metal target. Thus we will mostly derive our conclusions on base complexation by using Raman spectroscopy. Our IR analysis will only be used to bracket which bases are most likely to be complexed. The low frequency region is not discussed because the subtraction of the water liberation mode at 600 cm^{-1} induced background distortions comparable to the signal.

Results obtained for various Cd/DNA ratios gradually ranging from 1 to 4, show that when Cd is added to DNA, major changes occur in the spectra only for a metal/DNA ratio >3:1. However,

TABLE 1. The Main Bands Observed in the FT-IR Spectra of DNA and DNA + Cd^{2+} at a Metal/DNA Ratio Equal to 3, at pH *ca.* 6–7 in the 2200–500 cm^{-1} Region[a]

DNA	DNA + Cd^{2+}	Assignment	Reference
1716vs	1713vs	T, C	23
1690m	1691s	G, A	23, 25, 26
1661s	1670s	T, A, C, G	17, 23, 25
1649s			
	1610m	C, A	23, 26
1572w	1582m	A, C	17, 23
1527w	1530m	C, G	17, 23, 26
1494ms	1493ms	C, G	23, 26
1483sh		A, G	23, 26
1457w	1462w		
1425vw	1425vw	A, C, G	23, 25, 26
1395m	1400w	A, C, G, T	23, 25, 26
1387m	1387m		
1374m	1374m		
1322w	1327w	A	23
1294m	1295m	C, A	23
1281m	1281m	T, C	23
1225m	1223m	PO_2^-	23, 24
1088s	1087s	PO_2^- + C–O	23, 24
1053s	1053s	C–O ribose + ose ring + phosphate	23, 24
1016m	1016m	C–O ribose	24, 25
971s	970s	C–C + P–O single bond	24, 25
937w	937w		
896m	896m	ribose–phosphate main chain	24, 25

[a]Entries: s strong, m medium, w weak, v very, bd broad, sh shoulder.

the strong and sharp band at *ca.* 1716 cm^{-1} is only slightly modified, indicating that DNA is not significantly denatured and remains mainly under the B form, whatever the concentration of Cd^{2+} (see Fig. 2). This assumption is in disagreement with ref. 14 where the results are explained by invoking a B to C transition for an identical Cd/DNA ratio. However, we do not know the IR spectra of the C form of pure DNA in aqueous solution because nobody knows how to generate C DNA in solution. The slight modification of the 1716 cm^{-1} mode could also be due to the presence of the Z form, but we have already indicated that no quantitative Z formation was measured.

We focused our efforts on the 1700–1500 cm^{-1} part where major perturbations are observed at *ca.* 1690 and 1660 cm^{-1} upon metal addition. We should keep in mind that denaturation is a marginal process because our UV data indicate only a few % increase of the extinction coefficient. From the literature, it is known that full denaturation of DNA is followed by a 50% increase of the absorbance coefficient

(ϵ_{260} = 9700 *versus* 6500 for the native form [35, 36]). From our measurements, the ratio between the DNA absorbance and the DNA + Cd(II) absorbance is equal to 0.95. Meaning that if a linear relationship is assumed among the absorptivities of native and denatured DNA, about 10% of the molecule will be denatured or under another form.

According to previous remarks, the change in the 1500–1700 cm^{-1} region might be related to the N_7 complexation on guanine as it is suspected to be the first and the main target. When Gua is linked to a metal via N_7, the C=O stretch at *ca.* 1700–1690 cm^{-1} is perturbed and exhibits a downward frequency shift, followed by an intensity change. This behavior has been noticed on 5′-GMP [18]. But in the case of DNA, the base pairing does not allow such a straightforward interpretation. Thus, these features are not a clear evidence that the complexation takes place on the guanine base [16]. The decrease of the 1716 cm^{-1} mode remains one of the best evidences that Cd^{2+} is either bound to Gua, Thy or both according to similar studies done on the DNA–Ag^+ system [16]. The intensity increase at *ca.* 1660 cm^{-1} is, at this stage, also a good evidence as an enhancement of this mode is expected (involving NH_2). Other perturbations are also observed in the rest of the spectra possibly involving guanine, but they will not be discussed for the reasons already invoked. Therefore, these spectral particularities cannot be used either to ascertain the first statement or to rule out coordination on other bases that might occur in addition to the already identified PO_2^- binding.

