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EQUILIBRIUM AND HYDROLYSIS STUDIES OF PHOSPHATE ESTERS MODEL MOLECULES AND DNA CATALYZED BY OBISDIEN-Zn(II) COMPLEXES.

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ABSTRACT

Dinuclear Zn(II)-OBISDIEN complexes catalyze hydrolysis of DNA and a phosphate ester model molecule: bis(2,4-dinitrophenyl)phosphate (BDNPP). The increase in the rate of hydrolysis of BDNPP at p[H] values above 8.0 is attributed to the presence of a ternary hydroxide species, in which intramolecular catalysis is favored by the proximity of the hydroxide group coordinated to the bimetallic center of the receptor complexes, in agreement with equilibrium results of OBISDIEN-Zn(II)-ATP (1:2:1 molar ratio) system. In the treatment of pBR322 circular DNA with OBISDIEN-Zn(II) complexes, the results suggest that these complexes cleave DNA in a random and nonspecific manner, since no distinguishable low molecular weight bands were observed after treatment.

RESUMO

Complexos dinucleares Zn(II)-OBISDIEM catalisam a hidrólise do DNA e de uma molécula modelo: fosfato de bis(2,4-dinitrofenil) (BDNPP). O aumento da velocidade de hidrólise do BDNPP em valores de p[H] maiores do que 8,0 pode ser atribuído a presença de uma espécie ternária hidróxida, na qual catálise intramolecular é favorecida pela proximidade do grupo hidróxido coordenado ao centro bimetálico do complexo receptor, em acordo com os resultados de equilíbrio do sistema OBISDIEN-Zn(II)-ATP (razão molar 1:2:1). No tratamento do DNA circular pBR322 com os complexos OBISDEIN-Zn(II), os resultados sugerem que estes complexos hidrolisam o DNA de uma maneira aleatória e não específica, pois não foram observadas bandas de pesos moleculares baixos após o tratamento.

KEYWORDS: hydrolysis, DNA, bis(2,4-dinitrophenyl)phosphate, ATP

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INTRODUCTION

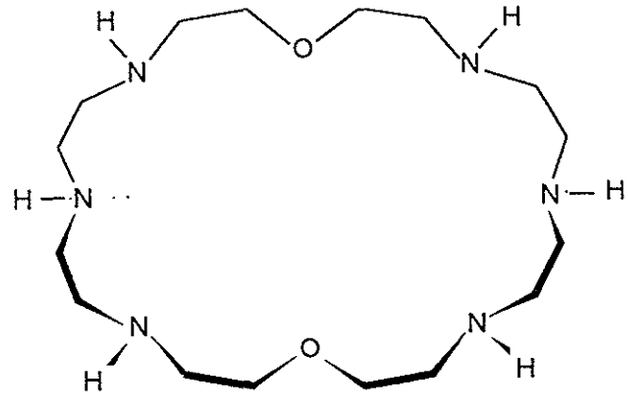
Metal ions are essential in a large variety of biological processes, including those with nucleic acids and their derivatives[1]. For example, both Mg^{2+} and Zn^{2+} are directly involved in the 3′- to 5′- exonuclease activity of the Klenow fragment of DNA polymerase I from *Escherichia Coli* [2,3]. DNA polymerase contains tightly bound Zn^{2+}, and there is evidence that this metal ion binds the enzyme to DNA[4,5].

DNA strand scission reactions are of considerable interest, both in understanding the ubiquitous phosphate ester hydrolysis reactions carried out in nature and in designing new artificial restriction enzymes[6]. Redox properties of a variety of metals have been exploited as DNA cleaving agents[7,8]. The full therapeutic potential of these compounds cannot be realized because these oxidative cleavage agents require activation by either light or an oxidant and tend to produce diffusible free radicals and products that are not amenable to further enzymatic manipulation. Hydrolytic cleavage agents do not require coreactants and therefore could be more useful in drug design[9].

Recently, Cu(II)[9-11], Zn(II), Ni(II)[11,12], Cd(II)[11] and Au(III)[13] complexes shown to hydrolyze DNA, and polyamines also possess potential as catalytic agents, in view of the propensity of polyammonium species for complexing phosphates and nucleotides[14,15]. Large ring macrocyclic exhibits a range of catalytic ability even in the absence of metal ion[16-18].

In order to identify possible mechanisms by which metal ions promote phosphodiester hydrolysis, model compounds, like bis(2,4-dinitrophenyl) phosphate and *p*-nitrophenyl phosphate, have been used to study the reactivity with metal complexes[19-22].

