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We very much regret that two pages of this paper were lost during printing of vol. 35 no. 4 therefore the paper in full length is reprinted in this issue

TOMASZ PAWŁOWSKI, BOGDAN GULANOWSKI, JOLANTA ŚWIĄTEK, KAZIMIERZ GASIOROWSKI and HENRYK KOZŁOWSKI[a]

SOME ASPECTS OF THE COPPER(II) - DNA INTERACTION*

Department of Basic Medical Sciences, Medical Academy of Wrocław, Kochanowskiego 14; 51-602 Wrocław, and
[a] Institute of Chemistry, University of Wrocław, F. Joliot-Curie 14; 50-383 Wrocław, Poland

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The Cu(II) ion interaction with calf-thymus DNA was studied by means of differential pulse polarography and sweep voltammetry as well as chromatography and viscosimetry. Most of the complexes formed at high ionic strength (0.2 M) and lower Cu(II) concentrations are of a nondenaturing nature. Their formation has but a minor effect on unwinding process of the DNA double helix. The excess of Cu(II) (P = 5) leads, however, to distinct denaturation of the DNA structure. Metal ions have little effect on the denaturation induced by the polarographic reduction of DNA on the mercury electrode. This conclusion is consistent with the character of the polarographic process and with the fact that Cu(II) ions are not very effective in the interaction with AT pairs. Cupric ions have no renaturing ability towards thermally denatured DNA at 0.2 M ionic strength but distinct renaturation was observed at low ionic strength (0.05 M).

The interaction of cupric ions with DNA is rather well established [1 - 7] although some controversions concerning its model still exist [6, 8 and references therein]. It is accepted that Cu(II) ions may influence the renaturation or denaturation of DNA molecule and that the concentration of metal ions and ionic strength are the critical factors for these processes. The binding between Cu(II) ion and DNA leads to formation of two different types

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of complexes, i.e. denaturing and nondenaturing ones, and the mode of coordination in both kinds of species was discussed recently [6]. Our previous studies on the metal - DNA interaction with the use of polarographic techniques [9 - 12] have shown that, e.g., the local labilizations of the double acid interaction. It was also shown that defects caused by the polarographic reduction of the nucleic bases could cause the labilization of the double stranded structure favouring the metal interaction with bases [10 - 13]. Cu(II) ions are very effective in the interaction with nucleic acids and they can play an important role in the DNA chemistry and biochemistry. The bases seem to be the most favourable binding sites for cupric ions though phosphates can play a critical role, especially during denaturation or renaturation of DNA molecule. In this work we have applied two polarographic techniques: differential pulse polarography (DPP) and sweep voltammetry (SV) as well as viscosity measurements and chromatography on hydroxyapatite columns to study the Cu(II) DNA system.

EXPERIMENTAL

Polarographic measurements. The measurements were carried out on native calf-thymus DNA (Serva, mean molecular weight 1.2×10^6 Da, protein content lower than 0.5%). The DPP measurements at pH 7 [14] showed less than 1% of denatured DNA in the samples used. All other chemicals were of analytical grade. DPP measurements were carried out on a pulse polarograph PP-04 (Telpod, Kraków, Poland) with X-Y recorder (Endim 620.02). The three electrode system with an MD-DME working electrode (drop time 3 s), a platinum wire auxiliary electrode and with a saturated calomel electrode as the reference electrode was used. In all measurements the modulation amplitude was 50 mV. SV measurements were carried out on a PA-4 polarographic analyser (Prague, Czechoslovakia). The three electrode system comprised a hanging mercury drop electrode (HMDE, 1.65 mm^2) as working electrode, a platinum wire as auxiliary electrode and saturated calomel electrode as the reference electrode. Scan rate of 0.2 Vs^{-1}, adsorption potential (U_i) from -0.1 to -1.3 V, switching potential (U_s) -1.85 V and accumulation time 120 s without stirring were applied in the SV measurements. The solution tested were deoxygenated with a slow stream of analytical grade nitrogen and, during the measurements, nitrogen was passed over the solution surface. All measurements were performed at room temperature. DNA was thermally denatured by heating in solution at 100°C for 30 min followed by rapid cooling in an ice bath. Acetate buffer (0.05 M), pH 5.6, or formate buffer (0.2 M), pH 6.4, was used as a supporting electrolyte. The concentrations of DNA evaluated spectrophotometrically were 50 or 100 mg dm^{-3} in DPP and SV measurements, respectively.

