

Electrochemistry of Osmium — Nucleic Acid Complexes: A Probe for Single-Stranded and Distorted Double-Stranded Regions in DNA

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Abstract. It was found that osmium introduced into a polynucleotide chain represents a suitable electroactive marker, which can be very sensitively detected by means of differential pulse polarography. Osmium-containing single-stranded DNA yields several reduction DPP peaks, one of them probably being of a catalytic nature. The height of this peak depends linearly on the concentration of denatured DNA in the range of 0.5—200 $\mu\text{g/ml}$. Single-stranded synthetic polynucleotides containing pyrimidine bases behave in a similar way. Homopolynucleotides containing only purine bases are inactive.

Up to now it has been supposed that native double-helical DNA (in contrast to single-stranded DNA) does not react with OsO_4 . It was demonstrated in this study that double-helical DNA reacts to a certain extent with OsO_4 : the extent of the modification of double-helical DNA depends on the content of structural defects (double-stranded distorted regions) in the DNA molecule. Suitable conditions for the modification of both native and denatured DNA with OsO_4 and conditions for their DPP measurement were found. The reaction of nucleic acids with OsO_4 and subsequent DPP detection of bound osmium can be exploited as a probe for the detection of single-stranded and double-stranded distorted regions in DNA.

Key words: Electrochemistry — DNA-osmium complexes — DNA secondary structure

Introduction

Single-stranded (ss) polynucleotides which contain electroactive base residues (adenine, cytosine) are polarographically reducible (Paleček 1969a, b, 1971, 1980). On the contrary, double-stranded (ds) polynucleotides behave as nonreducible compounds (Paleček 1976, 1980) or yield only relatively low polarographic currents under the same conditions. This phenomenon was exploited in structural analysis of nucleic acids in connection with modern electrochemical methods,

especially with differential pulse polarography (DPP) (Paleček 1971, 1976, 1980). Native DNA yields a very small peak (peak II) at room temperature and neutral pH. This peak increases in the case of damage of sugar phosphate backbone or other DNA constituents what enables the detection of slight perturbations in the DNA ds structure induced by various agents (Paleček 1976, 1980). Denatured DNA yields under the same conditions the well developed peak III at more negative potential. For calf thymus DNA the peak II yielded by the double-helical form of DNA is approximately 100 times lower than the peak III of ss DNA. The difference in the potentials of these peaks and the higher sensitivity of DPP for ss DNA enables the determination of the traces of ss polynucleotides in the samples of ds ones (Paleček 1971). The disadvantage of this method is that it can be used only for analysing pure samples of nucleic acids in solution. Therefore we endeavoured to propose a method that would have most of the advantages of the above-mentioned polarographic method (especially its sensitivity), but which would enable one to investigate properties and structural changes of ds nucleic acids *in situ* or in solution in a complex with other substances which interfere in polarographic analysis.

This effect could be realised by means of an electroactive marker which can be introduced into a nucleic acid *in situ* and whose presence could be detected polarographically in isolated nucleic acid. A suitable chemical agent should have the following properties: (a) the ability to bind specifically to ss or distorted (premelted) ds regions under conditions close to physiological ones, (b) to form a sufficiently stable bond even after the removal of the unreacted agent, (c) to be electroactive even after introduction into the polynucleotide chain. Among possible candidates we chose osmium tetroxide, which is known to bind at room temperature and neutral pH in the presence of some ligands such as CN^- , pyridine, dipyridine, etc. (Beer et al. 1966; Highton et al. 1968; Di Giamberlino et al. 1969; Chang Chien-Hsing et al. 1977) to pyrimidine bases of ss polynucleotides with formation of stable complexes. Free OsO_4 is polarographically reducible in several steps (Crowell et al. 1941; Meites 1957; Cover and Meites 1961; Connery and Cover 1968). Our preliminary results (Paleček et al. 1981) have shown that the osmium complex bound in a polynucleotide chain represents an electroactive marker which is markedly manifested in polarographic analysis.

In the present study we endeavoured to find optimum conditions for introducing this marker into the polynucleotide chain and to propose a new method for the determination of ss and ds distorted regions in DNA.

Material and Methods

Calf thymus DNA was isolated according to Zamenhof (1957) with the average molecular weight of about $3 \cdot 10^7$. The RNA content, estimated by orcinol method (Cerrioni 1956) was lower than 1%, the

protein content, estimated by Lowry's method (Lowry et al. 1951) was lower than 1%. The presence of ss DNA estimated by DPP method (Paleček 1971) was lower than 0.2%. Supercoiled DNA of the plasmid ColE1 was isolated by a modified phenol method (Vojtišková et al. 1980) and contained about 5% of open circular form. The content of proteins and RNA estimated by the same methods as in the case of calf thymus DNA was under the limit of detection of these methods. The presence of DNA from the bacterial chromosome estimated by DPP after thermal denaturation was lower than 1%. Endonuclease S_1 was isolated according to Vogt (1973). Polynucleotides poly(rC), poly(rG), poly(rU) and poly(rA), poly(dT) and OsO_4 were obtained from Serva. Sephadex G 25 medium was purchased from Pharmacia Fine Chemicals. Irradiation of DNA with ionizing radiation was performed with a ^{60}Co source. DNA modification with osmium tetroxide was performed in the following way: DNA solution in SSC/20 (7.5×10^{-3} mol. \cdot dm $^{-3}$ NaCl and 7.5×10^{-4} mol. \cdot dm $^{-3}$ sodium citrate) was mixed with the same volume of OsO_4 solution of concentration of 4×10^{-3} mol. \cdot dm $^{-3}$ containing 2% pyridine in SSC/20, unless stated otherwise; the reaction took place at 25°C for 20–24 hours. The nonreacted OsO_4 and pyridine were removed from the solution either by means of dialysis or on a Sephadex G 25 column (0.9×22 cm). The dialysis was performed in dialysing bags obtained from the Union Carbide Corporation against SSC/20 at 5°C for 24 hours. During the first four hours the dialysing solution was exchanged each 30 minutes, later at longer time intervals. Before use the dialysing bags were boiled in 5% solution of $NaHCO_3$ for 20 min, then thoroughly washed in distilled water. DNA concentration after the removal of OsO_4 was determined on the basis of phosphorus content by the method of Martin and Doty (1949) or spectrophotometrically at 260 nm.

