Osmium-Induced Alteration in DNA Structure

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Abstract. In the presence of pyridine and other ligands osmium tetroxide binds covalently to pyrimidine bases in DNA. Properties of osmium-modified native and denatured calf thymus DNA, and plasmid ColE1 DNA were investigated by means of differential pulse polarography, absorption spectrophotometry, circular dichroism, agarose gel electrophoresis, and nuclease S1 digestion. A great difference in the reaction kinetics of native and denatured DNAs with osmium, pyridine was observed. On the ground of the slow stepwise reaction kinetics of native DNA in the initial stage of its modification by osmium it has been suggested that the primary reaction sites do not include bases contained in the intact double helix. Osmium binding to sporadic primary reaction sites (represented e.g. by bases in the vicinity of a single-strand break) in native calf thymus DNA resulted in local changes in DNA conformation limited to a close neighbourhood of the binding site. At higher osmium/nucleotide ratios disordering of the DNA structure over a region extending beyond the immediate binding site was observed. With denatured DNA the same type of structure disordering was detected already in the initial stage of the reaction at osmium/nucleotide ratios as low as 0.01. Osmium binding to the supercoiled ColE1 DNA resulted in its relaxation without nicking and it increased its sensitivity to linearization by cleavage with nuclease S1. The behaviour of ColE1 DNA has been explained by the formation of a denatured region in the molecule (accompanied by a coupled loss of duplex and superhelical turns). It has been suggested that osmium can be used to label and to visualize distorted regions in the DNA double helix.

Key words: DNA-osmium complexes — Polarographic reduction — Absorption and CD spectra — Electrophoretic mobility — DNA structure probe.

Introduction

The study of heavy metal binding to nucleic acids and their constituents has attracted great attention for many reasons. Literature concerning various aspects of metal-ion interactions with nucleic acids has been recently summarized (Marzilli

1977; Hodgson 1977; Barton and Lippard 1980). Osmium labeling of nucleic acids has proved particularly useful and it has found application in x-ray crystallographic and electron microscopic research of polynucleotides. Osmium tetroxide selectively degrades pyrimidine bases (Burton and Riley 1967; Highton et al. 1968) and in the presence of ligands such as CN^-, CSN^-, pyridine and bipyridine stable osmate ester derivatives of pyrimidine bases are formed (Daniel and Behrman 1975; 1976; Chang et al. 1977). Until recently it has been assumed that reaction of osmium tetroxide takes place in single-stranded polynucleotides and not in native double-stranded (ds) DNA (Marzilli 1977).

Recently we have shown (Paleček et al. 1981; Lukášová et al. 1982) that osmium introduced into the polynucleotide chain in the presence of pyridine forms an electroactive marker, which can be sensitively detected by means of modern polarographic (voltammetric) methods. It has been demonstrated by means of the differential pulse polarographic (DPP) method that under suitable conditions osmium tetroxide also reacts with the native DNA in a limited extent. In this paper we have studied the reaction of osmium with ds DNA using DPP, gel electrophoresis and optical methods. Our results have shown osmium to be a convenient structural probe for ds DNA, potentially useful for visualization of single-stranded and distorted regions in the DNA double helix.

Materials and Methods

Calf thymus DNA was isolated by the method of Zamenhof (1957) and plasmid ColE1 DNA by the modified phenol method (Vojtíšková et al. 1980). Properties and purity of the DNA samplex were the same as described in our previous paper (Lukášová et al. 1982). Nuclease S1 was isolated from Takadiastase (Vogt 1973). Osmium tetroxide was purchased from Fisher Scientific Co., Sephadex G-50 medium from Pharmacia Fine Chemicals. Other chemicals were of analytical grade. DNA samples were irradiated using gamma-radiation from a ^{60}Co source and UV-radiation from a germicidal lamp Phillips TUV 15 W as described previously (Vorlíčková and Paleček 1974). Thermal denaturation was performed by heating the DNA sample in SSC/20 at 100 °C for 6 min followed by rapid cooling in an ice bath.

DNA modification with osmium tetroxide. A typical reaction mixture containing ds DNA at a concentration of 300 μg/ml in SSC (1.5×10^{-1} mol . dm^{-3} NaCl, 1.5×10^{-2} mol . dm^{-3} sodium citrate, pH 7), 2×10^{-3} mol . dm^{-3} OsO_4 and 1 % (v/v) pyridine was made by mixing an equal volume of an OsO_4-pyridine solution with a DNA solution. Concentration of the denatured DNA in the reaction mixture was the same or lower. The reaction mixture was incubated at 26 °C and the reaction was terminated either by passing the mixture through a Sephadex G-50 medium column or by ethanol precipitation and subsequent dialysis against SSC/20 for 20 hours at 4 °C or (at longer time intervals) only by dialysis with a frequent exchange of SSC/20 during the first 2 hours of dialysis. Modifications in the presence of other ligands and/or higher pyridine concentrations were basically performed under the conditions as described by Beer et al. (Highton et al. 1968; Chang et al. 1977). More details are given in the legend to the Figures.