Chromatography on hydroxyapatite was used for the separation of the single- and double-stranded DNA molecules [15]. The samples with metal free DNA and DNA containing Cu(II) ions were sonicated before being loaded on a column to reduce the molecular weight and to inhibit reassociation of complementary strands [16, 17]. Glass column of an inner diameter of 0.9 cm with glass frit was filled with a hydroxyapatite-Celite 545 (15:2, w/w) mixture suspended in 0.05 M sodium phosphate buffer (pH 6.8) to give about 10 cm of packed length. Since DNA may plug up the column, in addition to mixing Celite 545 with hydroxyapatite, a layer (0.5 cm) of Celite 545 was placed on glass frit [18]. The column was kept at 55°C by means of a thermostatically controlled water jacket. To eliminate nonspecific interactions between single stranded DNA molecules, the chromatography is usually performed at 50 - 60°C [19]. The 5 ml samples of DNA (50 mg dm^{-3}) in 0.05 M sodium acetate buffer, pH 5.6, with or without Cu(II) ions were loaded on a column after sonication (10×15 s, 22 kHz, amplitude 16 vm on ultrasonic disintegrator type UD 20), eluted with 30 ml of 0.05 M sodium phosphate buffer and then with a linear gradient of 0.05 - 0.5 M sodium phosphate buffer (pH 6.8). A constant flow rate of about 2 ml min^{-1} was maintained by means of a peristaltic pump. Fractions of about 2 ml were collected and absorbance at 260 nm was measured. The DNA samples were incubated at 25°C in acetate buffer with or without metal ion during 40 min (t_0) or 48 hours (t_{48}).

Viscosity measurements were performed on a circular channel crucible oscillating viscometer. This viscometer consists of an oscillating crucible, the liquid under study being contained in a half-filled U-shaped circular channel. The viscosity of the solution is determined by measuring the logarithmic decrement of the damped harmonic oscillation. The standardization of the relationship between logarithmic damping and viscosity was performed with the solutions containing sucrose in water at concentrations from 0 to 17% (w/w). The basic theory and instrument were described earlier [20, 21].

RESULTS AND DISCUSSION

In deoxyribonucleic acid the electroactive groups are bases. Adenine and guanine can be oxidized at graphite electrodes [22, 23], while adenine and cytosine can be reduced at mercury electrodes [24 - 26]. The relative height of a polarographic wave (limiting current) is used as a measure of locally unwound fragments of double stranded DNA, the bases of which adsorb at the mercury electrode and then undergo reduction [25, 27]. Our recent studies [10 - 13] revealed that the polarographic process can produce the defects in the DNA double helix which can labilize the double stranded DNA structure and, in consequence, increase the reduction wave of DNA. The amount of the local defects caused by a single polarographic reduction is rather low and

cannot be measured easily. Subsequent repetition of, e.g., DPP measurements leads, however, to a considerable increase of the DPP peak. Thus, the amount of unwound regions in DNA molecule increases with the number of consecutive measurements [13]. A detailed study has shown that induction of the local unwinding in the double helical DNA molecule requires only the adsorption of the nucleic acid at the charged surface of a mercury electrode. Consecutive reductions of DNA may lead, however, to more severe degradation of DNA molecule [13]. The DNA molecules adsorbed at a mercury electrode desorb very slowly. It takes several hours to observe the desorption of a considerable amount of damaged DNA molecules from a mercury surface (see also [28]). This was checked as follows: the DPP measurements were carried out in the solution containing 100 mg dm^{-3} of DNA for 1.5 h (20 consecutive measurements) and then the bulk solution was removed. The remaining pool of mercury derived from the electrode drops containing the DNA adsorbed and damaged by the reduction process, was covered with a DNA-free acetate buffer, and dinitrogen was bubbled through this solution. The DPP measurements of the latter solution showed increasing amounts of DNA in the solution; maximum DNA concentration was obtained about 5 - 7 h after adding the acetate buffer to mercury with adsorbed DNA. This experiment documented clearly that desorption of most of the DNA molecules adsorbed at the mercury electrode proceeds for several hours and these molecules reappear in the solution during the subsequent reduction process, as postulated earlier [10 - 13].