From these assumptions, we tried to assign spectral changes to other sites of complexation. We paid close attention in the region where adenine absorbs, namely at 1690, 1660–1640, 1600 and 1575 cm^{-1} [23–26] where changes partially correlate the results obtained with similar complexes on adenine [15]. For instance, the vibration located at *ca.* 1640 cm^{-1} is shifted to 1680–1700 cm^{-1} and exhibits a sharp intensity increase. Another vibration at *ca.* 1494 cm^{-1} can also be affected, but it is obscured by absorption from the rest of the molecule. However, the 1494 cm^{-1} band seems to gain some intensity as quoted in ref. 17 where complexation on Ade was reported. (Hg^+ coordination on adenine has induced similar perturbations of this particular mode [17].) As far as thymine is concerned, bands are present at *ca.* 1700 (C_2=O) and 1664 (C_4=O) cm^{-1}, and finally at 1400 and 1280 cm^{-1} where no specificity really exists for these two latter bands. From Raman studies, as it will be discussed below, there is some evidence of C=O perturbations in general. The absorptivity decrease at *ca.* 1716 cm^{-1} followed by an absorptivity increase at 1690 cm^{-1} could be related to an interaction located on the C_2=O, as has been mentioned above. However, this spectral change may simply be induced from the unavoidable increase of the ionic strength upon addition of metal salts rather than to pure coordination. Only the presence of a new feature at *ca.* 1550 cm^{-1} could be an indication of the binding of Cd^{2+} to thymine. This statement is true only if we assume that the similar spectral change observed on the uridine complex with Ag^+ can be correlated to the Cd^{2+} complex with thymine [19]. This hypothesis has been discarded because our own results on 5′-TMP did not confirm this assumption and we have estimated that the signal was too weak. In addition, guanine may induce an identical effect and already strong evidence indicates that this complexation takes place. Due to thermodynamic consideration, only the coordination on C_2=O is feasible and therefore C_4=O interaction or pure N_3H substitution seems unlikely. Coordination on C_4=O could also be invoked to explain the spectral change plotted in Fig. 2 and cannot be ruled out by simple inspection of IR data [17].

Guanine and cytosine are strongly related. Except for the two bands at *ca.* 1494 and 1294 cm^{-1} only due to cytosine, all the other features at *ca.* 1527, 1425, 1374 cm^{-1} derive from both residues. We do not think that cytosine is a major target for metal, because the strong 1494 cm^{-1} mode is almost cancelled upon mixing 5′-CMP with Cd^{2+}, but it remains untouched in DNA. In addition, thermodynamics show that a −19 kcal/mol barrier should be suppressed before the most reactive cytosine site becomes available, this being a value far larger than the −7.5 kcal/mol for the denaturation energy. It should be reminded that in the case of denaturation, the 1716 cm^{-1} mode will disappear. Thus, we think that cytosine is not concerned.

It is obvious that IR is not a good technique for investigating this type of complex except to investigate the PO_2^- moiety. Investigations on compounds such as phosphodiester in order to better characterize the affinity of PO_2^- have been considered, but readily discarded due to overwhelming experimental difficulties. Such experiments have been done on dimethylphosphite with lead and did not bring conclusive results [37]. At this stage, the spectra of DNA mixed with Cd(II) show that the bases are affected. The Gua, Ade and Thy are the targets and after inspection of the data at various concentrations, the first perturbation observed appears at 1690 and *ca.* 1660 cm^{-1}. This starts to be detectable for a Cd(II)/DNA molar ratio of 2:1 at 1690 cm^{-1} and for a Cd/DNA molar ratio of 3:1 at 1660 cm^{-1}. No conclusions can be drawn from that, except that at least two bases must be involved in coordination and should be the combination of either AT, AG or GT in order to explain the IR data. Of course, the three of them can also explain the data.

TABLE 2. The Main Bands Observed in the Raman Spectra of DNA and DNA + Cd^{2+} at a Metal/DNA Ratio Equal to 3, at pH *ca.* 6–7 in the 2000–600 cm^{-1} Region[a]