S1 nuclease digestion of plasmid ColE1 DNA was performed in 50μl drops (placed on parafilm M in a Petri dish) containing 3×10^{-2} mol . dm^{-3} sodium acetate (pH 4.6), 5×10^{-2} mol . dm^{-3} NaCl, 1×10^{-3} mol . dm^{-3} $ZnSO_4$, 5 % glycerol, 3.5 μg of DNA and various concentrations of the enzyme (0.5; 1.0; and 2.0 units). After 10 min of incubation at 37 °C the reaction was stopped by the addition of 4 μl of 1×10^{-1} mol . dm^{-3} sodium citrate and electrophoresed.

Gel electrophoresis of plasmid ColE1 DNA was carried out in 1 percent agarose containing 5×10^{-2} mol . dm^{-3} Tris, 2×10^{-2} mol . dm^{-3} sodium acetate, 2×10^{-3} mol . dm^{-3} Na_2EDTA, 1.8×10^{-2} mol . dm^{-3} NaCl, pH 8.0 in 10 cm glass tubes (gels 9.0 cm by 0.5 cm) at 100 V/5 mA/tube, or in a 1 % agarose slab gel containing 4×10^{-2} mol . dm^{-3} Tris-acetate, 2×10^{-3} mol . dm^{-3} EDTA, pH 8.0 for 18 hours at 2 V/cm at room temperature. Following electrophoresis, the gels were stained with ethidium bromide.

Differential pulse polarographic measurements were performed with a Model PAR 174A in connection with a PAR 315A automated electroanalysis controller using a Model 303 stationary mercury drop electrode (SMDE) as a dropping electrode. The three-electrode system included a Pt-wire as the auxillary electrode and a saturated calomel electrode K 77 as the reference electrode connected with the analysed solution via a K 65 bridge tube filled with the background electrolyte. Measurements were carried out in argon atmosphere. Polarograms were recorded on an Omnigraph Model 9002A Y—Y Recorder. Unless stated otherwise the instrument setting was: scan rate 2 mV/s, drop size L, drop time 1 s, modulation amplitude 50 mV.

Osmium content in DNA samples was determined from the height of the DPP peak C. The height of this peak of the fully labelled thermally denatured DNA,Os,py (modified under conditions as described by Chang et al. (1977), i.e. 24 hours in the presence of 3 mol . dm^{-3} pyridine) was taken as the standard in which one osmium atom is bound to each of the pyrimidine bases. Corrections for differences in the diffusion coefficients of denatured and native ds DNA samples were made when the osmium content was determined in ds DNA.Osmium content in some thermally denatured DNA,Os,py samples was determined colorimetrically using thiourea after decomposition of the DNA,Os samples with sodium peroxide (Beer et al. 1966; Chang et al. 1977). The amount of osmium in native ds DNA,Os,py samples was below the detection limit of the colorimetric method used.

Spectrophotometric measurements were carried out with a Zeiss VSU 2 and a Beckman DU 8 instruments. Circular dichroism spectra were measured on a Jobin-Yvon Dichrograph III in 0.5 cm cells at DNA concentrations of 25 μg/ml and corrected by subtracting blank solution. Phosphorus determination in DNA samples was performed by means of a colorimetric method according to Martin and Doty (1949).

Results

Differential pulse polarography. It has been shown earlier (Paleček et al. 1981; Lukášová et al. 1982) that denaturated calf thymus DNA,Os,py yields three DPP peaks; the most negative of them (Ep = −1.2 V) reduction peak C (Fig. 1*e*), is many times higher as compared with the other peaks, probably due to the catalytic nature of the former. Time dependence of the reaction of thermally denatured DNA with osmium (1 % pyridine, 26 °C) was determined (Fig. 2) in time intervals between 8 and 24 hours and a linear dependence of the DPP peak C height on time was observed. The same sample of denatured DNA was also modified under conditions (3 mol . dm^{-3} pyridine, 26 °C, 24 hours) where, all the