DPP of the Cu(II) DNA solutions in 0.2 M ammonium formate

Earlier DPP studies of the Cu(II) DNA system in acetate buffer, pH 5.6, at low ionic strength (0.05 M) [10] have shown that Cu(II) influences considerably the polarographic behaviour of DNA. Cu(II) forms various very strong complexes with DNA. Moreover, at low ionic strength the nucleic acid structure in solution is complicated. Therefore, it was very difficult to present a precise interpretation of DPP measurements basing only upon the variation of the peak height and peak potential changes [10, 13]. Higher ionic strength of 0.2 M ammonium formate seems to make DPP results more clear than those obtained with acetate buffer, probably due to the fact that at higher ionic strength the number of sites accessible to Cu(II) decreases and electrostatic interactions are considerably reduced (cf. [7] and references therein).

The changes of the DPP peak height of DNA reduction for metal-free DNA and DNA in the presence of different amounts of Cu(II) (metal to DNA phosphate ratio P = 0.5, 1 and 5) for different incubation times at room temperature and different numbers of the consecutive measurements are shown in Fig. 1. During the first measurement the effect of lower amount of metal

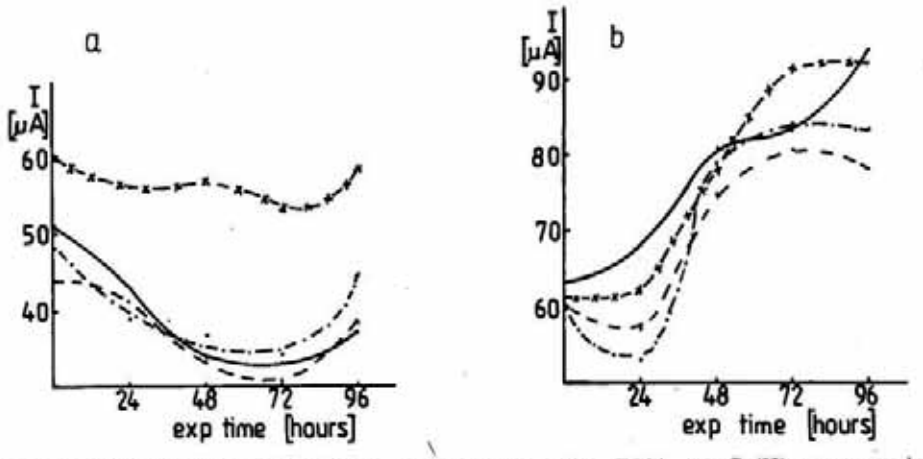

Fig. 1. Dependence of a DPP limiting current for native DNA on Cu(II) concentration at P = 0.5 (— — —), P = 1.0 (—·—·—) and P = 5 (—×—×—) and exposition time for the first and third successive measurement (a and b, respectively). [DNA] = 50 mg × dm^{-3}, 0.2 M ammonium formate buffer, pH 6.4, room temperature; solid line corresponds to metal-free native DNA

did not cause any appreciable change in the DNA reduction wave height. Only with a high excess of copper (P = 5) the DPP peak height increased by 20 to 40% as compared to that of the metal - free DNA. This indicates that most of the complexes formed at lower concentrations of metal are of a nondenaturing type and their formation does not cause any distinct DNA unwinding. Higher amounts of metal lead to formation of the unwound DNA regions, i.e. the complexes formed are of a denaturing character [6, 7]. These results are consistent with the earlier suggestion of Förster *et al.* [6] based on equilibrium binding studies. Distinctly different results were obtained for the samples in which DNA was partly damaged by DPP measurements (see above and ref. [13]), (Fig. 1b). The DPP peak height of DNA reduction changed with the exposition time in an S-like fashion for all the studied values of P (from 0 to 5). At shorter exposition times metal seemed to decrease the DNA reduction peak (possibly due to a renaturation process) while after 24 h a considerable increase of this peak was observed (Fig. 1b) to reach the maximum value after about 72 h. This indicates that the defects in a DNA molecule, the structure of which is not much affected by the presence of metal ions, induce its slow unwinding. Very similar results were obtained by the SV measurements (Fig. 2). The dependence of the SV peak height on the adsorption potential was similar to that found earlier and it was proportional to the amount of the unwound regions in the double helical DNA molecule [29 - 31]. Again, during the first measurement the amount of single stranded fragments in double helical DNA molecule was highest for P = 5, and the other concentrations used (P = 0.5 and 1) did not influence considerably the DNA SV reduction (Fig. 2a). When, however, several

