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In vitro studies on the DNA impairments induced by Cr(III) complexes with cellular reductants

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The influence of Cr(III) complexes with ascorbic acid, cysteine and glutathione on DNA has been studied spectrophotometrically and chromatographically. The toxic and genotoxic activities of these complexes were also investigated. It was found that these complexes bind to DNA weaker than hexaaqua Cr(III) complexes. It could be explained through the greater strength of the bi- and tridentate ligands coordinated to chromium in comparison to water molecules. The formation of DNA–DNA intermolecular bonds and DNA interstrand cross-linking has been also observed. These complexes were found to be non-toxic and non-genotoxic in the bacterial test.

Keywords: Cr(III) complexes, DNA, genotoxicity, hydroxyapatite chromatography, spectrophotometry

Introduction

The cancerogenic activity of Cr(VI) compounds was found both in epidemiological and animal studies (Langärd 1989). As the Cr(VI) species are inactive in the absence of red–ox systems, a key step in the toxicity of Cr(VI) compounds is the cellular reduction to Cr(III) (De Flora & Wetterhahn 1989). Reduction inside the cell may thus be a prerequisite for genotoxic action by the generation of highly reactive chromium species and various radicals (Farrell *et al.* 1989, Aiyar *et al.* 1991, Standeven & Wetterhahn 1991). Among intracellular low molecular weight molecules active in Cr(VI) metabolism, ascorbic acid, glutathione and cysteine are the most important (Connett & Wetterhahn 1985, Suzuki 1990). They are of significant reactivity toward Cr(VI) under conditions of physiological pH (Connett & Wetterhahn 1985). It was found that in mammalian tissues normal intracellular concentrations of ascorbic acid and glutathione are higher than those of any other reductants (Suzuki 1990).

Cr(VI) induced DNA impairments include Cr–DNA adduct formation (Tsapakos & Wetterhahn 1983, Borges & Wetterhahn 1989), strand breaks (Cupo & Wetterhahn 1985, Sugiyama 1991), DNA cross-linking (Cupo & Wetterhahn 1985), DNA–protein cross-links (Costa 1991, Sugiyama 1991) and the inhibition of the DNA synthesis (Ogawa *et al.* 1989).

In contrast to the chromate $[CrO_4]^{2-}$ anion, the Cr(III) ions are poorly permeated by the cell membranes (Beyersmann 1989). However, *in vitro* studies showed that the Cr(III) ions are bound to DNA in the cell-free system (Wolf *et al.* 1989, Snow 1991, Gulanowski *et al.* 1992) inducing DNA cross-linking (Snow & Xu 1989, Gulanowski *et al.* 1992), DNA–protein cross-links (Kortenkamp *et al.* 1992) and DNA condensation (Österberg *et al.* 1984), and also decreasing the DNA replication fidelity (Snow 1991). In the bacterial tests some Cr(III) complexes were found to be mutagenic (Warren *et al.* 1981, Snow 1991). At the same time the Cr(III) ions appeared to be essential for animals and humans (Morris *et al.* 1988). We do not know, as yet, which kinds of Cr(III) complexes are harmful and which ones are beneficial for living organisms.

Our recent work has focused on the isolation and physicochemical characterization of Cr(III) complexes which are products of Cr(VI) reduction with the main cellular reductants: ascorbic acid, glutathione and cysteine (Cieślak-Golonka *et al.* 1992).

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The aim of this paper is to present the results of the spectrophotometric and chromatographic examination of the interaction of Cr(III) complexes with DNA. The bacterial mutagenicity tests were carried out to study the toxic and genotoxic activities of these complexes.

Materials and methods

Materials

Common laboratory chemicals were of reagent grade and were used without further purification. Air stable Cr(III) complexes were synthesized by a method described in Cieślak-Golonka *et al.* (1992). Among various compounds prepared in Cieślak-Golonka *et al.* (1992) only the water soluble ones were used for our purposes: $K[Cr(asc)_2]\cdot 7H_2O$ (A1), $K_2[Cr(OH)(asc)_2]\cdot 6H_2O$ (A2), K[Cr(asc)(cys)]·$6H_2O$ (AC) and K[Cr(asc)(GSH)]·$6H_2O$ (AG). (In the original procedure we used AC1 and AG2 for AC and AG, respectively.) Calf thymus lyophilized 'high molecular' DNA (Type I) was obtained from Sigma (St Louis, MO). Hydroxyapatite was synthesized according to Hirano *et al.* (1985).

The DNA concentration was estimated spectrophotometrically at 260 nm with a VSU2-P Carl-Zeiss Jena spectrophotometer. All Cr(III) compounds were freshly dissolved before each experiment.

Spectrophotometric measurements

The absorption spectra were recorded with a M40 model Carl Zeiss Jena spectrophotometer. The spectra of the Cr(III) complexes (150×10^{-6} M) incubated with DNA (from 0 to 100×10^{-6} g ml^{-1}, i.e. to 300×10^{-6} M nucleotides) were carried out in the wavelength range 220–450 nm. The reference sample was 0.05 M acetate buffer solution (pH 5.6) or DNA in concentrations equal to the concentrations of DNA in the tested samples. Such acidity has been chosen to prevent the formation of insoluble Cr(III) hydroxides.

The absorption spectra were measured for (i) freshly dissolved Cr(III) compounds and Cr(III) compounds after adding DNA in various concentrations, and (ii) the same as (i) but after 24 h.