Polarographic measurements were performed on a PAR 174A apparatus (Princeton Applied Research) using a three-electrode system, including a dropping mercury electrode (the working electrode), a Pt wire (an auxiliary electrode) and a saturated calomel electrode (the reference electrode). The dropping mercury electrode had the following constants: capillary flow rate $m = 1.12$ mg/s, Hg column height $h = 40$ cm, drop time 2 s, scan rate 2.0 mV/s, modulation amplitude 25 or 50 mV. All measurements were carried out at 24°C. Spectrophotometric measurements were performed with a Zeiss VSU 2 apparatus. The digestion of DNA samples with endonuclease S_1 was performed in 0.03 mol. \cdot dm $^{-3}$ sodium acetate pH 4.6, 0.05 mol. \cdot dm $^{-3}$ NaCl, 1 m mol. \cdot dm $^{-3}$ $ZnSO_4$ and 5% glycerol; 50 μ g/ml of DNA and 40 units of the enzyme was incubated at 37°C for 30 min. The digestion was terminated by cooling in ice and by adding the same volume of glacial 1 mol. \cdot dm $^{-3}$ $HClO_4$. Content of the acid-soluble fraction was determined spectrophotometrically at 270 nm after removing precipitated polymeric DNA by centrifugation at 3000 r.p.m. for 5 min. The value of absorbance of a sample of this DNA of concentration of 25 μ g/ml, which was hydrolyzed in 0.5 mol. \cdot dm $^{-3}$ $HClO_4$ 30 min on a boiling water bath, was taken as 100% of the acid-soluble fraction.

Results

In a medium of 0.05 mol. \cdot dm $^{-3}$ sodium phosphate, pH 6.9, 5×10^{-5} mol. \cdot dm $^{-3}$ OsO_4 yielded two well-developed DPP peaks and an poorly developed inflex i at a potential of about -1.1 V (Fig. 1a). In the same medium the DPP polarogram of denatured DNA-Os in a concentration of 32.7 μ g/ml (i.e. at such a DNA concentration where the content of bound Os could correspond maximally to a concentration of 5×10^{-5} mol. \cdot dm $^{-3}$ under the assumption that each pyrimidine base residue in the polynucleotide chain binds one atom of osmium (Chang Chien-Hsing et al. 1977)) differed in the first place from the DPP polarogram of

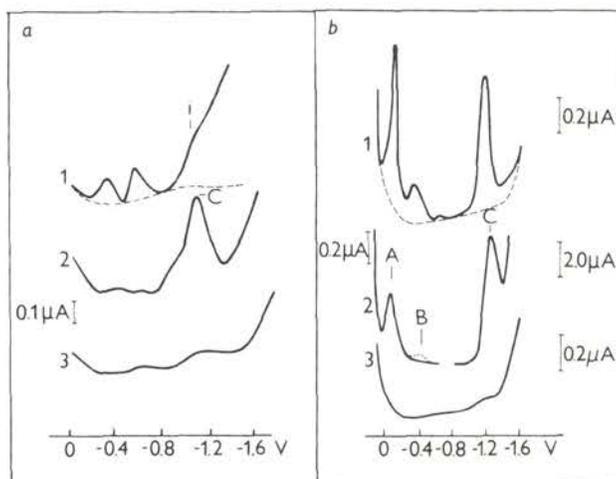


Fig. 1. DPP polarograms of free OsO_4 and native and denatured DNA-Os. a) in $0.05 \text{ mol. dm}^{-3}$ sodium phosphate pH 6.9; b) in 0.3 mol. dm^{-3} ammonium formate with $0.05 \text{ mol. dm}^{-3}$ sodium phosphate pH 6.9. (1) $5 \times 10^{-5} \text{ mol. dm}^{-3}$ OsO_4 ; (2) denatured DNA-Os in concentration of $1 \times 10^{-4} \text{ mol. dm}^{-3}$ (related to the monomer); (3) native DNA-Os in concentration of $1 \times 10^{-4} \text{ mol. dm}^{-3}$. - - - -, the background electrolyte. Pulse amplitude 25 mV. The modification was performed at DNA concentration of $150 \mu\text{g/ml}$ with $2 \times 10^{-3} \text{ mol. dm}^{-3}$ OsO_4 and 1% pyridine in SSC/20 at 25°C for 22 hours. Nonreacted OsO_4 and pyridine were removed by means of dialysis

OsO_4 by a marked high peak at potentials, where OsO_4 yielded only the inflex *i*. In a medium of 0.3 mol. dm^{-3} ammonium formate, i.e. in the medium which has been up to now normally used in the polarographic analysis of nucleic acids (Paleček 1969a, 1980), differences in DP polarograms of OsO_4 and denatured DNA-Os were also very expressive. The DP polarogram of OsO_4 (Fig. 1b) was roughly similar to the polarogram obtained in the medium of phosphate buffer (Fig. 1a); however, in the medium of ammonium formate a well-developed peak appeared instead of the slight inflex *i* (Fig. 1b). The corresponding peak (C) of denatured DNA-Os in a concentration of $32.7 \mu\text{g/ml}$ was, however, more than five times higher; on the other hand, the more positive peaks (A and B) of DNA-Os were lower than those of OsO_4 under these conditions. Native DNA yielded only very low peaks in both media under the same conditions (Figs 1a, b).