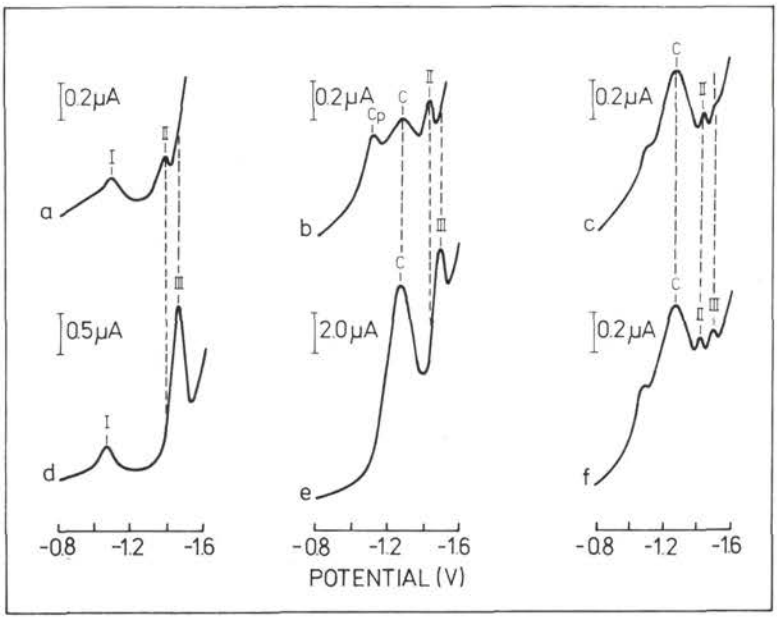

Fig. 1. Differential pulse polarograms of osmium-modified and unmodified DNAs. *a*, unmodified ds DNA; *b*, ds DNA,Os,py, 24 hours modification; *c*, *f*, ds DNA,Os,py, 50 hours modification; *d*, unmodified thermally denatured DNA; *e*, thermally denatured DNA,Os,py, 24 hours modification. Calf thymus DNA concentration: *a*, *b*, *c*, 105 μg/ml; *d*, 31 μg/ml; *e*, 54 μg/ml; *f*, 207 μg/ml. ds DNA at a concentration of 300 μg/ml and denatured DNA at a concentration of 100 μg/ml were treated with 2×10^{-3} mol dm^{-3} OsO_4, 1 % pyridine at 26 °C for intervals indicated above; DPP measurements were performed in 3×10^{-1} mol dm^{-3} ammonium formate, with 5×10^{-2} mol dm^{-3} sodium phosphate (pH 6.9). Samples were dialysed before the DPP analysis. (The decrease in peak II in Figs. 1*c* and 1*f* is at least partly due to the overlapping of this peak by a high peak C. At DNA concentrations exceeding 130 μg/ml the height of peak C was not proportional to the osmium content in ds DNA (Fig. 1*f*) due to the nonlinear dependence of the peak height on DNA,Os,py concentration in this concentration range.)

pyrimidine residues are labelled with osmium (Chang et al. 1977); the osmium content in this DNA,Os,py sample was determined colorimetrically (Beer et al. 1966; Chang et al. 1977). The obtained molar ratio Os/P = 0.5 agreed well with the published data (Chang et al. 1977). The height of the peak C of fully osmium-labelled DNA was thus taken as being equal to Os/P = 0.5 and it was used for calculations of Os/P from the heights of peaks obtained in the time dependence studies (Fig. 2). Osmium content was also determined colorimetrically in DNA, Os,py samples after 8 and 24 hours of modification (in 1 % pyridine). Good agreement was found between Os/P values obtained from DPP and those obtained from colorimetric measurements (Fig. 2).

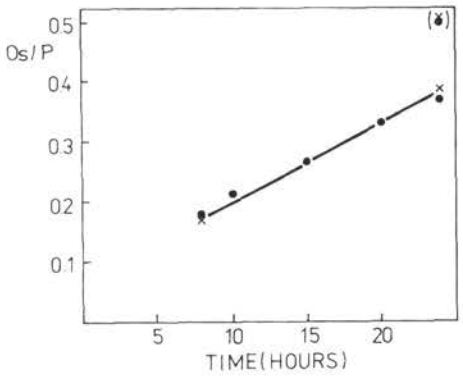

Fig. 2. Time-dependence of the OsO_4, pyridine reaction with thermally denatured DNA. Calf thymus DNA at a concentration of 150 μg/ml in SSC/20 was incubated with 1×10^{-3} mol dm^{-3} OsO_4, pyridine at 26 °C; ●,x, 1 % pyridine. (●), (x) 3 mol dm^{-3} pyridine. The content of osmium in DNA samples was determined by DPP (●) and colorimetrically (x) (see Materials and Methods.)

Osmium peaks in ds DNA. Modification of native calf thymus DNA with osmium under identical conditions as those used for thermally denatured DNA (24 hours, 1 % pyridine, 26 °C) resulted in the appearance of the DPP peak C (Fig. 1*b*); its height was, however, about seventy times smaller than that produced by denatured DNA at the same concentration (Fig. 1*e*). The reactivity of ds DNA with osmium, pyridine changed with both the ionic strength and the nature of salts present in the reaction mixture (while the reactivity of denatured DNA was only little influences under identical conditions), and it increased with the storage length of the given DNA sample (kept at 4 °C in SSC/10). Following gamma-irradiation of ds DNA with 100 Gy the peak C increased by a factor of 3, UV-irradiation with a dose of 1000 J/m^2 (10^4 erg/mm^2) resulted in an increase of this peak by a factor of 2.5.

In addition to the peak C ds DNA,Os,py also yielded a more positive peak Cp (Fig. 1*b*), which appeared close to potentials of the capacitive peak I produced by unmodified DNAs (Figs. 1*a*, *d*). The nature of the peak Cp (Fig. 1*b*) has not yet been fully understood and it will not be discussed in this paper.

