Unusual Catalyst Concentration Effects in the Hydrolysis of Phenyl Phosphate Esters and of DNA: A Systematic Investigation of the Lanthanide Series[1]

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Saturation kinetics are measured with all lanthanides and bis(nitrophenyl) phosphate BNPP as substrate. They show rather constant K_M values; the k_{cat} values, however, increase by up to 66 times for La^{3+} to Er^{3+} and decrease again for Yb^{3+} and Lu^{3+}. With all lanthanides, hydrolysis of the intermediate mononitrophenyl phosphate NPP is 2−30 times faster than that of BNPP. The k_{cat} values measured with BNPP correlate with the ion diameter of the lanthanides, in line with accepted mechanisms but with the notable exception of the higher lanthanides. A similar correlation holds for the cleavage rates with plasmid DNA, with striking differences again observed with the higher lanthanides, however. Thus, a concentration increase from 5×10^{-5} to 1×10^{-2} M leads to 64% and 84% more DNA cleavage with La^{3+} and Pr^{3+}, respectively, but to up to 68% less DNA cleavage, respectively, but with Yb^{3+}, Tm^{3+} or Lu^{3+}. In contrast to the BNPP cleavage, saturation kinetics derived k_{cat} values with DNA change little with the used cation, which on the other hand led to larger variations in the K_M parameters. Preliminary UV and CD studies with plasmid DNA indicate lanthanide-induced conformational changes with pseudo-first-order rate constants 10−100 times higher than the cleavage rate under the same conditions. Again, Yb^{3+} shows different effects than Eu^{3+}. The unusual behavior of the higher lanthanides is discussed on the basis of cation clustering, which, in contrast to earlier assumptions by Bamann et al., leads to *diminished* activities. Addition of salts such as of NaCl or $MgCl_2$ leads to distinct decrease of catalytic effects of for instance Eu^{3+}. The corresponding rates correlate well with Debye−Hückel ionic strength parameters. These as well as the effects of added amines are in line with a simple competition mechanism of the added cations for the anionic substrates.

Cleavage of nucleotides and of DNA or RNA by metal catalysts is an area of much current activity.[2] In particular, lanthanide ions and their complexes are known to be excellent catalysts for the hydrolysis of biozide-type phenyl phosphate esters, of DNA, and of related oligonucleotides.[3] Different ions such as Eu^{3+} or Yb^{3+} have been reported to show different efficiency,[4] but to our knowledge no quantitative and systematic study with all lanthanides has been carried out until now. In particular, the effect of varying Ln^{3+} concentrations has only been studied with Eu^{3+}, in which case we found regular saturation kinetics of the Michaelis−Menten type, both with phenyl phosphate esters[5] and with plasmid DNA.[3d] Preliminary experiments suggested that some lanthanides show a very surprising opposite concentration dependence (see below). Early reports claimed that gels, which form readily with many lanthanides at pH above 6.0,[6] are also responsible for the observed rate accelerations.[7,8] The aim of the present paper is the elucidation of these factors, which will shed light on the mechanisms involved and help to design better chemical nucleases of truly hydrolytic nature.

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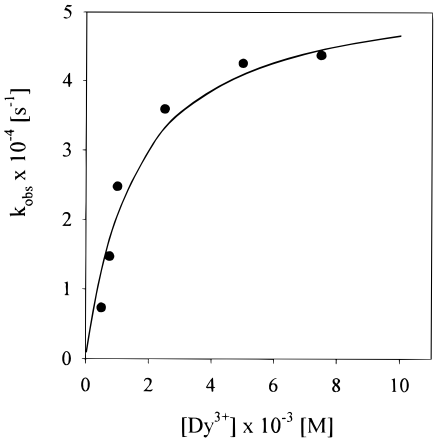

Figure 1. Saturation kinetics of the BNPP cleavage with Dy^{3+}; for conditions, see Table 3.