DNA	DNA + Cd^{2+}	Assignment	Reference
1674–1675bdm 1663bdm	1669bdm	T	21, 38–42
1602w		A	21, 38–42
1576m	1576m	G, A	21, 38–42
1510w	1510w	A	21, 38–42
1485ms	1485ms	G, A	21, 38–42
1450w	1446–1448w	R	21, 38–42
1417mw	1418mw	R, A, G	21, 38–42
1372s	1371s	T, A, G	21, 38–42
	1357m	A, G complexed	41
1337s	1339s	A, G	21, 38–42
	1329sh	A complexed	41
1300m	1301m	G, A	21, 38–42
1254m	1254m	C, A, T	21, 38–42
1093m	1094m	PO_2^-	21, 38–42
1047m	1047m	NO_3^-	
1014bdw	1014bdm	C–O ribose	21, 38–42
	875vw	phosphodiester C form	21, 38–42
830sh	831sh	phosphodiester B form	21, 38–42
784ms	785ms	C, T	21, 38–42
749m	749m	T	21, 38–42
735	735	A + plasma line (506.19 nm)	21, 38–42
682bdw	682bdw	G, T	21, 38–42

[a]Entries: s strong, m medium, w weak, v very, bd broad, sh shoulder.

Raman Results

We carried out Raman experiments at pH *ca.* 6–7 with a Cd/DNA ratio of 3, 2, 1 and 1/200. For the 3:1 ratio, we wanted to confirm the early IR interpretation that suggests interaction on both guanine, adenine and possibly thymine. The spectral features are assigned from recent reviews [38, 39] and articles [21, 40–42]. All the spectra are recorded in the presence of 0.01 M $NaNO_3$ used as an internal standard. During the preparation of the sample, no precipitation occurs as it is seen with DNA complexed by copper and lead [21]. The results are summarized in Table 2 and only the native DNA and Cd(II)–DNA 1/3 spectra are shown for the reasons already invoked (see Figs. 4 and 5).

First, we concentrate our efforts on the 830–784 cm^{-1} doublet which arises from the ν_{as} of the O–P–O diester group and from a C + T contribution respectively (see Fig. 4). When DNA is mixed with metal, it may undergo transitions. This will be indicated by the presence of a very intense peak at 810 cm^{-1} if a B to A transition takes place [38] and at 800 cm^{-1} for a Z form [41, 42]. The appearance of the A structure is easily noticed because

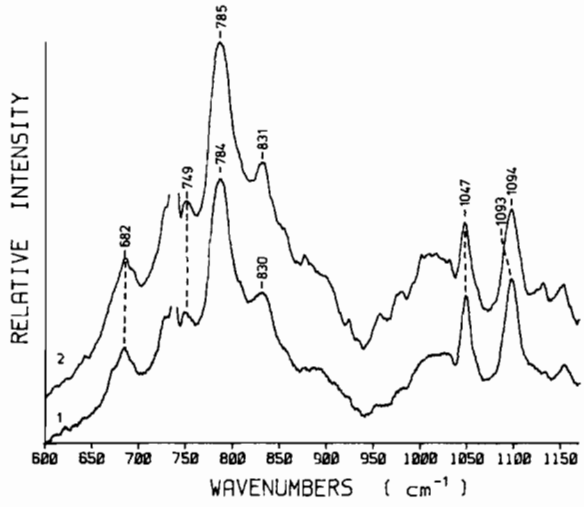

Fig. 4. Raman spectra of DNA (curve 1) and DNA + Cd^{2+} at a metal/DNA ratio equal to 3 (curve 2) at pH *ca.* 6–7 in the 600–1150 cm^{-1} region.

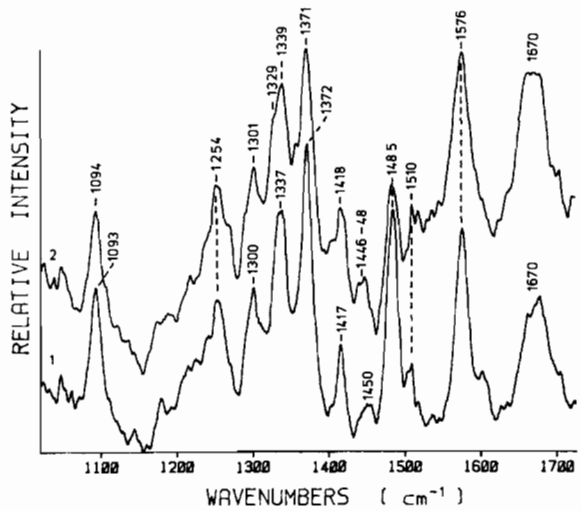

Fig. 5. Raman spectra of DNA (curve 1) and DNA + Cd^{2+} at a metal/DNA ratio equal to 3 (curve 2) at pH *ca.* 6–7 in the 1050–1700 cm^{-1} region.

of the large intensity increase observed at *ca.* 810 cm^{-1}. The peak height becomes then comparable to the 785 cm^{-1} band.