In agreement with the behaviour of denatured DNA,Os,py (Lukášová et al. 1982) the height of the peak C of ds DNA,Os,py (open circular DNA of plasmid ColE1) did almost not change in dependence on time (at 4 °C) during the first 24 hours after passing the sample through a Sephadex G-50 column. After a few days of storage of the ds DNA,Os,py solution at 4 °C, a marked decrease in the peak C was observed, probably due to the release of osmium from the DNA complex.

The dependence of the peak C height on the concentration of ds DNA,Os,py was measured at various Os/P (ranging from 0.005 to 0.012) (data not shown). A linear dependence of peak C height on the ds DNA,Os,py concentration up to

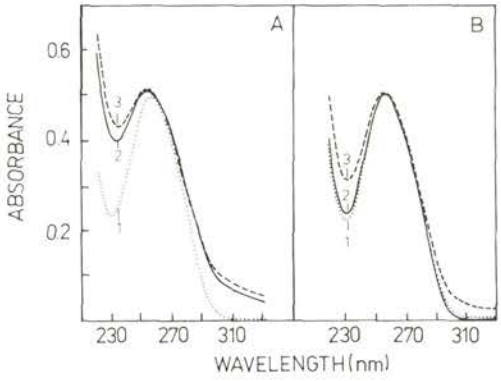

Fig. 3. Absorption spectra of osmium modified calf thymus DNA. *A*, thermally denatured DNA (100 μg/ml in SSC/20); *B*, native DNA (300 μg/ml in SSC). Reaction mixture contained: 2×10^{-3} mol dm^{-3} OsO_4 and pyridine at the concentration indicated below. A1 and B1, unmodified samples, A2 (Os/P = 0.32) and B2 (Os/P = 0.01), 24 hours modification with 1 % pyridine in the reaction mixture. A3, 24 hours modification with 3 mol dm^{-3} pyridine (Os/P = 0.50), B3 (Os/P = 0.15), 128 hours modification with 1 % pyridine. DNA concentration (based on phosphorus determination) was as follows: *A*, 20 μg/ml; *B*, 24 μg/ml (all the three spectra).

about 130 μg/ml could be observed. Any further increase in the DNA,Os,py concentration had little effect on the peak height, or a decrease in the peak height resulted. DPP can be thus used for the determination of both ds DNA,Os,py and the osmium content in this DNA (when the DNA concentration is known) provided the measurements are performed within an appropriate concentration range. No changes in the peak C potential could be observed in dependence on DNA concentration.

Changes in DNA non-osmium peaks. It has been shown earlier (Paleček 1971; 1980; 1983) that, in addition to the nonspecific peak I (Figs. 1*a*, *d*), unmodified DNA also yields a peak II, which is specific for ds DNA (Fig. 1*a*). Unmodified denatured DNA yields only peak I and peak III (Fig. 1*d*). Peak II increases due to the formation of distorted (premelted) ds regions induced by various physical and chemical agents (Paleček 1976; 1980; 1983).

Samples of ds DNA,Os,py modified over 24 hrs produced a peak II in addition to the peak C; this peak II was substantially higher than that of unmodified ds DNA under identical conditions (Figs. 1*a*, *b*); no peaks III appeared in these ds DNA,Os,py samples (Fig. 1*b*), even at a DNA,Os,py concentration of 235 μg/ml (not shown). Longer incubation (50 hours) of ds DNA with osmium, pyridine resulted in an increase in peak C and in the appearance of an inflection (Fig. 1*c*) at the potential of the peak III; a well-resolved peak III was observed at higher ds

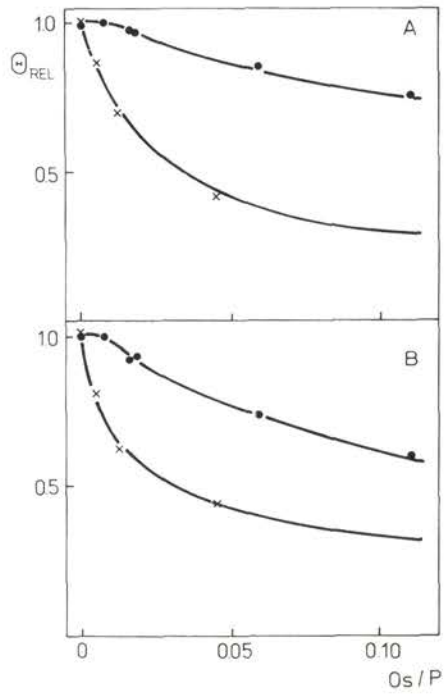

Fig. 4. Dependence of circular dichroism bands on the extent of modification of native and denatured calf thymus DNAs with osmium. ●—●, ds DNA,Os,py; ×—×, denatured DNA,Os,py; *A*, positive CD band (275 nm); *B*, negative CD band (245 nm); θ_{rel}, the ratio between the ellipticity of the osmium-modified and unmodified DNA samples.

DNA,Os,py concentrations (Fig. 1*f*). At low Os/P DPP thus may provide information about both the amount of the bound osmium and changes in accessibility of its inherent reduction sites (adenine and cytosine residues) (Paleček 1976; 1980; 1983) for the electrode process.