Table 1. Kinetic Data for the Hydrolysis of BNPP Derived from Michaelis−Menten-type Nonlinear Curve Fitting and Ionic Radii of the Lanthanides[a]

lanthanide	ionic radius[10] [Å]	K_M [M] × 10^3	k_{cat} [s^{-1}] × 10^4
La^{3+}	1.061	2.6 (±3%)	0.13 (±2%)
Pr^{3+}	1.013	1.6 (±14%)	0.63 (±4%)
Eu^{3+}	0.950	2.8 (±12%)	2.5 (±5%)
Gd^{3+}	0.938	1.6 (±23%)	5.4 (±7%)
Dy^{3+}	0.908	2.4 (±15%)	5.5 (±6%)
Er^{3+}	0.881	3.3 (±29%)	8.6 (±12%)
Tm^{3+}	0.869	–	–
Yb^{3+}	0.858	2.3 (±23%)	7.8 (±8%)
Lu^{3+}	0.848	3.2 (±54%)	5.0 (±24%)

[a] Rates measured at 50 °C in 0.01 M EPPS, pH 7.0.

Results and Discussion

Hydrolysis of Phenyl Phosphates. The pseudo-first-order rate constants of nitrophenolate liberation from bis(nitrophenyl) phosphate (BNPP), followed as usual[5] at pH 7.0, shows Michaelis−Menten type saturation curves with all lanthanides except with Tm^{3+} and Lu^{3+}, as illustrated with Dy^{3+} in Figure 1. With Tm^{3+} and to a lesser degree with Lu^{3+} turbidity of the solutions made it difficult or impossible to follow the reactions.

The Michaelis−Menten type of pretransition state association constants K_M (Table 1) show small changes between the lanthanides of between only 1600 and 3200 M^{-1}. The K_M values do not correlate with any property of the cations. The corresponding free energy, $\Delta G = 19$ or 22 kJ/mol, respectively, agrees with the formation of three to four salt bridges between substrate and the triply charged cation: analysis of over 100 ion pairs in water have yielded an increment of 5 ± 1 kJ/mol in water.[9] These are essentially independent of the nature of the cation or anion, again in accord with the small K_M variations observed here. In contrast, the catalytic rate constants k_{cat} derived from the saturation kinetics, with the exception of Lu^{3+} and Yb^{3+}, correlate well with the ionic diameter of the cations, independent of the underlying coordination number.[10] (Figure 2). Such a correlation has also been found for the catalytic effect of other metal cations[11] in substitution reactions at phosphorus. The ion diameter dependence is in accord with most of the known[12] possible mechanisms for the observed rate

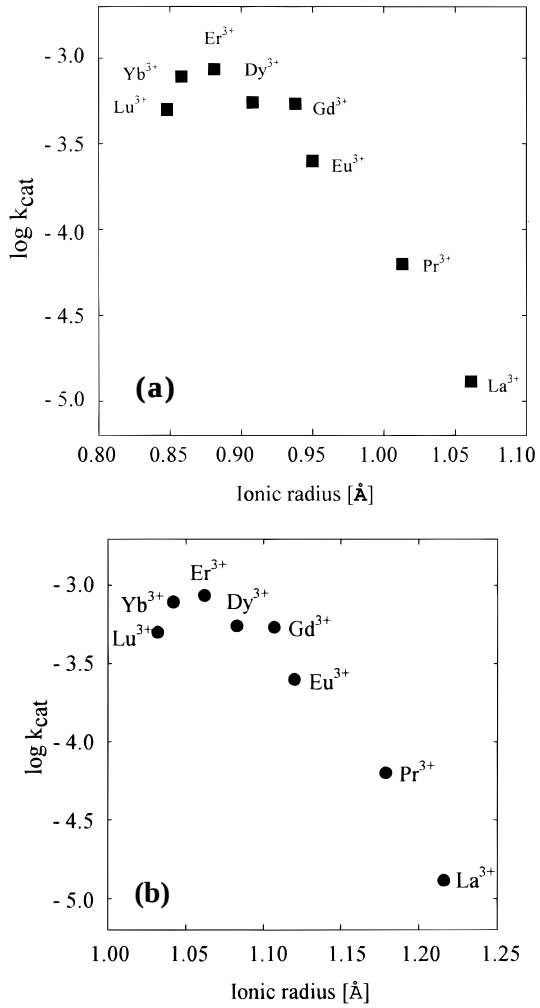

Figure 2. Dependence of the BNPP hydrolysis k_{cat} values on the ionic radii[10] of lanthanides. (a) Correlation with ionic radii for coordination number CN = 6 and (b) for CN = 9. For conditions, see Table 3.