We can thus conclude that a new electroactive centre is formed in the polynucleotide chain as a consequence of the binding of osmium to denatured DNA; its presence can sensitively be detected by means of DPP. Peak C, which can be only minimally influenced by the presence of any free OsO_4 , seems to be most suitable for the purposes of structural analysis. DP polarograms of DNA-Os can be distinguished from polarograms of OsO_4 on the basis of the comparison of the ratio of heights of peaks A and C of DNA-Os and the corresponding peaks (inflex) of

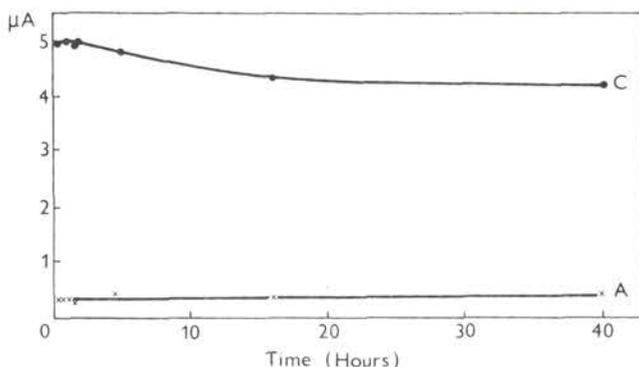


Fig. 2. Dependence of the heights of DPP peaks A and C of denatured DNA-Os on the time after removing the nonreacted OsO_4 . \bullet — \bullet , peak C; \times — \times , peak A. The conditions of the modification were the same as in Fig. 1. DPP measurement was carried out in 0.3 mol. dm^{-3} ammonium formate with $0.05 \text{ mol. dm}^{-3}$ sodium phosphate, pH 6.9, pulse amplitude 25 mV. DNA concentration was the same as that given in Fig. 1. Nonreacted OsO_4 was removed on Sephadex G 25

OsO_4 . A more detailed characterization of DPP peaks of denatured DNA-Os from the electrochemical point of view will be published elsewhere.

We investigated the height of DPP peaks A and C as a function of time starting with the moment when free osmium and pyridine were separated from DNA on a Sephadex column. No changes in the height or potential of either peak were observed during the first two hours (Fig. 2). After five hours' standing the height of peak C decreased by about 5%, after 15 hours by about 14% and after 40 hours' standing the height of peak C corresponded to approximately 86% of its original height value; on the contrary, the height of peak A increased slightly in the same interval. As follows from Figs. 1 and 2, the decrease of peak C and a small increase of peak A could indicate the release of osmium from some binding sites in the polynucleotide chain. If the incubation of the DNA-Os solution was carried out in the presence of metallic mercury on the bottom of the vessel, the changes in the height of peak C were somewhat greater. It is therefore recommendable to avoid a long-term contact of a DNA-Os solution with mercury. The results of this experiment show that the binding of the electroactive centre in a polynucleotide chain is sufficiently stable from the point of view of the objectives set out in the introduction to this paper.

Conditions of the reaction of osmium with DNA

We studied the dependence of DPP behaviour of DNA-Os on the conditions of reaction of DNA with OsO_4 , such as time of modification, OsO_4 concentration, type and concentration of the ligand, pH and ionic strength of the medium. Peak

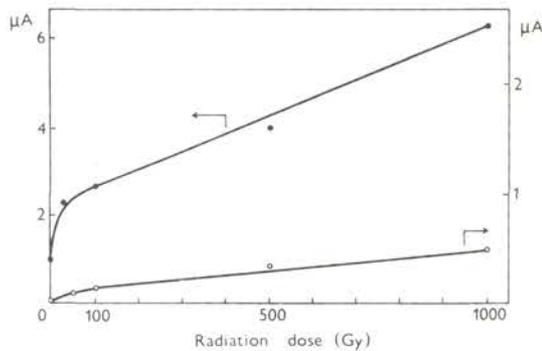


Fig. 3. Dependence of the heights of peaks C and III of denatured DNA-Os on the time of modification. \bullet — \bullet , peak C; \times — \times , peak III. DPP measurement was performed at DNA concentration of $80 \mu\text{g/ml}$ and amplitude 50 mV in $0.3 \text{ mol}\cdot\text{dm}^{-3}$ ammonium formate with $0.05 \text{ mol}\cdot\text{dm}^{-3}$ sodium phosphate pH 6.9. The modification was carried out at DNA concentration of $250 \mu\text{g/ml}$, other conditions were the same as those given in Fig. 1. In given time intervals samples were taken in which nonreacted OsO_4 and pyridine were removed on a Sephadex G 25 column

C grew in a dependence on the time of modification of denatured DNA by osmium (Fig. 3); after approximately 20 hours the height of this peak reached under the given conditions maximum value and did not change further. At shorter time intervals a more negative peak could be observed in addition to peak C; its potential corresponded to the potential of peak III of unmodified denatured DNA (Paleček 1980). The height of this peak decreased with time.

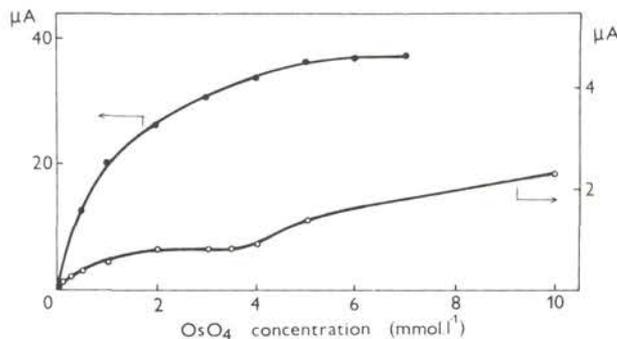


Fig. 4. Dependence of the height of peak C yielded by native and denatured DNA-Os on the OsO_4 concentration during the modification. \bullet — \bullet , denatured DNA-Os, \circ — \circ , native DNA-Os. DPP measurement was performed at DNA concentration of $115 \mu\text{g/ml}$, other conditions as those given in Fig. 3. DNA modification: DNA concentration $165 \mu\text{g/ml}$, 1% pyridine. Nonreacted OsO_4 and pyridine were removed by means of dialysis

If polarographic measurements of native DNA-Os were carried out at high concentration, native DNA also yielded a marked peak C.