Electronic absorption spectra. Spectral data on fully labelled DNA,Os (containing pyridine or other ligands) were reported by Chang et al. (1977). These data showed a marked red shift of λ max (310 nm) in bipyridine-labelled DNA. On the contrary, in pyridine-labelled DNA only a slight blue shift in λmax was observed, accompanied by a small hyperchromic effect and a marked decrease in the εmax/εmin ratio. Our measurements of fully labelled denatured DNA agreed with results reported by Chang et al. (1977) (Fig. 3). Our samples of DNA,Os,py were prepared with various Os/P (by treating native and thermally denatured DNAs with OsO_4 for various periods of time and at various pyridine concentrations) and their circular dichroism (see below) and absorption spectra were determined.

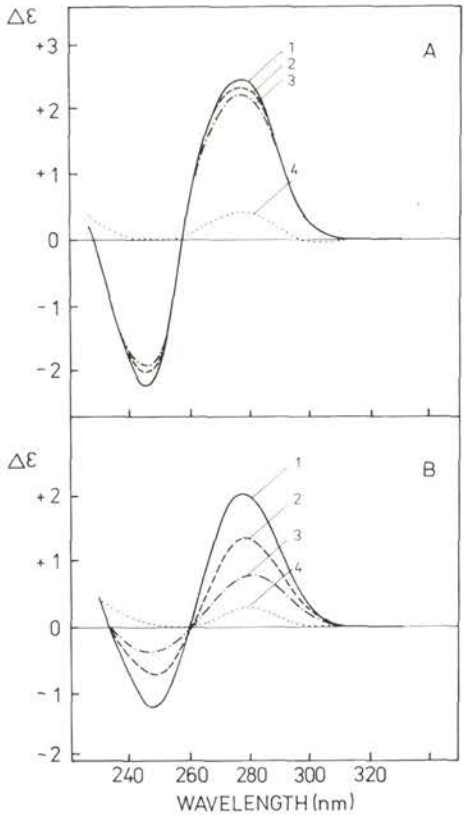

Fig. 5. Circular dichroism spectra of native and thermally denatured calf thymus DNA modified with osmium at various concentrations of NH_4CSN. *A*, ds DNA; *B*, denatured DNA. 1, unmodified control, 2,3 DNA,Os,CSN^- modified 24 hours at 55 °C with 2×10^{-3} mol dm^{-3} OsO_4 in the presence of 2, 7.5×10^{-2} mol dm^{-3} NH_4CSN, 3, 2.5×10^{-1} mol dm^{-3} NH_4CSN at a DNA concentration of 300 μg/ml, 4, ds DNA,Os,py modified 24 hours at 26 °C in 10 % pyridine; thermally denatured DNA,Os,py modified 24 hours at 26 °C in 1 % pyridine. No attempt has been made to determine Os/P in DNA,Os,CSN^- samples as the DPP method was not suitable for the osmium determination in these samples having high negative charge densities (see the text).

εmax/εmin decreased nonlinearly with the increasing Os/P ratio. As a result of modification εmax of native DNA,Os did not substantially change up to an Os/P ratio of 0.15 (Fig. 3) (no measurements were performed at higher Os/P). Changes in εmax/εmin were thus due mainly to an increase in εmin. In denatured DNA,Os εmax increased with the extent of modification (Chang. et al. 1977); ε_{260} was, however, little changed. At higher Os/P ratios spectra of both ds and denatured DNAs were complicated by a tail (Fig. 3) around 310 nm, i.e. in the region where the DNA,Os, bipyridine complex displayed its εmax. At low Os/P absorbance

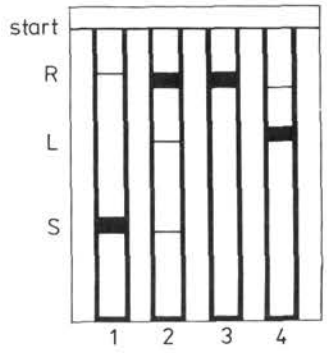

Fig. 6. Schematic presentation of electrophoretic migration of plasmid ColE1 DNA modified with osmium and/or cleaved by S1 nuclease. Lanes: 1, control supercoiled DNA; 2, supercoiled DNA cleaved by S1 nuclease (2 units, DNA at a concentration of 60 μg/ml); 3, osmium-modified DNA (60 μg/ml, 2×10^{-3} mol dm^{-3} OsO_4, 1 % pyridine, 48 hours); 4, osmium-modified DNA (as with lane 3) cleaved by S1 nuclease (0.5 unit).
Symbols: S, supercoiled; L, linear; R, relaxed DNA.

measurements at 260 nm can be used instead of more laborious phosphorus determination to determine DNA,Os,py concentration.