accelerations, as both the water and the phosphoryl activation should be enhanced by higher charge density of the cation. In view of their small ionic radii, the unexpectedly *lower* efficiency of the higher lanthanides Lu^{3+} and Yb^{3+} is likely the result of aggregations, which are known to occur even before they become visible.[6] As will be seen more dramatically with the DNA hydrolysis (see below) such aggregations have an effect *opposite* that believed earlier.[7] Rate increases similar to those with BNPP, although smaller in magnitude, were observed with the mononitrophenyl ester NPP (Table 2). No saturation kinetics were studied here, as the major point was to secure that the hydrolysis of NPP is faster with all lanthanides than that of BNPP; it was found earlier[5] that the BNPP reaction is the rate-determining step.

The effect of other cations with small charge densities on the Eu^{3+}-catalyzed rates was studied with sodium and magnesium chloride (Table 3) and found to be a linear function of the corresponding Debye−Hückel ionic strength parameters[13] (Figure 3). The slope of the correlations with added Na^+ or Mg^{2+} is not very different and is in line with simple ion pair competition between Eu^{3+} *and* Na^+ (or Mg^{2+}) and the substrate BNPP, and with the relatively small variations in ion pairing

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Table 2. Rate Constants for NPP Hydrolysis with Lanthanides[a]

lanthanide	k_{obs} $[s^{-1}] \times 10^3$	$[k_{obs}/k_o]$ $\times 10^4$	relative enhancement $\times 10^{-6}$
–	$k_0 = 6.00 \times 10^{-5}$	–	(1×10^{-6})
La^{3+}	0.3	0.5	0.5
Pr^{3+}	0.7	1.2	1.2
Eu^{3+}	0.8	1.4	1.4
Gd^{3+}	1.5	2.4	2.4
Dy^{3+}	1.7	2.9	2.9
Er^{3+}	1.7	2.9	2.9
Tm^{3+}	1.6	2.7	2.7
Yb^{3+}	1.1	1.8	1.8
Lu^{3+}	–[b]	–	–

[a] Conditions: $[Ln^{3+}] = 0.01$ M, 50 °C, pH 7.0 in 0.01 M EPPS buffer. [b] Not detecable because of precipitation.

Table 3. Influence of Metal Ions on the Eu^{3+}-Catalyzed Hydrolysis of BNPP[a]

added salt	concentration $[M] \times 10^3$	k_{obs} $[s^{-1}] \times 10^4$	reactivity against Eu alone [%]
–	–	1.67	100
NaCl	1	1.59	98
	5	1.53	94
	50	1.26	78
	500	7.85	48
$MgCl_2$	1	1.50	92
	5	1.28	79
	50	0.81	49
	500	0.27	16

[a] Conditions: $[Eu^{3+}] = 5 \times 10^{-3}$ M, 50 °C, pH 7.0 in 0.01 M EPPS buffer.

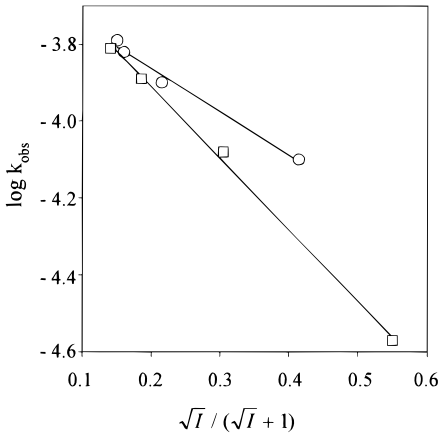

Figure 3. Salt effects. BNPP hydrolysis rate constants with Eu^{3+} versus ionic strength (○, Na^+; □, Mg^{2+}). For conditions, see Table 3.