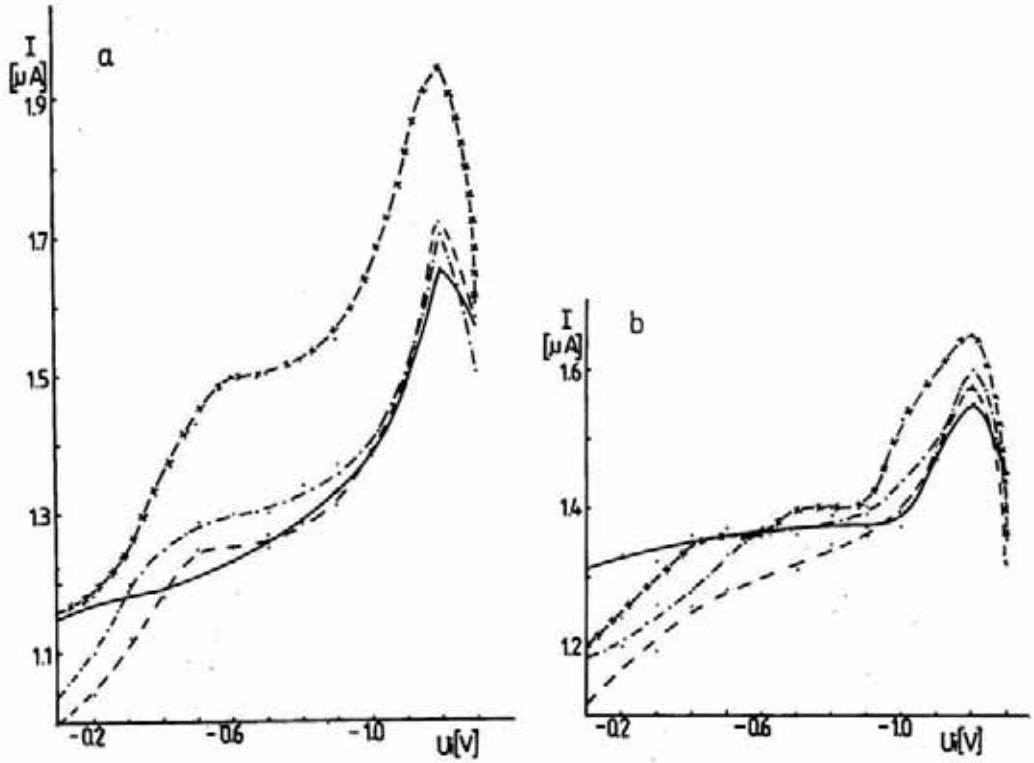

Fig. 2. Dependence of a SV reduction peak height of native DNA on Cu(II) concentration and on initial potential U_i for the first measurement (a) and for the samples measured several times (b). The waiting time 120 s, scan rate 200 $mV \times s^{-1}$, [DNA] = 100 $mg \times dm^{-3}$; other details and designations as in Fig. 1.

measurements were made with the same solution and the solution was left for several hours to allow desorption of the damaged DNA molecules from the mercury pool, the results of renewed measurements were different (Fig. 2b). Similarly as it was found in the DPP measurements, the effect of the P value on the SV reduction peak was much less distinct than that found in the experiment shown in Fig. 2a. The lack of a considerable influence of the metal ion even for P = 5 on the polarographic picture of the electrochemically damaged DNA molecules presented in Figs. 1b and 2b may be explained as follows: The bases which undergo reduction on an electrode (adenine and cytosine) initiate the unwinding around the reduction site. Since AT (adenine-thymine) rich regions of DNA molecule are more easily unwound due to weaker hydrogen bond system as compared to GC (guanine-cytosine) pairs, the opening of AT pairs is more likely (as a consequence, reduction of adenine is also more likely). The binding of Cu(II) ions to the latter bases is much less effective than binding to GC pairs [6, 7] and it has not any distinct renaturing or denaturing properties. Thus the obtained results are in

very good agreement with the polarographic features of DNA as well as with the previous results obtained for the coordination of DNA with cupric ions with the use of widely varying techniques [6 - 8].