Circular dichroism spectra. As a result of labelling DNA with osmium a decrease in both the positive and negative CD bands was observed (Figs. 4, 5). This decrease was accompanied by a slight red shift of the positive and a blue shift of the negative CD bands. At low Os/P the same amounts of osmium bound to DNA resulted in different changes in CD of native and denatured DNA,Os,py. In thermally denatured DNA modification of 1 % of all the pyrimidine bases led to an about 12 % decrease in the positive and to an 18 % decrease in the negative band (Fig. 4), while in native ds DNA (modified to the same extent) CD spectra remained practically unchanged. At Os/P > 0.01 (more than 2 % of the pyrimidine bases modified) both the positive and negative CD bands of ds DNA decreased with the increasing Os/P. At Os/P > 0.3 the difference between the CD spectra of equally modified ds and thermally denatured DNAs almost disapeared. CD spectrum of fully labelled denatured DNA,Os,py did almost coincide with the background curve.

Electrophoretic mobility of circular duplex DNA,Os,py. Circular duplex (cd) and open circular (oc) forms of plasmid ColE1 DNA were treated with OsO_4 and 1 % pyridine for 24 hours at 26 °C; they were then separated from the reaction mixture on a Sephadex G-50 (medium) column and analysed by agarose gel electrophoresis and DPP. As a result of osmium treatment both DNA forms showed small peaks

C; an accurate evaluation in terms of Os/P was not possible (due to low DNA concentration). In electrophoretic experiments osmium treatment resulted in a transfer of all cd DNA to the nicked band (Fig. 6) with little or no alteration in the electrophoretic mobility of the oc form. The change in the mobility of the osmium modified cd DNA does not appear to be due to a simple chain scission since this DNA did not denature as a result of heating to denaturation temperature. On the contrary, the oc form was denatured under these conditions. This implies that no thermostable interstrand cross-links were formed as a result of osmium reaction. Denaturation was followed by electrophoresis and DPP (not shown). Similar results had been obtained with platinum complexes covalently bound to phage PM2 cd DNA (Howe-Grant et al. 1976).

Both modified and unmodified cd DNAs were digested with single-strand specific (region-specific) nuclease S1 at different enzyme concentrations. While S1 treatment of unmodified DNA transferred all the closed DNA to the nicked band, the treatment of DNA,Os,py resulted in a mobility change of the latter corresponding to that of the linear species (Fig. 6).

Influence of other ligands. Native and thermally denatured calf thymus DNAs were modified with osmium, KCN (or NH_4CSN) at room temperature and at 55 °C under conditions used previously (Highton et al. 1968; Di Giamberardino et al. 1969). Changes in CD spectra due to DNA modification (Fig. 5) were similar to those observed with DNA,Os,py. Fully labelled denatured DNA,Os,CN^- did not yield any DPP peak C at DNA concentrations at which DNA,Os,py produced a well-developed peak C; ds DNA and thermally denatured DNA modified to a small extent with osmium, KCN or NH_4CSN produced, however, small DPP peaks whose potential corresponded to that of peak C of DNA,Os,py (Fig. 1). The observed inactivity or small DPP activity of DNA,Os,CN^- and DNA,Os,CSN^- samples may be brought in association with a great negative charge density at the DNA molecule (due to the introduction of additional negative charges of CN^- or CSN^-) resulting in a strong repulsion between the DNA molecule and the negatively charged electrode, thus preventing interactions necessary for the appearance of a reduction DPP peak. In contrast to the DPP behaviour of DNA,Os,CN^- and DNA,Os,CSN^- native and denatured DNAs modified with Os, bipyridine produced DP polarograms similar to those produced by DNA,Os,py (Fig. 1).

The time course of the ds DNA reaction with osmium tetroxide, pyridine was investigated by means of DPP (Fig. 7). During the first 10—15 hours the DPP peak C increased markedly in dependence on time (alpha phase); the height of the peak C changed only little within the next 30 hours (beta phase), and this peak was growing steeply with time afterwards (gamma phase). In the gamma phase a small

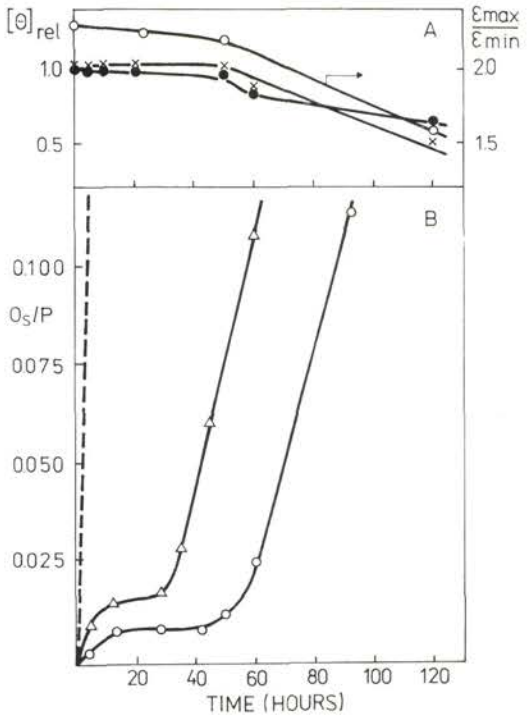

Fig. 7. Time-dependence of the OsO_4, pyridine reaction with native and gamma-irradiated calf thymus DNA: DPP, CD and spectrophotometric measurements. *A*, ○—○, $\varepsilon max/\varepsilon min$, ×—×, positive CD band (275 mm), ●—●, negative CD band (245 nm), native DNA; *B*, DPP: ○—○, native DNA; △—△, DNA irradiated with a dose of 50 Gy (5000 rad), – – –, denatured DNA (taken from Lukášová et al. 1982); Θ_{rel}, the ratio between the ellipticity of unmodified and osmium modified DNA samples. The content of osmium in DNA samples was determined from the height of the DPP peak as described in Materials and Methods. ds DNA at a concentration of 300 μg/ml in SSC was incubated with 2×10^{-3} mol dm^{-3} OsO_4, 1 % pyridine at 26 °C.