Table 4: Influence of Amines on BNPP Hydrolysis with Eu^{3+} [a]

ligand	k_{obs} $[s^{-1}] \times 10^4$	reactivity against Eu alone [%]
–	1.67	100
ethylendiamine	0.22	12
propylendiamine	0.29	17
Cyclen	0.64	38
Trpn	0.83	49

[a] Conditions: 50 °C, pH 7.0 in 0.01 M EPPS buffer, $[Eu^{3+}] = 5 \times 10^{-3}$ M, [ligand] $= 5 \times 10^{-2}$ M.

due to the nature of ions as mentioned above. Such competition must also be responsible for the rate-decreasing effect of some polyamines (Table 4), which with some transition metal ions can produce opposite accelerations.[14] These observations are significant for the design of ligands, which are only expected to further increase the efficiency of lanthanides if the ligands are neutral, bind stronger to the cation, and/or have additional side groups which can act as cofactors.[15] The observed rate decreases caused by added salts also limit the use of buffers, particularly of those with multiple charges.

Table 5. DNA Cleavage Rates [%RF I][a] with Lanthanides at Different Metal Concentrations

metal	concentration [mol/L] $\times 10^3$ 10	5	1	0.5	0.1	0.05
La^{3+}	58	74	83	87	92	95
Ce^{3+}	63	69	81	86	95	98
Pr^{3+}	43	60	63	65	73	79
Eu^{3+}	54	59	63	64	82	85
Gd^{3+}	54	56	64	67	76	86
Dy^{3+}	43	50	58	63	68	77
Er^{3+}	47	49	51	53	62	74
Tm^{3+}	54	50	39	32	26	27
Yb^{3+}	56	49	43	44	23	18
Lu^{3+}	44	40	32	31	31	20

[a] The values of [% RF I] are corrected for different stainability of RF I (supercoiled form) and RF II (open circular form) and the amount of RF II in the DNA. Relative error is ±2.5%. Conditions: pH 7.0 in 0.01 M EPPS buffer, incubation time 2 h, 37 °C, [DNA] = 1.9 × 10−5 M (bp).

Cleavage of DNA. The cleavage of the supercoiled plasmid DNA pBR322 form RF I was followed by its conversion to the open circular form RF II as described earlier[3e,13,16] Careful calibration and densitometry after electrophoretic separation of RF I and RF II allowed us to derive clean first-order rate constants for this biopolymer as well. The hydrolytic nature of the process is evident (i) by the absence of any other cleavage products such as RF III which are observed with redox reactions,[15b] (ii) by the absence of effects of radical scavengers, initiators, or added hydrogen peroxide,[3e,15b] (iii) by the observation of typical hydrolysis products from dinucleotides,[15b,17] and recently (iv) by successful religation of cleaved plasmid DNA.[15b]

The cleavage rates observed under the same conditions (Table 5) showed with the higher lanthanides Tm, Yb, and Lu a hitherto unknown decrease with increasing catalyst concentration. Using our invariably pseudo-first-order rates established earlier,[3e,14] with up to seven points with linear correlation coefficients $r <$ 0.98, we calculated rate constants for the present whole lanthanide series from the single point measurements listed in Table 5. Lanthanides such as La^{3+} or Er^{3+} Michaelis−Menten type saturation curves showed for the rate constant k/concentration profiles (Figure 4), similar to those obtained earlier[3e] with Eu^{3+}, allowing us to derive k_{cat} and K_M values (Table 6). The three cations Tm^{3+}, Yb^{3+}, and Lu^{3+} showed an "inverse" curve (Figure 5), indicating that the lanthanides with the highest charge density are only the most effective ones if they are applied at very low concentrations, as expected. Indeed, at $[Ln^{3+}] = 1$ mM one observes a roughly linear correlation between cleavage rate and ion diameter[10] (Figure 6), similar to the correlation found with the BNPP (Figure 2). However, there are no such correlations with the K_M and k_{cat} values derived from the saturation kinetics (Table 6). In contrast to the BNPP hydrolysis, the K_M values decrease significantly from La^{3+} to Dy^{3+}, whereas the k_{cat} remains almost constant within error.