Denatured DNA molecules are reduced at a potential of −1.45 V, i.e. lower than that of the native DNA (−1.40 V) (DPP) and, since only bases of the single stranded fragments of DNA undergo reduction, the reduction peak height of denatured DNA is much larger (by one order of magnitude). The DPP and SV results presented in Figs. 3 and 4 show

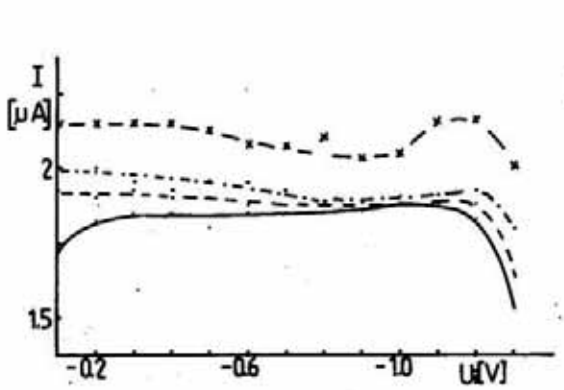

Fig. 3

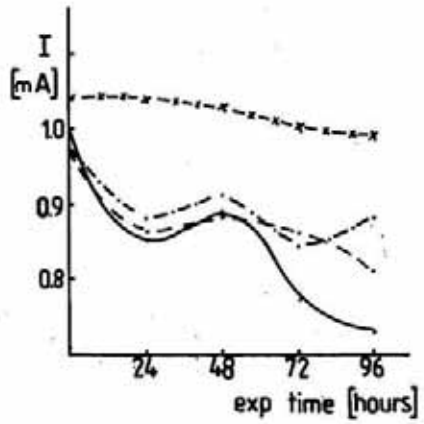

Fig. 4

Fig. 3. Dependence of the limiting current of thermally denatured DNA on Cu(II) concentration and exposition time. Other details and designations as in Fig. 1

Fig. 4. Dependence of the SV reduction peak height of thermally denatured DNA on Cu(II) concentration and on initial potential U_i; other details and designations as in Fig. 2

that the complex formed between denatured DNA and Cu(II) ions has no significant renaturing abilities (see also [6]). The SV data obtained for the denatured DNA - Cu(II) system in 0.05 M sodium acetate buffer (Fig. 5) show, however, that at low ionic strength cupric ions can induce some renaturation (P = 0.5 and 1). Metal ions like Cu(II) forming strong bonds with nucleic bases produce cross-links in the unwound regions preventing renaturation unless a chelating agent or strong electrolyte is involved [6, 32]. At low ionic strength Cu(II) acts also through electrostatic interactions with phosphates. These interactions may induce the recovery of the helical structure.

Chromatography on hydroxyapatite was performed for the P values of 0.05, 0.5, 1 and 5. The amount of single stranded DNA strongly depended on the concentration of copper (Fig. 6). For metal concentrations up to P = 1 the amount of single stranded DNA was decreasing distinctly and it increased slightly for P = 5. The exposition time up to 48 h had only

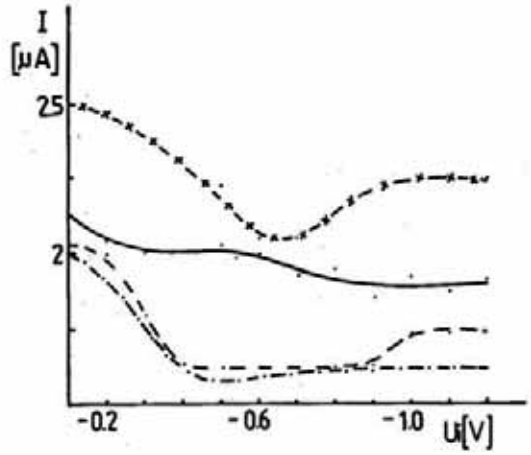

Fig. 5. Dependence of the SV reduction peak height of thermally denatured DNA on Cu(II) concentration and on initial potential U_i in 0.05 M acetate buffer, pH 5.6; other details and designations as in Fig. 2

a minor effect on the amount of single stranded DNA released from the hydroxyapatite (Fig. 6). It should be mentioned that although the DNA was incubated with Cu(II) ions and the respective complexes were formed as described above, the nucleic acid leaving the hydroxyapatite column was practically metal-free. This indicates that Cu(II) forms with DNA denaturing