The 24-membered macrocycle 1, 4, 7, 13, 16, 19-hexaaza-10,22-dioxacyclotetracosane (OBISDIEN) has been discovered to be an exceptional mimic for enzymes that hydrolyze adenosine triphosphate (ATP), i.e., the ATPases[16,23]. The protonated forms of the macrocyclic OBISDIEN catalyze ATP hydrolysis through a nucleophilic pathway wich involves a phosphorylated intermediate[24,25].



1. OBISDIEN

We have previously demonstrated that OBISDIEN-Cu(II) complex interacts with dipeptides[26] and that OBISDIEN-Zn (II) complexes hydrolyze glycylglycine[27]. In this paper we report the discovery that binuclear OBISDIEN-Zn(II) complexes also cleaves DNA and bis(2,4-dinitrophenyl)phosphate. The formation constant of Zn(II)-OBISDIEN complexes with ATP were investigated and the results were used to interpret the cleavage ability of these complexes towards DNA.

EXPERIMENTAL

The OBISDIEN. 6 HBr in the form of colorless hexahydrobromide was synthesized by the method described in literature[28,29]. The adenosine 5′-triphosphate disodium salt hydrate and pBR322 plasmid DNA from *Escherichia Coli* were obtained from Aldrich Chem. Co. and used without further purification. The stock solution of $ZnCl_2$ was standardized by titration with EDTA (ethylenediaminetetraacetic acid)[30]. Carbonate-free solution of 0.100 M KOH were prepared from Dilut-it (Baker) ampoules and were standardized by titration with potassium acid phthalate. Potassium chloride, the supporting electrolyte, was obtained as reagent grade quality.

Potentiometric equilibium measurements

Potentiometric studies of OBISDIEN, in the absence and presence of Zn (II), and ATP were carried out with a Micronal-B 375 research pH meter fitted with blue-glass and Ag-AgCl reference electrodes. The potentiometric apparatus was calibrated with standard HCl and KOH solutions to read $-\log[H^+]$ directly and pK_w for water at $\mu = 0.100$ M was 13.78 [31]. The temperature was maintained at 25.00 °C and the experimental solutions, adjusted to 0.100 M in ionic strength by addition of KCl, were titrated with 0.100 M CO_2-free KOH solution. Equilibrium measurements were made on solutions containing 2:1 molar ratio of metal ion to OBISDIEN, 1:1 molar ratio of metal ion to ATP, and the ternary system containing OBISDIEN, Zn (II) and ATP were studied at molar ratio of 1:2:1 with 0.050mmol of OBISDIEN, 0.100 mmol of Zn (II) and 0.050 mmol of ATP. Potentiometric studies were carried out on 50.00 mL of experimental solution in a thermostated cell, purged with argon cleaned by an alkaline solution of KOH. Each system was titrated at least three times. The range of accurate p[H] measurements was considered to be 2.7 – 11. All stability constants were determined using procedures outlined in detail in the literature[31].

Computations

Computations were all carried out with the BEST7 program and species diagrams were obtained with SPE and SPE-PLOT programs[31].

Cleavage of bis(2,4-dinitrophenyl) phosphate

The instrument utilized for absorption measurements was a diode array spectrophotometer (Hewlett-Packard), model 8452A, equipped with a thermostated compartment at constant temperature (50°C). This instrument was attached to a microcomputer HP Wectra 386/33N and a HP Deskjet 500 printer.

Samples containing 1*m*M of OBISDIEN and 1.9 *m*M of Zn(II) with $\mu = 0.100$ M (KCl) have the p[H] adjusted by small increments of 1.0 M KOH or 1.0 M HCl. After that, solution of bis(2,4-dinitrophenyl) phosphate (BDNPP) was added to the experimental solution containing OBISDIEN-Zn. The final concentration of BDNPP was 7.0×10^{-5}M. The absorbance measurements as a function of time were read after addition of BDNPP to the experimental solutions. The hydrolysis was monitored by following the visible absorbance change at 400 nm due to the release of 2,4-dinitrophenolate anion.

Cleavage of DNA and Electrophoresis analysis

DNA degradation assay: Two different sources of DNA, a circular plasmid DNA (pBR322, Sigma D 9893), or a linear double stranded genomic DNA purified from mussels, was treated at a concentration of 10ng/μl in a final volume of 400 μl and pH 8.0 at 25°C or 40°C. The OBISDIEN-Zn(II) concentration varied from 0 to 25 μM, as indicated on figure legends. The reaction was stopped with loading buffer 2X (20mM Tris-Cl pH 8.0, 10mM borate, 2mM EDTA and 20% glycerol). An aliquot of the sample (10 μL) was applied to a 0.8% agarose gel, and an electric field (5V/cm) applied for 1-2h. The DNA was visualized by ethidium bromide staining or silver staining[32].