The effects of added cations with small charge densities such as Na^+ or Mg^{2+} are similar to those observed with BNPP. There is also a linear Debye−Hückel dependence on salt concentration

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(16) Hettich, R.; Schneider, H.-J. *J. Chem. Soc., Perkin Trans. 2* **1997**, 2069.
(17) See also ref 8a.

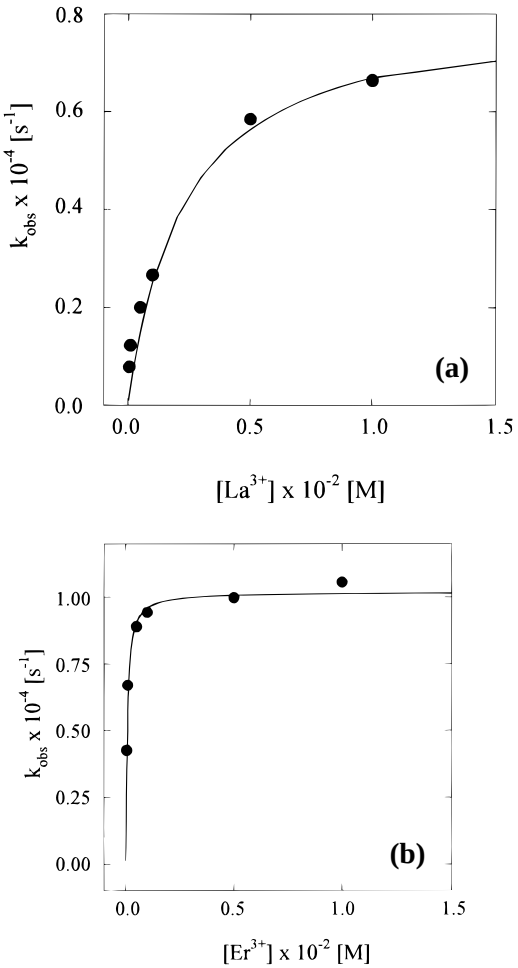

Figure 4. Saturation kinetics of DNA hydrolysis with La^{3+} (a) and Er^{3+} (b). For conditions, see Table 5.

Table 6. Kinetic Data of DNA Cleavage from Nonlinear Curve Fitting[a]

metal	k_{cat} [s^{-1}] × 10^5	K_M [M] × 10^4	enhancement
La^{3+}	9.24 (±10%)	19.8 (±3%)	9×10^{-6}
Ce^{3+}	6.96 (±5%)	12.4 (±17%)	1×10^{-7}
Pr^{3+}	8.90 (±12%)	1.32 (±13%)	9×10^{-6}
Eu^{3+}	6.97 (±6%)	1.03 (±10%)	7×10^{-6}
Gd^{3+}	8.13 (±5%)	1.53 (±5%)	8×10^{-6}
Dy^{3+}	10.0 (±8%)	1.18 (±9%)	1×10^{-7}
Er^{3+}	10.2 (±2%)	0.64 (±1%)	1×10^{-7}

[a] For conditions, see Table 5.

(Figure 7), indicating competing ion pairing of Na^+ and Mg^{2+} with the DNA backbone phosphates. Also for practical applications one should note that Mg^{2+} in concentrations as low as 5×10^{-5} M decreases the catalytic efficiency of Eu^{3+} by at least 10%.

What could be the reason for the dramatic efficiency decrease with Tm^{3+}, Yb^{3+}, and Lu^{3+} at higher concentrations? Microscopic inspection showed no insoluble material after incubation of plasmid DNA with these cations at pH 7.0, although measurements by Suzuki et al.[6] yielded a drop in the precipitation pH from 7.58 for La^{3+} to 5.85 for Lu^{3+} as a function of the ion diameter. However, there is evidence for cluster formation of cations such as Ce^{3+}, which Komiyama et al.[18] characterized by potentiometric measurements as $(Ce_3OH_5)^{4+}$

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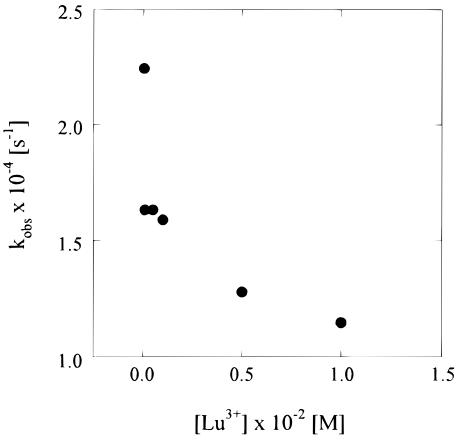

Figure 5. Inverse catalyst concentration profile with Lu^{3+} and DNA. For conditions, see Table 5.