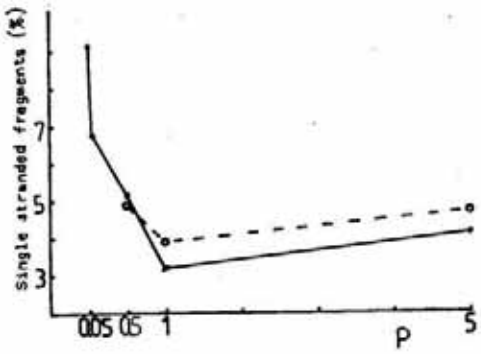

Fig. 6

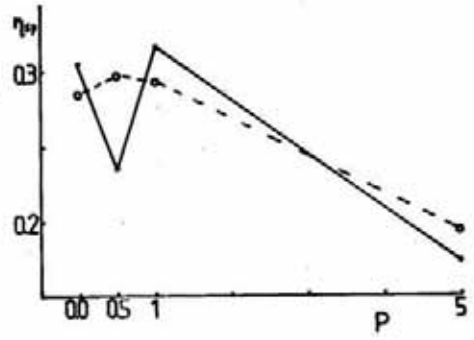

Fig. 7

Fig. 6. Dependence of the amount of the single stranded fragments of DNA on Cu(II) concentration. The data were obtained from hydroxyapatite chromatography for exposition time 0 (solid line) and 48 h (dashed line); [DNA] = 50 mg × dm^{-3} in 0.05 M acetate buffer, pH 5.6

Fig. 7. Dependence of the specific viscosity of native DNA on Cu(II) concentration for exposition time 0 (solid line) and 48 h (dashed line); [DNA] = 25 mg × dm^{-3} in 0.05 M acetate buffer, pH 5.6

and nondenaturing complexes but metal removal leads to considerable renaturation of the labilized segments of double helical DNA molecule. Thus, it could be concluded that even high excess of copper does not denaturate DNA molecules in the same sense as high temperature does. Metal ions bound to nucleic acid labilize some fragments of double helical structure (denaturing type of complexes) which can be recovered when metal ion is removed. In the case of Cu(II) denatured DNA mostly intrastrand metal coordination takes place. At low ionic strength, when still some GC pairs keep two strands of the DNA molecule together, metal ion can induce some renaturation process most likely by forming additional interstrand bonds between GC pairs (see above). The renaturation of the single stranded fragments of native DNA molecule by a low amount of Cu(II) was suggested to proceed due to interstrand binding between cytosine and guanine donor set [6, 8], to electrostatic interactions with phosphates as mentioned above, and weakening of the intramolecular electrostatic interactions between DNA fragments. The chromatographic results also indicated that exposition of metal-free DNA in acetate buffer for 48 h raised the amount of the single stranded fragments to about 3% while in the presence of Cu(II) this increase was less than 1%.

The results of the specific viscosity measurements of metal-free DNA and DNA incubated with Cu(II) are shown in Fig. 7. For the DNA incubated with copper ions for 48 h the viscosity was slightly increased for $P = 0.5$ and 1 and then it decreased considerably (32.4%) when $P = 5$. The viscosity decrease can be due, among others, to the fact that metal ions neutralize negative charges in a nucleic acid molecule, and this leads to a decrease of contour length of DNA [8, 33, 34]. Also formation of the DNA - Cu(II) complexes alters the hydrodynamic properties of a nucleic acid molecule. The slight increase of specific viscosity for $P = 0.5$ and 1 may be the result of the renaturing ability of Cu(II) ions discussed for these P values above.

Conclusions

The results presented show that:
(i) at 0.2 ionic strength, at low Cu(II) to phosphate ratio $P \leqslant 1$, nondenaturing Cu(II)-DNA complexes are formed, whereas the complexes formed at the high ratio $P = 5$ cause distinct denaturation of DNA molecules;
(ii) the presence of Cu(II) does not significantly influence the polarographic behaviour of DNA molecules locally denatured by polarographic reductions;
(iii) the effect of renaturation of single stranded DNA was observed for low P values at 0.05 M ionic strength.

Summing up these results and those obtained by other authors, we can conclude that cupric ions interact with DNA through formation of strong bonds with nucleic acid bases (preferentially guanosines) as well as, at low

ionic strength, also through electrostatic interactions with phosphate chains. The mode of interaction depends strongly on the state of DNA structure, ionic strength, temperature and amount of cupric ions, pH and nucleotide sequence, as well as the amount of the respective nucleic bases (see e.g. [6-8]). Controversies found in the literature are most likely due to this variety of influencing factors. As it was suggested for other systems [10 - 13], the structural state of the DNA molecule may have a decisive influence on the mode of metal ion - DNA interaction.

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