RESULTS AND DISCUSSION

Equilibrium measurements in the OBISDIEN-Zn(II)-ATP system

The protonation and Zn (II) binding constants of OBISDIEN and its interactions with bromide ions were reported earlier[33-35]. The protonation constant of ATP and its binding constants with zinc(II) ion were redetermined under the present experimental conditions from titration data. Figure 1 shows the titration curves OBISDIEN, OBISDIEN-Zn(II) 1:2 molar ratio, OBISDIEN-ATP 1:1 molar ratio and OBISDIEN-ATP-Zn(II) 1:1:2 molar ratio. The equilibrium constants determined are reported in Table 1 and 2, which includes comparison with the values reported in the literature[24,34].

The equilibrium constants found for the formation of ternary systems are defined by Eqs. (1) – (5), where LZn_2 is the binuclear OBISDIEN-Zn(II) complex, the receptor complex, and A^{4-} is the basic deprotonated form of ATP.

		K	
$H_nL + A^{4-} \rightleftharpoons H_nLA^{n-4}$		$\frac{[H_nLA^{n-4}]}{[H_nL][A^{4-}]}$	(1)
$H_6L + HA^{3-} \rightleftharpoons H_7LA^{3+}$		$\frac{[H_7LA^{3+}]}{[H_6L][HA^{3-}]}$	(2)
$LZn_2^{4+} + A^{4-} \rightleftharpoons LZn_2A$		$\frac{[LZn_2A]}{[LZn_2^{4+}][A^{4-}]}$	(3)
$LZn_2A + nH^+ \rightleftharpoons H_nZn_2A^{+n}$		$\frac{[H_nZn_2A^{+n}]}{[LZn_2A][H^+]^n}$	(4)
$LZn_2A \rightleftharpoons LZn_2(OH)A^- + H^+$		$\frac{[LZn_2(OH)A^-][H^+]}{[LZn_2A]}$	(5)

Equilibrium studies of the ternary system show that the binuclear OBISDIEN-Zn(II) complexes hold on ATP molecule coordinated in their cavities at p[H] values above 5.5.

Table 1. Log values of protonation constants of ATP, their binding constants with Zn(II) and the stability constants for the complexes ATP-OBISDIEN at 25.0 °C (μ = 0.100 (KCl))

Equilibrium quotient	log K	
	This work	Others [24,34]
$[HA^{3-}]/[A^{4-}][H^{+}]$	6.76 (0.01)	6.50
$[H_2A^{2-}]/[AH^{3-}][H^{+}]$	4.05 (0.04)	4.00
$[AZn^{2-}]/[A^{4-}][Zn^{2+}]$	4.34 (0.13)	4.87
$[HAZn^{-}]/[AZn^{2-}][H^{+}]$	4.76 (0.12)	4.57
$[AZn(OH)^{3-}][H^{+}]/[AZn^{2-}]$	-8.36 (0.62)	-8.76
$[H_4LA]/[H_4L][A^{4-}]$	5.75 (0.02)	4.80
$[H_5LA]/[H_5L][A^{4-}]$	7.83 (0.12)	8.15
$[H_6LA]/[H_6L][A^{4-}]$	10.14 (0.11)	11.0
$[H_6LHA]/[H_6L][HA^{3-}]$	7.01 (0.06)	7.85

Table 2. Log values of protonation and stability constants for the ternary species formed by Zn(II) complexes of OBISIDIEN with ATP at 25.0 °C (μ = 0.100 (KCl))

Equilibrium quotient	log K
$[LZn_2A]/[LZn_2^{4+}][A^{4-}]$	5.97 (0.18)
$[HLZn_2A^{+}]/[LZn_2A][H^{+}]$	6.98 (0.09)
$[H_2LZn_2A^{2+}]/[HLZn_2A^{+}][H^{+}]$	6.47 (0.22)
$[LZn_2A(OH)^{-}]\ [H^{+}]/[LZn_2A]$	-9.50 (0.12)