OsO₄ concentration. For denatured DNA the height of peak C increased with the OsO₄ concentration used for the DNA modification up to 5×10^{-3} mol.dm⁻³ (Fig. 4); further increase of OsO₄ concentration did not influence the height of peak C under the given conditions. The height of peak C of native DNA increased with OsO₄ concentration up to 1×10^{-3} mol.dm⁻³; in the range of $1-3 \times 10^{-3}$ mol.dm⁻³ the height of peak C was independent of OsO₄ concentration. At higher OsO₄ concentration the peak height again increased with concentration. This increase could be conditioned by structural changes in DNA induced by osmium binding, in analogy to other agents (e.g. formaldehyde), which are bound predominantly to ss DNA (Brown 1974).

Influence of the nature and concentration of the ligand

In the labelling of DNA with osmium, other ligands, such as dipyridine and ammonium rhodanide (Chang Chien-Hsing et al. 1977; Kistenmacher et al. 1976), were used besides pyridine to stabilize the ester linkage of osmium to the base. DPP peaks obtained with a complex DNA-osmium-rhodanide at 25°C were substantially less developed than peaks of analogous complexes with pyridine. A complex of DNA-dipyridine-osmium yielded well-developed DPP peaks, similar to those of the complex with pyridine. However, dipyridine itself is polarographically reducible and yielded a DPP peak in the region of potentials of peak C under conditions of our measurement. Considering the fact that we could not exclude the possibility that dipyridine could also be bound to DNA by a type of bond other than in the complex with osmium and that the complex containing bipyridine represented a more complicated system from the polarographic point of view, we preferred in our study the polarographically nonreducible and DPP-inactive pyridine.

The height of peak C of denatured DNA-Os grew markedly with pyridine concentration in the range of 0.1–2% (Fig. 5): further increase of pyridine concentration had only a slight influence on the height of peak C. For native DNA, changes in pyridine concentration in the range of 0.1–0.8% had only a slight influence on the height of peak C. An increase of pyridine concentration above 1% led to a steep rise in peak C; at a 10% concentration of pyridine the height of peak C of native DNA approached the peak height of denatured DNA.

Influence of ionic strength. We investigated the dependence of the height of peak C on the CsCl concentration used in the reaction of native and denatured DNA with OsO₄-pyridine (Fig. 6). The height of peak C of denatured DNA-Os was nearly independent of CsCl concentration in the range of 0.5–2.0 mol.dm⁻³. (In the range of 0.1–0.5 mol.dm⁻³ the height of peak C grew with CsCl

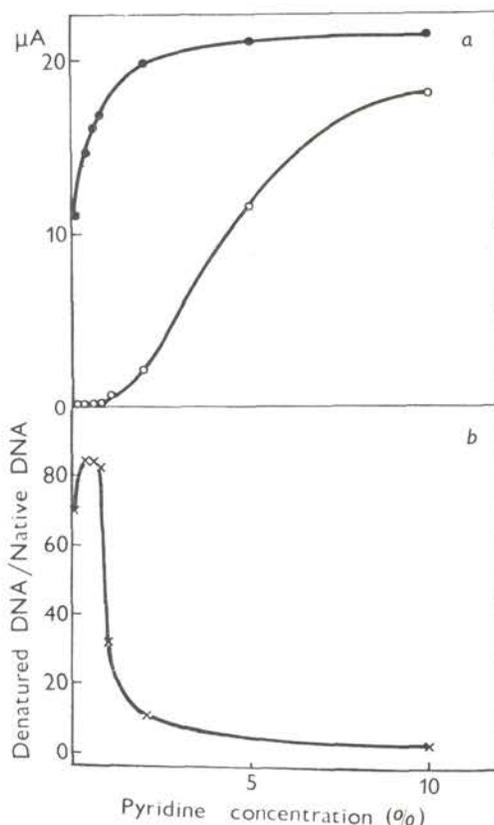


Fig. 5. a) Dependence of the height of peak C of denatured and native DNA-Os on pyridine concentration in the reaction mixture. ●—●, denatured DNA-Os, ○—○, native DNA-Os. DPP measurement was carried out at DNA concentration of 83 $\mu\text{g/ml}$, other conditions as given in Fig. 3. DNA concentration at the modification was 150 $\mu\text{g/ml}$, other conditions as those given in Fig. 1. Nonreacted OsO_4 and pyridine were removed by means of dialysis
b) The ratio of the height of peak C of denatured DNA-Os to the height of peak C of native DNA-Os at various concentrations of pyridine in the reaction mixture

concentration.) For native DNA the height of peak C decreased with increasing concentration in the range of 0–0.1 $\text{mol}\cdot\text{dm}^{-3}$, it was nearly independent of CsCl concentration in the region of 0.1–0.3 $\text{mol}\cdot\text{dm}^{-3}$ and increased at concentrations higher than 0.3 $\text{mol}\cdot\text{dm}^{-3}$. This growth of peak probably reflects higher accessibility of bases to an interaction with the environment, which was also observed in the polarographic analysis of nonmodified DNA (Paleček 1980).