DPP peak III (Figs. 1*c*, *f*) appeared, suggesting that some single-stranded regions had been formed. The reaction kinetics of ds DNA thus markedly differed from that of thermally denatured DNA (Lukášová et al. 1982); the latter showed a much faster kinetics with no steps in the initial stage of the reaction. The time dependence of ds DNA damaged with a dose of 50 Gy (5000 rad) of gamma-rays differed from that of native DNA; peak C of the former reached higher values in the beta phase, and the gamma phase of damaged DNA started earlier than that of native DNA (Fig. 7). Time course of the reaction of native DNA was observed by means of absorption spectrophotometry ($\varepsilon max/\varepsilon min$, t_m) and CD in parallel with DPP. In agreement with DPP measurements the $\varepsilon max/\varepsilon min$ ratio decreased only

slightly in both the alpha and beta phases, while it decreased very markedly in the gamma phase. Similarly, almost no changes in t_m (not shown) and CD (Fig. 7) were observed in the alpha and beta phases, respectively; in the gamma phase both CD bands decreased.

Discussion

Primary binding sites in ds DNA. It has been shown earlier (Marzilli 1977; Hodgson 1977; Barton and Lippard 1980) that in the presence of pyridine osmium binds covalently to thymine and cytosine residues in single-stranded DNA. The structure of the thymine,Os,py complex was determined by means of crystal X-ray diffraction analysis (Kistenmacher et al. 1976; Neidle and Stuart 1976). The experimental results presented here as well as in our previous communications (Paleček et al. 1981; Lukášová et al. 1982) show that, under suitable conditions, osmium also binds to ds DNA to a limited extent. Experiments with molecular models (based on the assumption that the osmium,py complex formed in DNA is identical to that found with monomeric thymine (Kistenmacher et al. 1976; Neidle and Stuart 1976))have demonstrated that in an intact B form of the DNA double helix the formation of an osmium,py complex with a thymine residue is not possible for steric reasons (H. Sobell, personal communication). Taking into consideration the small reactivity of ds DNA in the initial stage of its modification by osmium (Fig. 7), it may be concluded that the primary reaction sites in ds DNA do not include bases contained in the intact B form. In principle, the primary reaction site in the double helix might be formed as a result of any permanent or transient (if the opening/closing rates are sufficiently slow) distortion which makes the 5,6 double bond of the pyrimidine residue available for osmium complex formation. Osmium can thus bind to single-stranded regions (Lukášová et al. 1982) but a sufficient condition for osmium complex formation may even be certain kind of bending or kinking (Sobell 1981; Lozansky and Sobell 1981) of the double helix, including β-kink formation.

Changes in DNA conformation due to osmium binding. It has been shown earlier (Marzilli 1977; Barton and Lippard 1980) that metal binding to bases in polynucleotides seeks to order the nucleotide residues in a manner dictated by the stereochemistry of the metal, which may be different from the way in which the sugar-phosphate backbone orients the bases with respect to each other. This seems to be the case with osmium binding to DNA, which results in a counter-acting of the constraints imposed by the phosphodiesteric linkages and causes disordering to occur. Disordering of the structure of single-stranded DNA had already been revealed by electron microscopy (Cole et al. 1977), showing a large increase in the

mean spacing between the bases. Our CD measurements (Figs. 4, 5) give further support to disordering of DNA by osmium binding. A large decrease in CD bands of thermally denatured DNA observed at low Os/P (Fig. 4) suggests that osmium distorts the helical structure of denatured DNA over a region extending beyond the immediate binding site. For example, binding of osmium to less than 1 % of the pyrimidine bases in denatured DNA resulted in an about 10 % decrease in the DNA positive CD band.