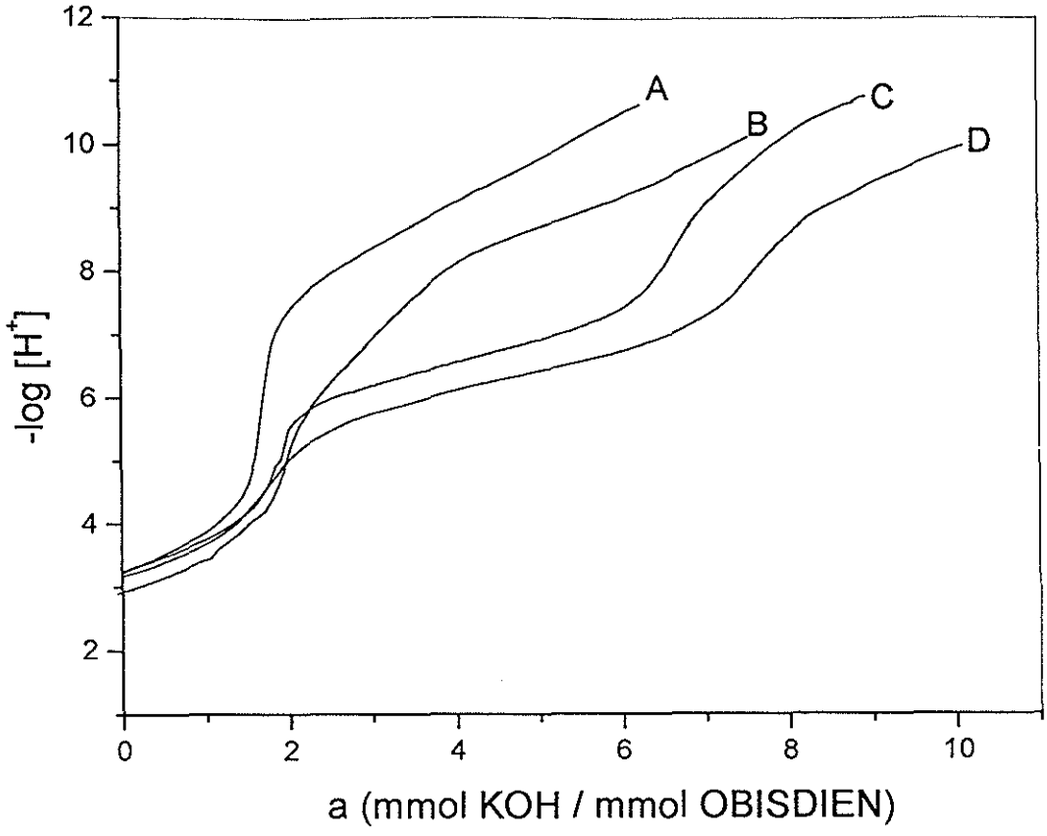


Figure 1. Potentiometric p[H] profiles for solutions containing A) 0.05 mmoles OBISDIEN; B) 0.05 mmoles OBISDIEN and ATP; C) 0.05 mmoles OBISDIEN and 0.1 mmol Zn(II); D) 0.05 mmoles OBISDIEN, ATP and 0.1 mmol Zn(II) at 25.0 °C and μ = 0.100 M (KCl).

This is clearly seen by analysis of the species distribution diagram shown in Figure 2. The diprotonated species, H_2LZn_2A, is 27 % formed at p[H] = 6.3. Above this p[H] it decreases giving place to the monoprotonated species, $HLZn_2A$. This species is 31 % formed at p[H] 6.9 and decreases at higher p[H] values. The normal species, LZn_2A, is 77 % formed at p[H] 8.2. This species has ATP coordinated to the bimetallic center. The hydroxide species appears at p[H] values above 8 and it is 38% formed at p[H] 9.8.

The capacity of OBISDIEN in acting as a receptor of anionic molecules is fundamental on its ability to interage with ATP. The presence of zinc (II) ions in its cavity facilitates the coordination of ATP, favoring the formation of ternary species at neutral and alkaline p[H].

Lehn, et al,[24] and Mertes, et al,[23] relate that OBISDIEN can hydrolyze the phosphoric ester bond of ATP molecule. In p[H] 7.6 and 70°C the $k_{obs} = 25 \times 10^{-3}$ min^{-1}. However, the OBISDIEN-Zn(II) at 1:1 molar ratio diminishes the hydrolysis rate of ATP comparing with OBISDIEN without metal ion ($k_{obs} = 3.2 \times 10^{-3}$ min^{-1})[23]. Potentiometric studies and ^{13}C NMR indicated strong interactions between the metal and macrocycle, the retardation in rates for these ternary macrocycle-ATP-Zn(II) system is probably the result