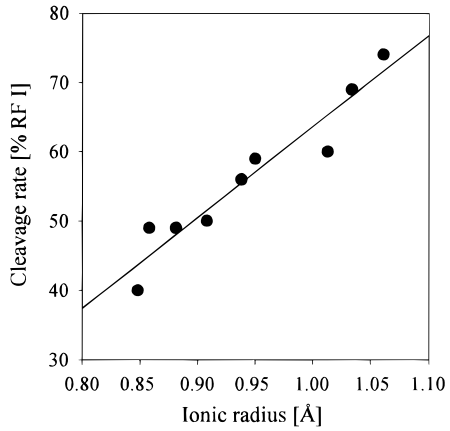

Figure 6. Cleavage rate [%RF I] versus ionic radius. For conditions, see Table 5; $[Ln^{3+}] = 5 \times 10^{-3}$ M.

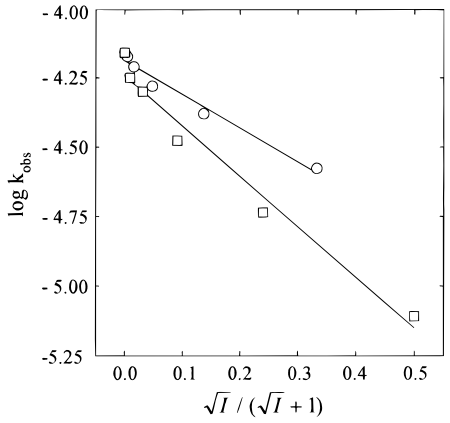

Figure 7. Salt effects: DNA hydrolysis rate constants with Eu^{3+} versus ionic strength (○, Na^+; □, Mg^{2+}). For conditions, see Table 5.

species. The resulting effective decrease in the catalytically active Ln^{3+} concentrations may thus be the origin of the observed effects with the higher lanthanides.

Komiyama et al.[19] have used addition of cyclodextrins to solubilize Pr^{3+}, for examples, even at pH 11.5, and report fully retained catalytic activity against DNA and peptides in the case of Ce^{4+}. In our hands, addition of γ-cyclodextrin in concentra-

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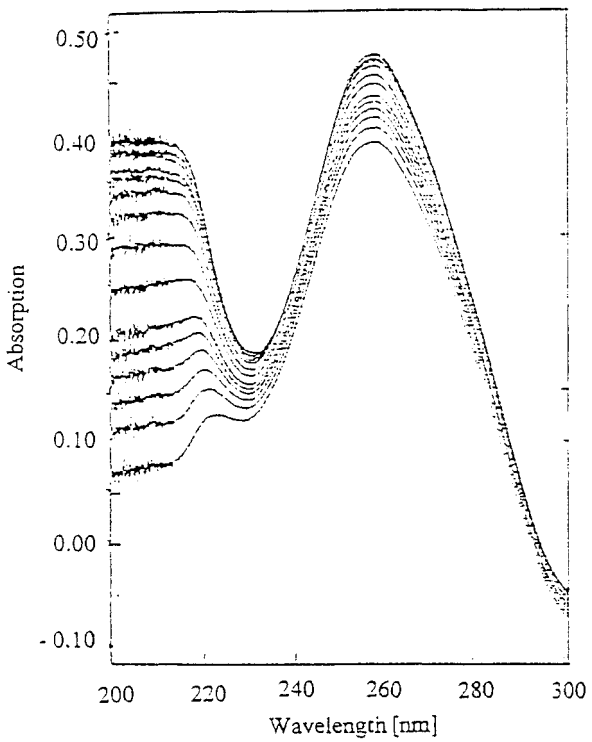

Figure 8. UV−vis spectra of DNA after adding 5×10^{-5} M Eu^{3+}.