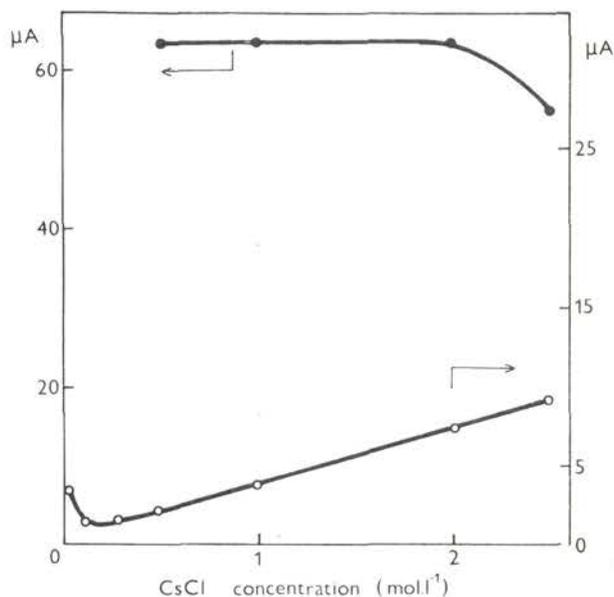


Fig. 6. Dependence of the height of peak C of native and denatured DNA-Os on CsCl concentration during the modification. \bullet — \bullet , denatured DNA-Os; \circ — \circ , native DNA-Os. DNA concentration in the DPP measurement was $164 \mu\text{g/ml}$, other conditions were the same as those given in Fig. 3. The DNA modification was performed in $0.02 \text{ mol} \cdot \text{dm}^{-3}$ sodium phosphate pH 7 at 25°C for 22 hours. Concentrations: DNA — $200 \mu\text{g/ml}$, $2 \times 10^{-3} \text{ mol} \cdot \text{dm}^{-3}$ OsO_4 , 1% pyridine, and variable concentration of CsCl. The reaction was terminated by dialysis

Conditions of polarographic measurement

In the present study we aimed only at finding suitable conditions for the polarographic measurements of DNA-Os. Characteristics of processes to which polynucleotides, labelled with osmium, are subjected at an electrode will be the object of a further communication. In the analysis of denatured DNA-Os we also used, besides DPP, normal pulse polarography (NPP), DC polarography and linear sweep voltammetry (LSV) (Fig. 7). Among these methods DPP appeared to be the most suitable one for analytical purposes under the given conditions. The height of DPP peak C grew linearly with the pulse amplitude (Fig. 8); for peak A this dependence was linear only up to 25 mV. In order to reach the optimum sensitivity it is thus suitable to carry out the measurement of peak C at the amplitude of 50–100 mV, whereas peak A should be measured at lower amplitudes.

The height of DPP peak C of denatured and native DNA-Os remained almost unchanged with pH in the range of 6.0–7.6. Our measurements were performed mostly at pH 6.9. At this pH the height of DPP peak C of denatured DNA-Os

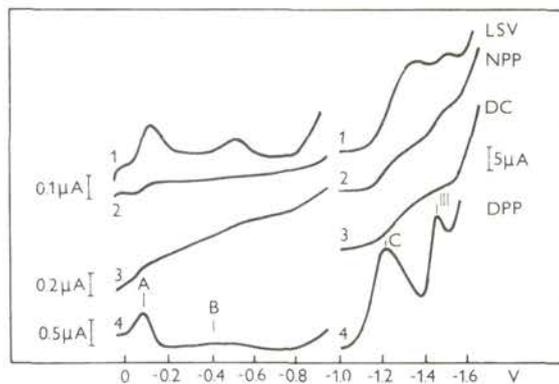


Fig. 7. Polarograms of denatured DNA-Os obtained by different techniques. DPP — differential pulse polarography, DC — direct current polarography, NPP — normal pulse polarography, LSV — linear sweep voltammetry. DNA concentration during measurements was $100 \mu\text{g/ml}$, other conditions were the same as those given in Fig. 3. Scan rate in LSV was 0.2 V/s . DNA concentration in the modification was $300 \mu\text{g/ml}$, other conditions were the same as those given in Fig. 1

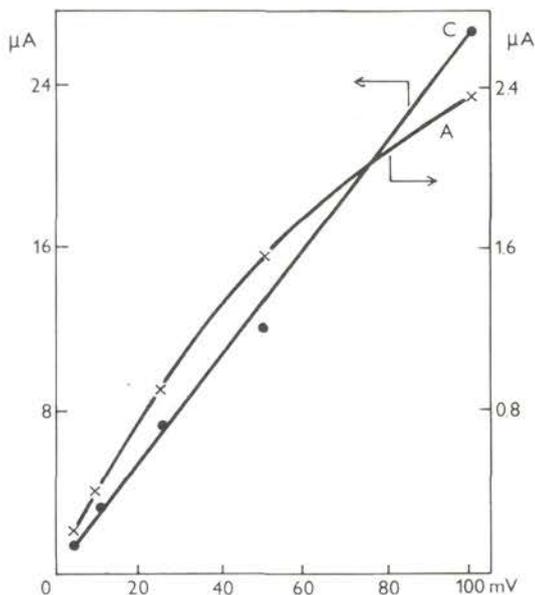


Fig. 8. Dependence of peaks A and C of denatured DNA-Os on pulse amplitude. x—x, peak A; •—•, peak C. Conditions of both DPP measurement and modification were the same as those given in Fig. 1b. DNA concentration in DPP measurement was $50 \mu\text{g/ml}$

depended on the nature of salt in the background electrolyte. The height of this peak was approximately three times higher in a medium containing ammonium ions ($0.1 \text{ mol} \cdot \text{dm}^{-3} \text{ HCOONH}_4$ or HN_4Cl) than in a medium with $0.1 \text{ mol} \cdot \text{dm}^{-3} \text{ NaCl}$. Substitution of NaCl with CsCl led almost to a duplication of the peak height. We investigated the dependence of peak C on the concentration of ammonium ions (Fig. 9). The height of this peak increased steeply with NH_4Cl