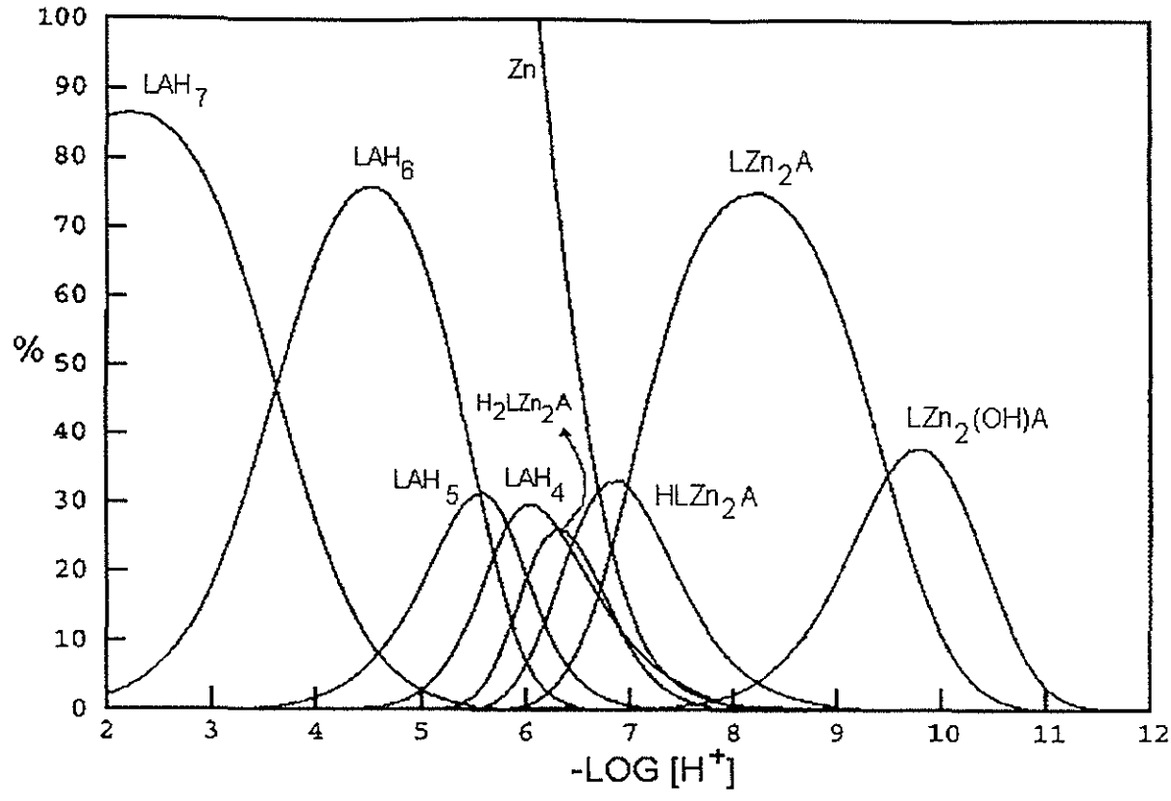


Figure 2. Species distribution curves of the 1:1:2 OBISDIEN-ATP-Zn(II) system in aqueous solution, at 25.o °C and μ = 0.1 M (KCl).

of competitive inhibition. Kinetic studies were done in the 1:1.5:1.5 molar ratio of ATP:OBISDIEN:Zn(II), respectively. However, our results show ATP coordinated to the binuclear OBISDIEN-Zn(II) complexes. The two Zn(II) ions in the cavity of OBISDIEN are in favorable arrangement interacting with the substrate as in a binuclear arrangement, predominating species 1:1 molar rate of OBISDIEN:Zn(II). On the other hand, OBISDIEN-Zn(II) system, studied by Mertes, et al, does not have a complex in an appropriate association with ATP. The binuclear arrangement leaves coordination sites in the metal center occupied by water molecules that can be substituted by an ATP molecule during its association.

The results of equilibrium studies of OBISDIEN-ATP-Zn(II) system show the predomination of two species near physiological p[H] values (Figure 2), LZn_2A and $HLZn_2A$. These results prompted us verify the DNA cleavage by binuclear OBISDIEN-Zn(II) complexes.