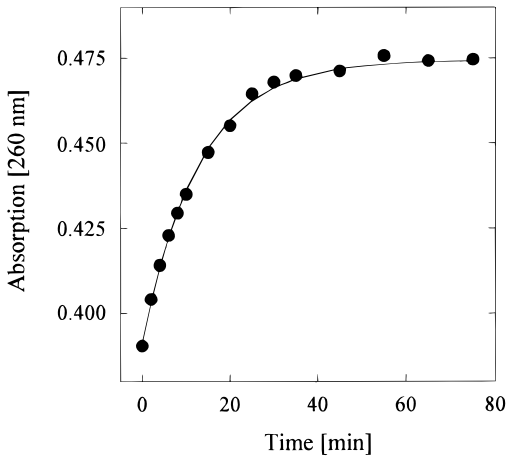

Figure 9. Maximum absorption from UV spectra versus time for 5×10^{-5} M Eu^{3+}; experimental points (●) and computer fit for first-order reaction.

tions from 0.0001 to 0.01 M showed decreased activity for Eu^{3+}, Pr^{3+}, and Yb^{3+}.

UV and CD Investigations of DNA−Lanthanide Interactions. It is known that supercoiled plasmid DNA can at least partially interconvert from the dominating right-handed B form to the left-handed Z form, particularly in dGdC-rich sequences.[20] Such interconversions, which can imply intermediate forms, are also promoted by cations, particularly by those with high charge densities.[20] The questions raised then, were as follows: (i) does the native B form undergoes cleavage by the lanthanides; (ii) can a cation-induced conformational change be rate-determining;

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Table 7: Comparison of Rate Constants for Widening and for Cleavage of DNA from UV Measurements[a]

	Eu^{3+}	Yb^{3+}
k (widening) [s^{-1}]	1.3×10^{-3}	1.1×10^{-3}
k (cleavage) [s^{-1}]	2.3×10^{-5}	2.4×10^{-4}

[a] $[Ln^{3+}] = 5 \times 10^{-3}$ M, [DNA] $= 1.9 \times 10^{-5}$ M, 0.01 M EPPS, pH 7.0, 37 °C.