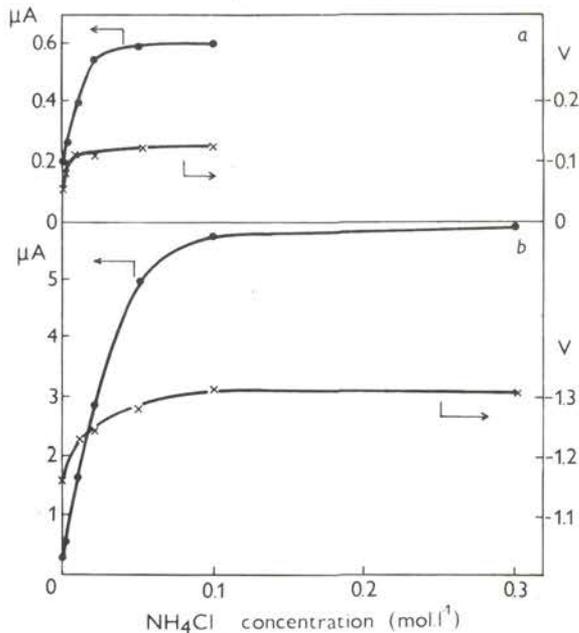


Fig. 9. Dependence of the height of peaks A and C of denatured DNA-Os on concentration of NH_4Cl in the background electrolyte at DPP measurements. a) peak A; b) peak C; \bullet — \bullet , the peak height; \times — \times , the peak potential. DPP measurement was carried out in $0.05 \text{ mol} \cdot \text{dm}^{-3}$ sodium phosphate pH 6.9 with varying NH_4Cl concentration, and DNA concentration of $50 \mu\text{g}/\text{ml}$ and pulse amplitude 50 mV . Modification procedure was the same as that described in Fig. 1.

concentration up to $0.05 \text{ mol} \cdot \text{dm}^{-3}$ and the peak potential was shifted to more negative values. Further growth of the peak height was already less steep and in the concentration range of 0.1 – $0.3 \text{ mol} \cdot \text{dm}^{-3} \text{ NH}_4\text{Cl}$ the peak height did not increase and its potential scarcely changed.

Homopolynucleotides

Besides native and denatured DNA, ss poly(rA), poly(rG), poly(rC), poly(rU) and poly(dT) were treated with osmium-pyridine. Pyrimidine polynucleotides yielded

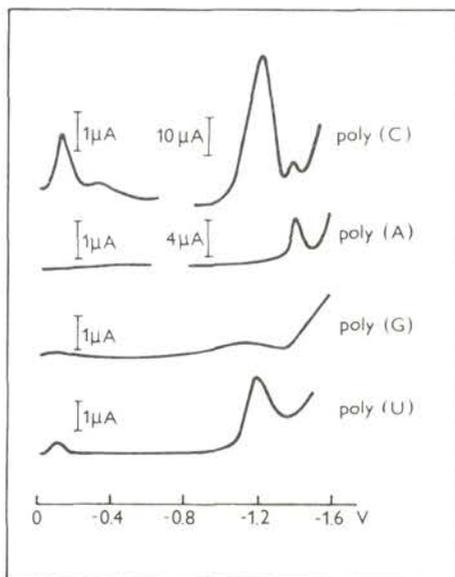


Fig. 10. DP polarograms of polynucleotides. Poly(rC) concentration was $3.5 \times 10^{-4} \text{ mol. dm}^{-3}$, poly(rU) and poly(rA) were in concentration of $5 \times 10^{-4} \text{ mol. dm}^{-3}$. Conditions of DPP measurements were the same as those given in Fig. 3. Polynucleotide concentration during the modification was $1 \times 10^{-3} \text{ mol. dm}^{-3}$, other conditions were the same as those given in Fig. 1

DPP peaks (Fig. 10) similar to those of denatured DNA. Purine polynucleotides, i.e. poly(rG) and poly(rA), remained practically intact after the action of osmium. Poly(rG) behaved as DPP inactive under conditions used in the DPP measurement of poly(rC) (Fig. 10); at high instrument sensitivity poly(rG) yielded a small peak C corresponding in height approximately to 1/100 of the peak of poly(rC). Poly(rA) yielded a single peak conditioned by the reduction of adenine residue, which is usual for unmodified poly(rA) (Paleček 1969a, 1971, 1980); otherwise its DPP behaviour was in agreement with that of poly(rG).

Peak C of poly(rU) modified with osmium-pyridine is substantially smaller than that of poly(rC). This is in agreement with the results of Chang Chine-Hsing et al. (1977), demonstrating lower stability of poly(rU) osmium pyridine complex as compared with that of poly(rC). Similar results were obtained also with poly(dT). It can be thus concluded that the DPP behaviour of synthetic polynucleotides agrees basically with the fact, known from literature (Chang Chien-Hsing et al. 1977), that OsO_4 reacts under the given conditions with the residues of pyrimidine bases and not with those of purine bases.

Determination of *ss* and *ds* distorted regions in DNA

We investigated the dependence of the height of peak C on the concentration of native and denatured DNA-Os (Fig. 11). In the range of 0.5–200 $\mu\text{g/ml}$ the peak