Cleavage of bis(2,4-dinitrophenyl) phosphate (BDNPP)

The rate of binuclear OBISDIEN-Zn(II) complex promoted cleavage of BDNPP was monitored by following the increase in the visible absorbance at 400 nm due to the release of 2,4-dinitrophenolate (see UV-vis spectra in Figure 3) at different pH's. Figure 4

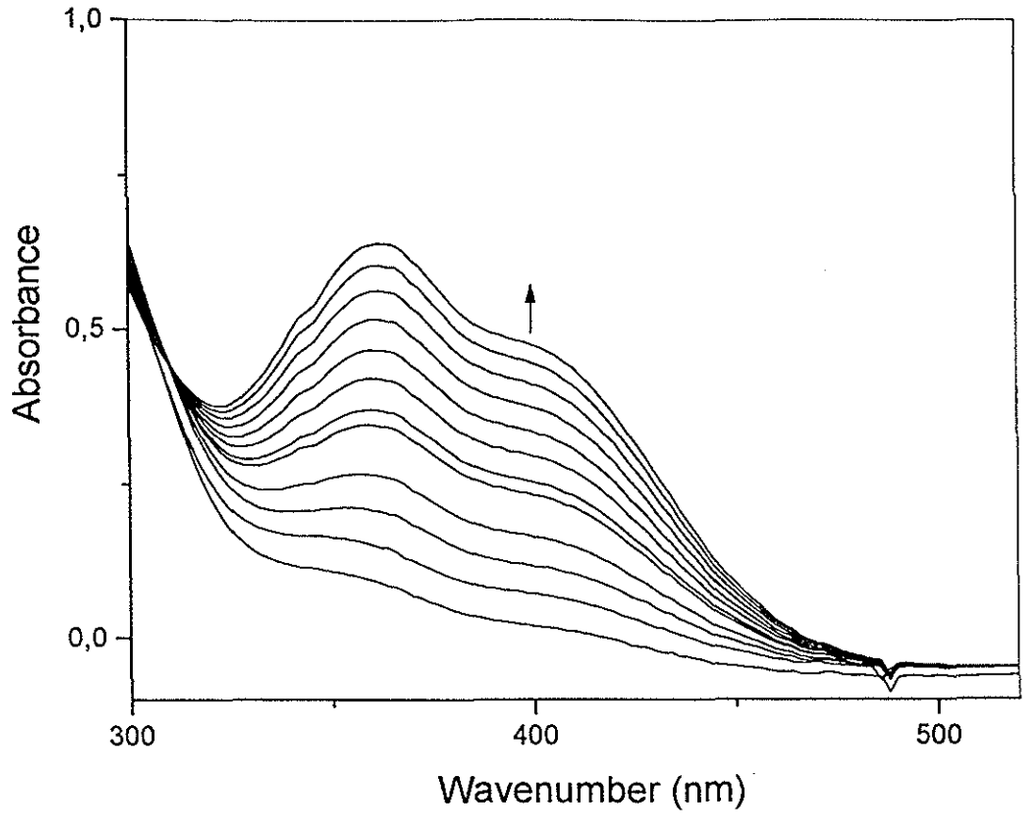


Figure 3. UV-Vis spectra of a solution containing 1 x 10^{-3} M of OBISDIEN, 1.9 x 10^{-3} M of Zn(II) and 7 x 10^{-5} M of BDNPP at pH 8.5, μ = 0.1 M (KCl) and 50 °C. Spectra measured during 7 h.

shows the pseudo-first order plot[36] for the hydrolysis reaction in pH 8.5 with a rate of 2.42 x10^{-5} s^{-1} and a correlation coefficient R = 0.9998. The observed rate constants determined in this way are summarized in Table 3.

It is known from the species distribution curves of OBISDIEN-Zn_2-ATP system that as the p[H] is increased above 8, the hydroxide species appears. The normal complex is maximum near p[H] 8 and below p[H] 7 the protonated species are the major ones. The increase of rate from p[H] 6.5 to 8.2 is mostly due to increase of binuclear OBISDIEN-Zn(II) complexes rather than many differences in the catalytic actives of protonated and normal ternary complex. However, at higher p[H] values the increase in the rate can be attributed to the presence of a ternary hydroxide species. In this species intramolecular catalysis is favored by the proximity of the hydroxide group coordinated to the bimetallic center. This interpretation is in agreement with potentiometric results of OBISDIEN-Zn(II)-ATP (1:2:1 molar ratio) system.