Upon metal complexation, the C form for DNA has also been invoked in order to explain modifications [14, 43]. In this latter case, a new peak appears at *ca.* 875 cm^{-1} [44, 45]; this is not the case, as shown in Fig. 4. Besides no significant upward shift is observed on the PO_2^- stretching mode (usually located at *ca.* 1104 cm^{-1} instead of *ca.* 1094 cm^{-1}). One can assign the shoulder on the right hand of the PO_2^- stretch to this particular geometry, but it is unlikely. Therefore it seems reasonable to claim that the molecule is mostly under its native B form.

We ruled out the presence of the Z form by comparison with data obtained on model compounds published in the literature [41,42]. Usually, in addition to the already quoted disappearance of the 830 cm^{-1} peak at the expense of a new shoulder at *ca.* 800 cm^{-1}, the Z form is characterized by a frequency shift of the 682 cm^{-1} peak to *ca.* 622 cm^{-1} (this mode is related to Gua). None of these modifications is monitored. Additional features found between *ca.* 1300 and 1400 cm^{-1} have been attributed to the complexation of either Gua or Ade (see Table 2) and could also be attributed to the presence of a Z conformation [41]. However, after close inspection, this was discarded as a poor correlation was found among all the spectral features related to a Z form. This completes our early IR interpretation, where some ambiguities could have remained.

The fact that the DNA is still under its B conformation is not surprising. Literature mentioned that the presence of divalent metals is not sufficient to induce a transition and if that happens, it may be a tricky process [46]. The conformation is often dependent on the metal concentration and, upon metal addition, can pass through various geometries and finally end up back with the original conformation, meaning that the details of the process are in part hidden.

Only one question is left unanswered, namely the measure of the degree of denaturation. About a 20% intensity increase is observed on the C + T mode at *ca.* 784–785 cm^{-1}. This increase is also observed upon thermal denaturation. There is no direct relationship between the relative intensity of the 785 cm^{-1} of the native and complexed DNA and the degree of complexation, but this band is weakly affected as long as the Cd(II) is kept below the critical value ratio Cd(II)/DNA = 3/1. The 830 cm^{-1} mode did not significantly change, meaning that as mentioned before, DNA is under its B form. This will indicate that the base stacking is retained and similar conclusions have been revealed on Pb^{2+}–DNA complexes [21].

Then, we investigated the symmetric PO_2^- stretch. This vibration is known to be fairly insensitive to the DNA secondary structure, therefore a change will be an evidence of an interaction. No frequency shifts are observed in the spectra of the complex, but a decrease in intensity is computed after normalization to the 1047 cm^{-1} mode of NO_3^- (about 20%). A broadening of the band is also observed, meaning that possibly two non-equivalent PO_2^- components are present. This correlates well with the previous conclusions derived from IR studies.

Other major differences are readily seen from inspection of the data in Fig. 5. Especially between 1400 and 1650 cm^{-1}, where the modes involved mostly derive from guanine and adenine [47,48]. The modifications cannot be assigned to denaturation, as we have already established that it is a marginal effect. In addition, upon thermal denaturation these particular modes are intensity enhanced [38] and in this work a sharp decrease in intensity is seen. The 1485 cm^{-1} vibration is accounted for by 26% of N7–C8, 27% C4–C5 plus 12% N1–C2 motion of Gua and should partly disappear upon complexation, as observed during the protonation of guanine [38]. This is obviously the case. The 1576 cm^{-1} mode is accounted for by 28% of N3–C4, 20% of N9–C4 and 17% of N9–C4–C motion of Gua. This latter band presents some kind of mixing with the 1597–1600 cm^{-1} adenine mode (see Fig. 5 and Table 2).

The 1576 cm^{-1} guanine vibration is slightly sensitive to bonding. However under denaturation, an intensity increase is observed. Our data suggest that there is a small intensity loss (about a 7% loss is recorded, located within the experimental error). Therefore we conclude that the combination of N_7 complexation and denaturation partially hides the real intensity changes as they are predicted to be opposite. We ruled out the N1 substitution even if it has been suggested that an intensity decrease of this particular vibration is associated to N1 binding [21,38]. We would rather think that the intensity decrease of this vibration is due to the previously reported coordination on N_7 as the expected spectral change is identical [21,38].