Contrary to this no detectable changes in CD (Figs. 4, 7) and t_m was observed and no DPP peak III (characteristic for single-stranded regions) appeared (Fig. 1*b*) with ds DNA at the same Os/P; however, the DPP peak II doubled (Figs. 1*a*, *b*). These results suggest that osmium binding to sporadic primary reaction sites in native DNA results in local changes in DNA conformation limited to the close neighbourhood of the binding site. Greater structural disordering does not occur because at this stage the double helical structure (in contrast to denatured DNA) is stable enough to resist the strains induced by osmium binding. At greater Os/P stability of the double helix is disturbed and a major disordering extending far beyond the immediate binding site occurs. This process is accompanied by a loss of helicity and by the formation of single-stranded regions (manifested by a decrease in CD bands — Figs. 4, 5 and by the appearance of DPP peak III — Figs. 1*c*, *f*). The single-stranded and distorted ds regions are targets of further osmium binding and the disordering process proceeds to further polynucleotide regions. The possible reason for DNA disordering might be seen in stacking interactions of the pyridine moiety and the adjacent bases as suggested earlier (Daniel and Behrman 1976; Cole et al. 1977) on the basis of studies of osmium bipyridine labelled ApU. However, this type of interaction cannot be expected in DNA,Os,CN^- and DNA,Os,CSN^-; nevertheless their CD spectra are basically the same (Fig. 5) as those of DNA,Os,py. The main reason for the DNA disordering thus seems to be the bound osmium itself.

Recently disordering of both single-and double-stranded polynucleotides due to binding of other ligands has been observed (Marzilli 1977; Barton and Lippard 1980). For example, covalent binding of a derivative of the carcinogenic drug benzo (α) pyrene distorts the structure of ds DNA over a region extending beyond the immediate binding site (Hogan et al. 1981). Disordering of single-stranded polynucleotides by Cu (II) is very cooperative Rifkind et al. 1976).

Is it possible to label distorted regions in ds DNA with osmium? In recent years evidence has accumulated (Paleček 1976; Sobell 1981; Lozansky and Sobell 1981; Wells et al. 1980) suggesting that transiently or permanently distorted regions may exist in ds DNA. Formation of these regions may be conditioned by a specific nucleotide sequence, by anomalies in the DNA primary structure, by the occurrence of boundaries between the B form and some other less usual forms in

DNA, etc. As the local conformational changes may be of great biological significance it would be convenient to have a method available that would make possible the detection, visualization and nucleotide sequencing of distorted regions. Osmium binding to ds DNA seems to be a suitable tool for this purpose. It has been shown in this paper that the kinetics of osmium binding shows well-resolved alpha and beta phases (Fig. 7). A very small time dependence of the osmium binding in the beta phase suggests that under the given conditions the observed osmium binding is little influenced by the DNA dynamics, including breathing and various kinds of fluctuation. The results thus suggest that in alpha and beta phases osmium binds predominantly to pre-existing distorted regions in ds DNA and that it acts as a denaturing agent in the gamma phase. Only if overlapping of the beta and gama phases is minimized, labelling of the primary distorted regions with osmium becomes possible. So far it has been demonstrated that osmium binds preferentially to single-stranded regions in ds DNA (Lukášová et al. 1982), in the vicinity of damage induced in the polynucleotide chain by ionizing (Fig. 7) or UV radiations, i. e. probably in the vicinity of single-strand breaks and close to thymine dimers.

Changes in the electrophoretic mobility of plasmid ColE1 cd DNA (Fig. 6) suggest that the initial sites of osmium binding might be associated with relatively destabilized regions (e.g. cruciforms) contained in tightly-wound superhelical DNAs (Paleček 1976; Wells et al. 1980; Lebowitz et al. 1977; Howe-Grant et al. 1976). As it follows from our CD measurements (Figs. 4 and 5) osmium binding results in the formation of permanent non-helical regions which may cause a coupled loss of duplex and superhelical turns in ColE1 DNA. Osmium binding is probably assisted by a high free energy of supercoiled DNA until the molecule becomes fully or partially relaxed. As a result of this process a denatured region large enough to be visualized by the conventional electron microscopy may be formed. Recently such a denaturation "bubble" has been observed with osmium-modified plasmid cDm 506 DNA (E. Paleček and G. Glikin, unpublished); mapping of the osmium binding site has revealed one major site which differred from that found by nuclease Sl cutting in the absence of osmium. Moreover, thanks to the work of Beer et al. (Marzilli 1977; Barton and Lippard 1980; Cole et al. 1977) the visualization of individual osmium atoms in distorted regions of double-stranded DNAs should be possible by means of special electron microscopic techniques.

The methods used so far for the determination of osmium bound to DNA (Beer et al. 1966; Chang et al. 1977) have been suitable for the analysis of single-stranded DNAs. Their sensitivity is, however, insufficient for the determination of osmium attached to a few percent of bases during the initial stage of modification of ds DNA. Methods of electrochemical analysis are sufficiently sensitive for this purpose and they do not require destruction of the DNA sample. Our quite recent measurements (Paleček and Hung 1983) have shown that the

application of stripping voltammetry increases the sensitivity of the determination of DNA,Os,py by two orders of magnitude as compared with DPP.

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Abbreviations used: DPP, differential pulse polarography; CD, circular dichroism; cd DNA, closed duplex DNA; oc DNA, open circular DNA; ds, double-stranded; DNA, Os, py, the reaction product of DNA with OsO_4-pyridine purified on Sephadex column or by dialysis. Os/P varied in DNA,Os,py samples from 0.008 to 0.5; DNA,Os,CN^-, (DNA,Os,CSN^-), the purified reaction product of DNA with OsO_4, KCN (NH_4CSN).

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