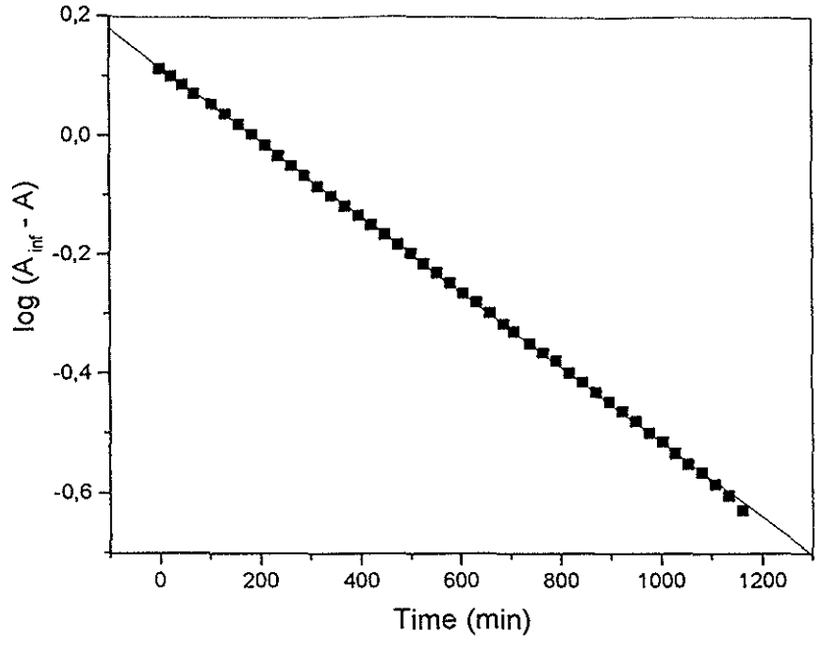


Figure 4. Pseudo-First-order plot for the hydrolysis of bis(2,4-dinitrophenyl)phosphate by OBISDIEN-Zn. The solution containing 1 x 10^{-3} M of OBISDIEN, 1.9 x 10^{-3} M of Zn(II) and 7 x 10^{-5} M of BDNPP at pH 8.5, μ = 0.1 M (KCl) and 50°C. A_{inf} represent the absorbance in infinite time and A the absorbance in each measurement.

Table 3: Observed rate constants for the pseudo-first order reaction of the binuclear OBISDIEN-Zn(II) (1:2 molar ratio) complex with BDNPP, at 50.0°C, μ = 0.100 M (KCl).

p[H]	k_{obs} (10^5 s^{-1})
6.5	0.80
7.4	1.30
8.2	1.80
8.5	2.42
9.5	5.22

DNA cleavage analysis

A typical assay to demonstrate DNA cleavage is the transformation of supercoiled circular double stranded DNA on its relaxed form by a single strand nick[37,38].

In Figure 5 the effect of treatment of pBR322 circular DNA with OBISDIEN-Zn(II) complex (25 μM) at 25°C is observed. The supercoiled DNA was transformed to a relaxed form (single strand nicked DNA) only in the presence of the OBISDIEN-Zn(II) complex in a time dependent manner. The effect is also dependent on temperature, at 40°C the DNA is completely degraded in 1h (not shown). In Figure 6 the source of DNA was a double stranded, linear genomic DNA, and the complex was also capable of degrading this DNA as evidenced from disappearance of the high molecular weight band, the reaction was strictly dependent on OBISDIEN-Zn(II) concentration. The observed smear on the gel is