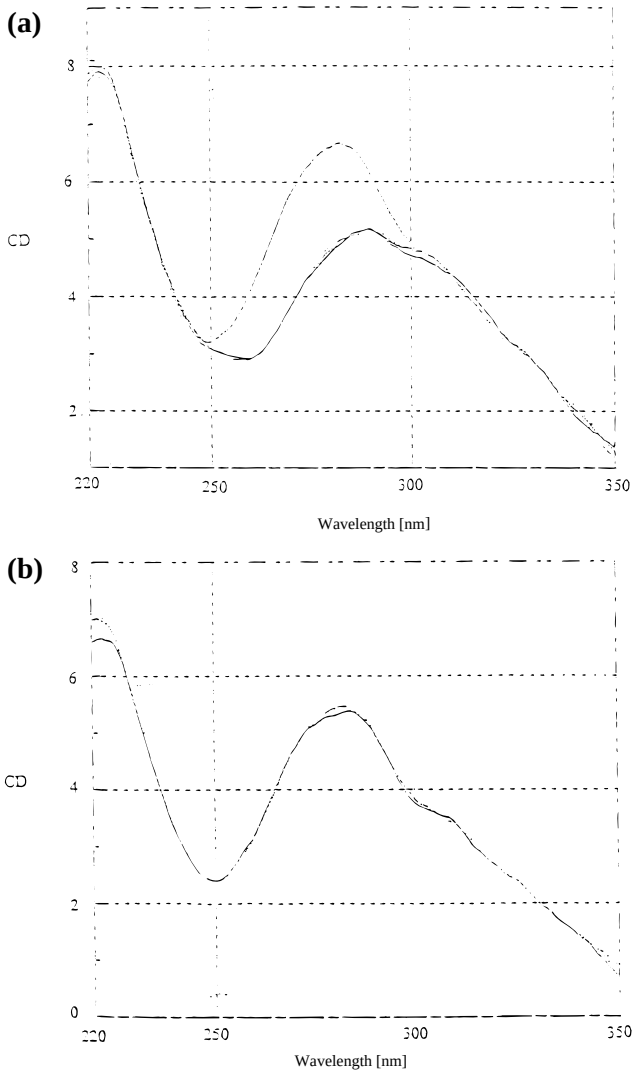

Figure 10. CD spectra of (a) DNA without metal and with 5×10^{-5} M Eu^{3+} and (b) DNA without metal and with 5×10^{-5} M Yb^{3+} [upper traces, DNA alone; lower traces, after addition of metal each after 1, 10, and 20 min, respectively].

and furthermore (iii) is the exceptional concentration dependence of the higher lanthanides related to specific conformational effects? A preliminary spectroscopic study was undertaken to clarify these points.

Incubation of plasmid DNA with 5×10^{-5} M $EuCl_3$ or $YbCl_3$ under the usual cleavage conditions leads to slow changes in the UV absorptions (Figure 8), which can be fitted to pseudo-first-order rate constants k (Figure 9). The results (Table 7) show that this conformational change is substantially faster than cleavage under the same conditions and that the corresponding rates are similar with Eu^{3+} and Yb^{3+}. However, an increase of the lanthanide concentration to 1×10^{-4} M leads only with Eu^{3+}, and not with Yb^{3+}, to a further time-dependent change ($k = 6.53 \times 10^{-2}$ s^{-1} with Eu^{3+}). Control experiments with

native DNA samples containing up to 2.5% RF II showed no difference in the UV spectra in comparison to RF I + Eu^{3+}.

The observed UV hypochromicity decrease is in line with a destacking and widening of the native supercoiled form. Furthermore, that Yb^{3+} in contrast to Eu^{3+} is not efficient in concentrations above 5×10^{-5} M suggests clustering effects beginning even at high dilution.

A striking difference between Eu^{3+} and Yb^{3+} is also seen in the CD spectra taken under the same conditions (Figure 10). With Yb^{3+} there is no change even after 20 min, whereas Eu^{3+} induces a rapid change. It seems to be that Eu^{3+} could effect a fast B−Z transition and Yb^{3+} could not.

The preliminary conclusion is that with Eu^{3+} and related lanthanides there are at least biphasic transitions of DNA conformations prior to hydrolysis.

Experimental Section

Disodium 4-nitrophenyl phosphate (NPP),[21] bis(4-nitrophenyl) phosphate (BNPP), pB*R*322 plasmid DNA, reagent grade inorganic salts, and the buffer (*N*-(2-hydroxyethyl)piperazine-*N*′-propanesulfonic acid (EPPS) were purchased from commercial sources and used without further purification. The lanthanide salts used were always $LnCl_3 6H_2O$.

Reaction solutions were prepared by combining appropriate amounts of metal stock solutions, ligand, and buffer. A Knick digital pH meter 646 was used for pH measurements.

Kinetic analysis with BNPP and with plasmid DNA were carried out as described previously.[14,16] The rate of *p*-nitrophenolate ($\epsilon = 6430$ M^{-1} cm^{-1}) release was monitored spectrophotometrically at 400 nm. The pH of the solutions was adjusted with NaOH or HCl and checked at 25 °C. Plasmid DNA experiments were conducted at 37 °C in a constant temperature bath. Samples were incubated at 37 °C for 2 h in 10 μL samples (Eppendorf tubes). Electrophoresis was conducted with 0.9% agarose in a horizontal gel apparatus (Stratagene) at 20 V for 16 h. Quantification after electrophoresis was performed with an "Eagle Eye II" densitometry system (Stratagene), using the "Zero-Dscan" software from Scanalytics.[14] All measurements were carried out at pH 7.0 in EPPS buffer.

UV spectra were taken with a Varian BioCary1 instrument at 37 °C; data collection and nonlinear least-squares curve fitting to pseudo-first-order equations were performed with suitable PC programs. Circular dichroism spectra were measured with a Jasco J-715 spectropolarimeter at 37 °C. For measuring conditions for all experiments, see footnotes to tables and/or figures.

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