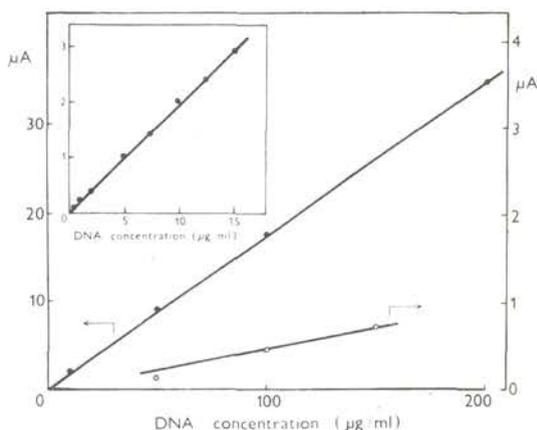


Fig. 11. Dependence of the height of peak C of native and denatured DNA-Os on the DNA concentration at DPP measurement. \circ — \circ , native DNA-Os; \bullet — \bullet , denatured DNA-Os. Conditions of DPP measurement were the same as those described in Fig. 3. DNA concentration during the modification was 400 $\mu\text{g/ml}$. other conditions were the same as described in Fig. 1

height depended linearly on the concentration of denatured DNA-Os; with a further increase of DNA concentration the peak height did not change any more. The sensitivity of the determination of denatured DNA-Os is thus approximately one order of magnitude higher than the sensitivity of direct DPP determination without Os (Paleček 1971). Native DNA-Os yields peak C only at concentrations of about 50 $\mu\text{g/ml}$ and higher under the given conditions; the height of this peak grew linearly with increasing concentration of DNA-Os. The great difference in the heights of peak C of native and denatured DNA-Os offers the possibility of exploiting this peak for the determination of *ss* regions in DNA, or even for the investigation of other changes in the conformation of *ds* DNA. To test this possibility we followed the kinetics of renaturation of plasmid ColE1 DNA and the damage to native calf thymus DNA by ionizing radiation in the range of doses where *ss* segments had not yet been formed (these doses cause the formation of distorted *ds* regions in the vicinity of sites where a chemical change has taken place as a consequence of irradiation, e.g. in the vicinity of a *ss* break, these changes bring about the increasing of peak II). The dependence of peak C on renaturation time (Fig. 12) agreed reasonably with the course of renaturation monitored by means of endonuclease S_1 specific for *ss* regions. The height of peak C grew linearly with the

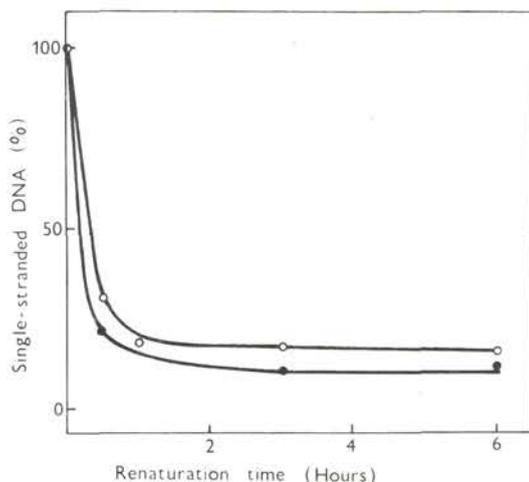


Fig. 12. The course of renaturation of DNA of plasmid ColE1 irradiated with gamma rays. The renaturation was monitored by means of peak C of DNA-Os, ●—●, and by means of the digestion of nonmodified DNA with endonuclease S₁, ○—○.

DNA of plasmid ColE1 was irradiated with a dose of 30 Gy at a concentration of 50 $\mu\text{g/ml}$ in SSC/20, then denatured by heating to 100°C for 6 min., cooled in an ice bath and divided into test tubes of 1.2 ml each. To each test tube 0.3 ml of 5 times concentrated NaCl solution of 0.3 $\text{mol}\cdot\text{dm}^{-3}$ with 0.05 $\text{mol}\cdot\text{dm}^{-3}$ phosphate buffer pH 7.2 were added and the samples were equilibrated at 60°C. At given time intervals the test tubes were cooled in an ice bath and samples were taken for the modification with osmium and for digestion with endonuclease S₁. DNA modification was performed at 25 $\mu\text{g/ml}$, other conditions were the same as those given in Fig. 1. DPP measurement was carried out at DNA concentration of 20 $\mu\text{g/ml}$, other conditions were the same as described in Fig. 3

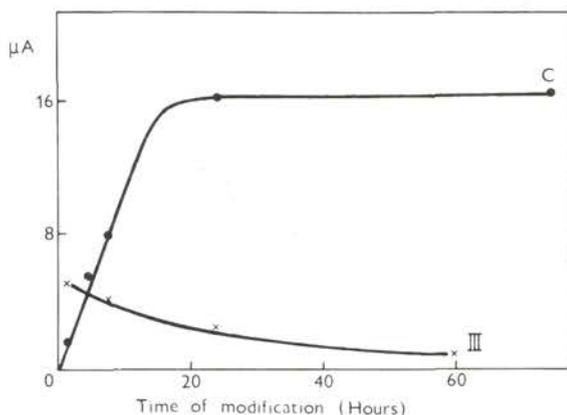


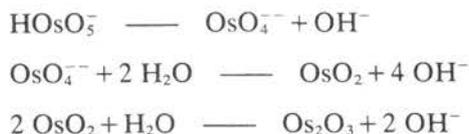
Fig. 13. Dependence of the height of peak C of irradiated DNA-Os and of peak II of nonmodified irradiated DNA on the dose of gamma radiation. After irradiation the DNA sample was divided into two parts. In the first one peak II was measured immediately after irradiation, the other one was treated with OsO₄ as described in Fig. 1, at DNA concentration of 380 $\mu\text{g/ml}$. DPP measurement was carried out at DNA concentration of 300 $\mu\text{g/ml}$, other conditions were the same as in Fig. 3. ○—○, peak II; ●—●, peak C of DNA-Os

dose of gamma radiation in the range of 100—1000 Gy (10^3 — 10^4 rad) (Fig. 13), in analogy to the studies of damage to DNA induced by ionizing radiation carried out by other methods (Paleček 1976; Lukášová and Paleček 1971). It may be thus concluded that the content of ss and distorted ds regions in DNA can be determined by means of peak C of DNA-Os.