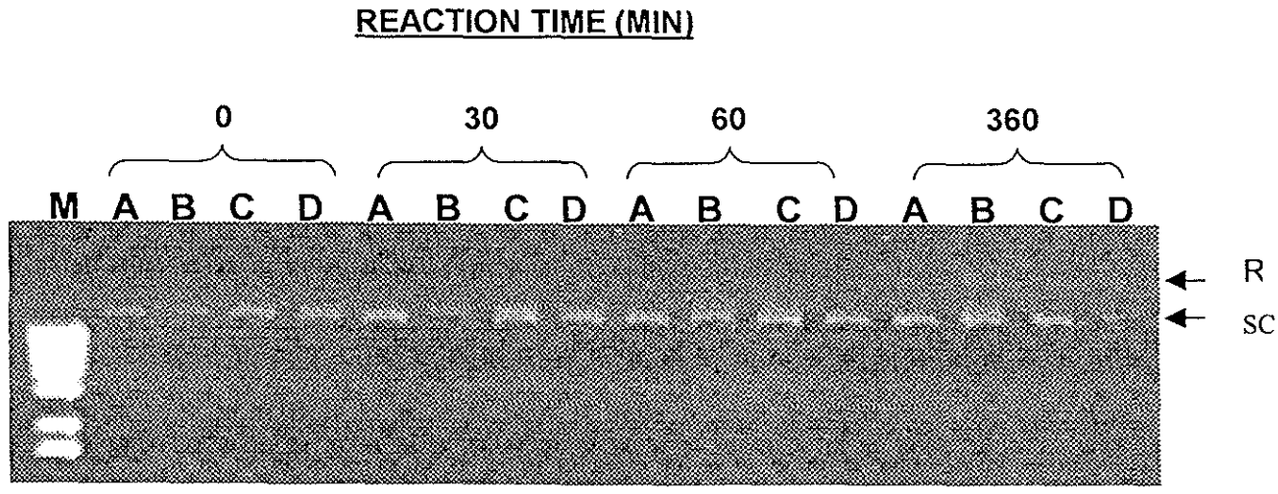


Figure 5. Plasmid DNA (pBR322) incubated at 25°C for 0, 30, 60 and 360 min as shown. R and SC at the right of the figure refer to 'relaxed' and 'supercoiled' forms of pBR322. A) DNA at pH 8.0; B) DNA-OBIS-Zn(II), C) DNA-OBIS; D) DNA-Zn(II), M) molecular weight marker (Gibco BRL 1kb ladder).

due to numerous different fragments appearing as a result of OBISDIEN-Zn(II) cleavage reaction.

As evidenced from these results, OBISDIEN-Zn(II) may be considered a "chemical nuclease" since it is able to cut DNA directly under physiological conditions by apparently all positions regardless of the nucleotide linked to the deoxyribose as for example Fe-EDTA[39], various metalloporphyrins[40] and octahedral complexes of 4,7-diphenyl-1,10-phenanthroline[41].

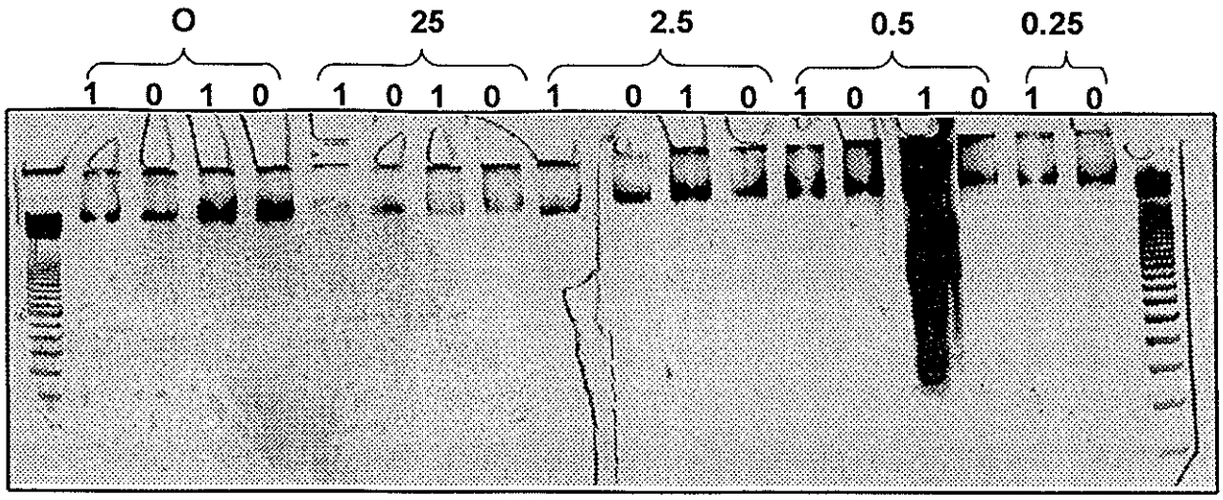


Figure 6. OBIS-Zn(II) complex treatment on genomic DNA. Various concentrations of OBISDIEN-Zn(II) (0 to 25 μM) were added to genomic DNA, at 40 °C and incubated for 1h, 0 and 1, on the top of the figure refers to incubation time (h), 0 is the control. At each side of the figure the molecular weight marker (100bp, Gibco) is observed. On lane 0.5, 1 an artifact of silver staining is observed.

The reaction mechanism of these 'chemical nucleases' distinguish them from other chemical modification reagents widely used in nucleic acid chemistry, for example, dimethyl sulfate, diethyl pyrocarbonate, osmium tetraoxide, and permanganate react preferentially with the various bases and do not cause strand scission without subsequent base treatment (e.g., piperidine as in Maxam-Gilbert sequencing)[42].

The results shown suggest that OBISDIEN-Zn(II) cleave DNA is in a random and nonspecific manner, since no distinguishable low molecular weight bands were observed after treatment, the staining method employed detected only uncut genomic DNA or the supercoiled and relaxed forms of pBR322 plasmid DNA, suggesting again the randomness of the cleavage reaction. Further studies may confirm this hypothesis and should introduce OBISDIEN-Zn(II) as a novel footprinting reagent.

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