A semi-quantitative approach can be used by plotting the behavior of the normalized 1485 cm^{-1} mode of Gua (+ a small contribution of Ade) that seems the most affected by the complexation as a function of the Cd(II) concentration. The plot (Fig. 6) suggests that for low Cd^{2+} concentrations, no significant complexation takes place, but suddenly at a Cd/DNA ratio of 3, a sharp intensity change is experienced by the Gua 1486 cm^{-1} vibration. This seems to be an indication that a cooperative effect is present and is consistent with the results reported in ref. 14. This cooperativity has

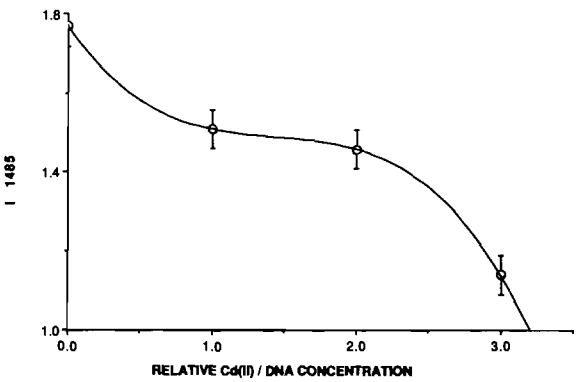

Fig. 6. The intensity of the DNA 1485 cm^{-1} Raman band as a function of Cd^{2+} concentration.

been recently mentioned [49] and, in our sense, this is not detected with UV techniques because base coordination does not induce any variation in the absorption.

According to previous assumptions [33, 34], adenine should be the second target. One can notice that the 1600 cm^{-1} mode is cancelled upon complexation. This vibration involves mostly the N_7 position (C5–C6 37%, C5–N7 20% motion), but other spectral features like the 1372 cm^{-1} (ring), 1337 cm^{-1} (39% N7–C5, 12% C8–N7 motion) are obviously perturbed. The modifications are more subtle but can be monitored and the assignment is done (*vide infra*) in Table 2. N_1 of Ade will also be a good candidate for complexation, but if N_7 is accessible in the major groove, N_1 will be available only after denaturation which has been considered negligible so far. Therefore, from this particular statement, the N_1 coordination is rejected. It should be added that the cancellation of the 1600 cm^{-1} band occurs simultaneously with the cancellation of the 1486 cm^{-1} Gua mode.

The only site left is the C_2=O of the thymine residue. Small frequency shifts, band broadenings and intensity increases are observed in the 1670 cm^{-1} region, but as already mentioned, this can be easily associated to a change in the ionic environment of the C_2=O moiety. If the spectroscopic measurements on guanine and PO_2^- have been easily monitored, it is not possible to find quantitative marker bands for thymine and adenine.

The combination of the two techniques can prove that upon metal addition, PO_2^- is the first target, quickly followed by complexation on both Ade and Gua in a cooperative way. Spectral changes associated to Thy are attributed to the variation in the ionic strength experienced by the exposed C_2=O. These modifications recorded at low metal concentrations on both the Raman and IR spectra in the 1700–1650 cm^{-1} region are attributed to Thy and cannot be confounded with coordination on other bases when both techniques are used. (For instance, in Raman spectroscopy the 1650–1700 cm^{-1} is only concerned with Thy modes.)

Conclusions

From spectroscopic measurements, the binding sites of Cd^{2+} have been identified to be on both bases and PO_2^- moieties. Cd^{2+} is known to be a borderline case between the hard and soft species as defined by the Lewis theory. Therefore this result is not surprising. However, its affinity for PO_2^- is slightly larger than the one for the bases, but remains low. No significant denaturation is detected, and even a large amount of Cd^{2+} does not induce a conformation transition. When bound to the base residues, the cation seems to complex first on N_7 of guanine and N_7 of adenine to a lesser extent, inducing a slight denaturation of the DNA. The C_2=O is not directly involved, but due to the change of its ionic environment, spectral lines appear in addition to the ones related to complexed Gua and Ade. The complexation starts to be quantitative for a Cd^{2+}/DNA ratio between 1 and 2 on PO_2^- and for a ratio between 2 and 3 on bases and seems to be a cooperative process for these latter.

At a Cd/DNA ratio = 1/200, the concentration usually met in real biological systems for toxicological tests, the IR, UV and Raman spectra of the complexes and the native DNA are similar. This suggests that at these concentrations, the interaction, if any, between this molecule and the cation is purely electrostatic. As a consequence, it seems unlikely that the carcinogenic effect of Cd^{2+} derives from its direct action on DNA, but should rather derive through other types of mechanisms.

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