Discussion

The reaction of OsO_4 with polynucleotides has been studied in the past mainly because this agent represented a suitable marker detectable by means of electron microscopy and was potentially exploitable in nucleic acid sequence analysis (Beer et al. 1966; Highton et al. 1968; Di Giamberlino et al. 1969). It was found that in the presence of cyanide ions, thymine residues in ss DNA were converted into a product carrying one osmium atom and two cyanide ions (Highton et al. 1968); under the same conditions the reaction with adenine and guanine was negligible and cytosine reacted only slightly. It was further shown that due to the modification with osmium both the DNA density and the sedimentation coefficient increased (Di Giamberlino et al. 1969). The chemical alteration of DNA was not accompanied by aggregation, fragmentation or cross-linking even if the reaction was carried out at 55°C. Later it was shown (Daniel and Behrman 1975, 1976; Chang Chien-Hsing et al. 1977) that stable osmate derivatives were formed, if instead of CN^- , pyridine or 2.2'-bipyridine were used as ligands. The reaction product was an adduct on the 5,6 double bond of pyrimidine bases. Crystallographic investigations demonstrated (Neidle and Stuart 1976; Kistenmacher et al. 1976) the overall geometry for thymine and 1-methylthymine derivatives. The OsO_4 -pyridine reagent reacted selectively with pyrimidines (cytosine, thymine and uracil) and offered the advantage of lower temperature of reaction and shorter reaction times as compared with OsO_4 -cyanide.

Polarographic reduction of OsO_4 was demonstrated in 1941 by Crowell, Heyrovský and Engelkemeir. In a saturated solution of calcium hydroxide these authors observed three DC polarographic waves, corresponding to



Polarographic characteristics of osmium were further investigated (e.g. Meites 1957; Cover and Meites 1961; Connery and Cover 1968). In ammoniacal ammonium chloride media Meites (1957) observed well-defined DC polarographic waves; the first arose from zero applied electromotoric force, $U_{1/2}$ of the second and third were -0.32 nad -1.58 V respectively (in 1 mol. dm^{-3} ammonia and

0.1 mol.dm⁻³ ammonium chloride). The third wave was too high to correspond even to an eight-electron reduction of the osmium. It was therefore believed to be a catalytic hydrogen wave. The experiments (Meites 1957) were performed in alkaline media; it can however, reasonably be assumed that the most negative peak of OsO₄ we observed in neutral media containing ammonia ions (Fig. 1b) is also of a catalytic character. Introduction of osmium into the polynucleotide chain results in an increase of the most negative peak (peak C) (Fig. 1b); it appears probable that the catalytic effect is enhanced due to strong polynucleotide adsorption on the electrode. Electrochemical aspects of this problem will be discussed in greater detail elsewhere.

It has been believed until now that OsO₄ does not react with native ds DNA (Beer et al. 1966; Brown 1974). The results presented in this paper clearly show a limited binding of osmium to ds DNA (Fig. 4). This observation has been made possible due to the higher sensitivity of DPP as compared with the methods hitherto used. Further experiments will be necessary to determine accurately the binding sites in ds DNA from the point of view of dynamics and local conformational changes of DNA. It follows from the present data (Fig. 13) and from our unpublished results that in addition to ss regions osmium reacts preferentially with distorted ds regions e.g. in the vicinity of a ss interruption. The results so far obtained thus show a certain similarity between the interaction of ds DNA with the mercury electrode (Paleček 1976, 1980) and with osmium. The reaction of ds DNA with osmium takes place, however, in the absence of the electrode and thus cannot be influenced by factors which may act on the electrode surface during the adsorption and electroreduction of DNA (e.g. the electric field of the electrode). Osmium reacts with cytosine and thymine residues while cytosine and adenine represent the reducible species in DNA (Paleček 1969, 1980). The direct polarographic analysis of unmodified DNA and the polarographic investigation of the DNA interaction with osmium can thus complement each other. An advantage of the latter method is higher sensitivity and the possibility to derive DNA under conditions not suitable for direct electrochemical measurements. On the other hand, the fact that the potentials of peak C produced by native and denatured DNAs do not differ from each other (Fig. 1b) can be considered a relative disadvantage (compared with ds DNA peak II and peak III of denatured DNA). Quite recently a method has been designed for the determination of ss DNA in ds DNA samples based on the terbium fluorescence enhancement (Ringer et al. 1980; Topal and Fresco 1980) in the presence of ss DNA. This method appears to be slightly less sensitive as compared with the polarographic analysis of DNA-Os presented in this paper; besides, the former method seems to be suitable only for the determination of the ss DNA content and not for the detection of ds distorted regions. Ringer et al. (1980) claim that the terbium fluorescence enhancement for native DNA at 20°C increases 13.6-fold after thermal denaturation. The ratio of

the heights of peak C of native and denatured calf thymus DNA is about 1:30 if the modification is performed in the presence of 1% pyridine (at room temperature) (Fig. 5). It has, however, been shown that the height of peak C of ds DNA is, in the range of 1–10%, strongly dependent on the pyridine concentration probably due to the destabilization of the DNA double helix by pyridine. If ds DNA is treated with osmium in the presence of 0.6% pyridine (Fig. 5) the ratio of the heights of peak C of native and denatured DNA is markedly lower (about 1:80). It is probable that further variation of conditions of DNA modification may result in even more sensitive determination of minor structural changes resulting in an increased accessibility of bases in DNA for their reaction with osmium.

Abbreviations

DPP	— differential pulse polarography
LSV	— linear sweep voltammetry
DC	— direct current
NPP	— normal pulse polarography
$U_{1/2}$	— half-wave potential
DNA-Os	— DNA in which the osmate ester of pyridine is bound to some pyrimidine residues by means of esteric bonding
ds	— double-stranded
ss	— single-stranded
SSC/20	— 7.5×10^{-3} mol. dm ⁻³ NaCl and 7.5×10^{-4} mol. dm ⁻³ sodium citrate

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