Hydroxyapatite chromatography

Hydroxyapatite $[Ca_{10}(PO_4)_6(OH)_2]$ column chromatography has been applied for the isolation and separation of nucleic acids (Bernardi 1971). Incubations of the sole 50×10^{-6} g ml^{-1} DNA sample and that with a Cr(III) complex ($0–300 \times 10^{-6}$ M) were carried out in sodium acetate buffer (0.05 M, pH 5.6) at 25 °C for 0–72 h. Samples were loaded on the hydroxyapatite bed (1 × 6 cm) and eluted with sodium phosphate buffer (pH 6.8) of increasing concentration (0.01–0.5 M). Flow rate was maintained at 2 ml min^{-1}. The fraction volume was 2 ml. The collected fractions were spectrophotometrically analyzed at 260 nm. The unbound Cr(III) complexes, the single-stranded and the double-stranded DNA were eluted at 0.01, 0.18 and 0.22 M buffer, respectively. The samples containing DNA were, if necessary, sonicated before loading on the column. The sonication conditions were: 4 × 15 s in an ice bath, frequency 22 kHz, amplitude 16 μm on an ultrasonic disintegrator type UD 20 (Techpan).

In the experiments estimating the DNA interstrand cross-linking the DNA was incubated with Cr(III) compounds in 0.05 M sodium acetate buffer (pH 5.6, at 25 and 37 °C) for 24 h. The DNA concentration was 50×10^{-6} g ml^{-1} and that of the Cr(III) complex was 150×10^{-6} M. The samples were sonicated and loaded on a hydroxyapatite column followed by DNA elution to remove unbound Cr(III) compounds. The fractions containing DNA were collected and were diluted with water at a ratio of 1:4. Then the DNA was denaturated for 20 min in a boiling water bath. The sample was cooled immediately in an ice bath and sonicated (to avoid DNA renaturation). To separate single- and double-stranded DNA the sample was re-chromatographed on hydroxyapatite.

Bacterial test

The genotoxic and toxic activities of Cr(III) complexes A1, A2, AC and AG were tested with the *Bacillus subtilis* assay system described by Kada *et al.* (1980), using freshly grown vegetative cells of H 17 (rec$^+$) and M45 (rec$^-$) strains. This method is recomended for the genotoxicity study of metal compounds (Kanamatsu *et al.* 1980). The concentrations of the tested complexes were: 50, 100, 200, 400, 600 and 800×10^{-9} mol in 50 μl per disk, and for $K_2Cr_2O_7$ (positive control) at 50×10^{-9} mol in 50 μl per disk.

Results and discussion

Spectrophotometric studies: Cr(III) complexes binding to DNA

It was found that the biological activity of Cr(III) ions studied both *in vitro* and *in vivo* depends on the nature of the complex used (Warren *et al.* 1981, Kortenkamp *et al.* 1986). The complexes we used precipitated in the form of potassium salts of the following anions: $[Cr(asc)_2]^-$(A1), $[Cr(OH)(asc)_2]^{2-}$ (A2), $[Cr(asc)(GSH)]^-$ (AG) and $[Cr(asc)(cys)]^-$ (AC). (In the coordination sphere the remaining places are occupied by water.) The complexes crystallized as hydrates with six water molecules (seven for A1).

The complexes in the solid state and freshly dissolved showed a tetragonal deformation around the Cr(III) ion presumably with the $[MO_4XX']$ chromophore (X, X'=O', N, S) (Cieślak-Golonka *et al.* 1992).

No qualitative changes of the spectra in solution in comparison to the solid state have been observed for freshly dissolved samples.

In this work we have studied spectrophotometrically the following effects: (i) the changes in time of the spectra without DNA and (ii) the changes of the spectra upon DNA addition.

The analysis of the spectra was performed in the range of the ascorbate (asc) anion absorption (240–270 nm) through the observation of the changes of the band position and its intensity. The UV high intensity of absorption has been assigned to the transition in the C=C bond in the ascorbic acid ring which may be conjugated to the C=O bond (Ogata & Kosugi 1970). The variation of the dissociation degree of ascorbic acid can be observed through the changes of the band position from 243.5 to 265.5 nm and finally to 265.5–267.5 nm for H_2asc, $Hasc^-$ and asc^{2-} species, respectively (Yatsimirski 1977). For these complexes in the solid state we have previously observed the O-(1) and O-(3) type coordination (Cieślak-Golonka *et al.* 1992). However, other types of coordination, e.g. O-(2) and O-(3), have been found for metal ascorbate complexes (Tajmir-Riahi 1991, Tajmir-Riahi & Boghai 1992). Although in the aqueous solution the formation of the chelates is more difficult, there are documented examples of bidentate metal ascorbate coordination in such solution (Tajmir-Riahi 1991, Tajmir-Riahi & Boghai 1992).

Cr(III) complexes. The position of the band for the freshly dissolved complexes suggests that, at least in complexes A1, A2 and AG, ascorbic acid retains its solid state coordination to chromium. However, both in time and after adding DNA we have observed slight but significant changes both in the asc^{2-} band position and its intensity.

The variation of the λ_{max} and its intensity in time showed significant water interaction in these systems. The strongest effect was found to be in A2 (see Figure 1). The following order of the magnitude changes has been observed: band position, $A2 \gg A1 \simeq AG$ (for AC the band was weakly resolved); band intensity, $A2 > A1 \gg AG \simeq AC$.

The results show that the chemical processes in water disturb most seriously the hydroxoascorbatochromate(III) (A2) anion. The complicated equilibria which are expected in these system can occur due to hydrolysis and further polymerization reactions (Larkworthy & Nolan 1987). The hydrolysis of various Cr(III) complexes is a very complicated

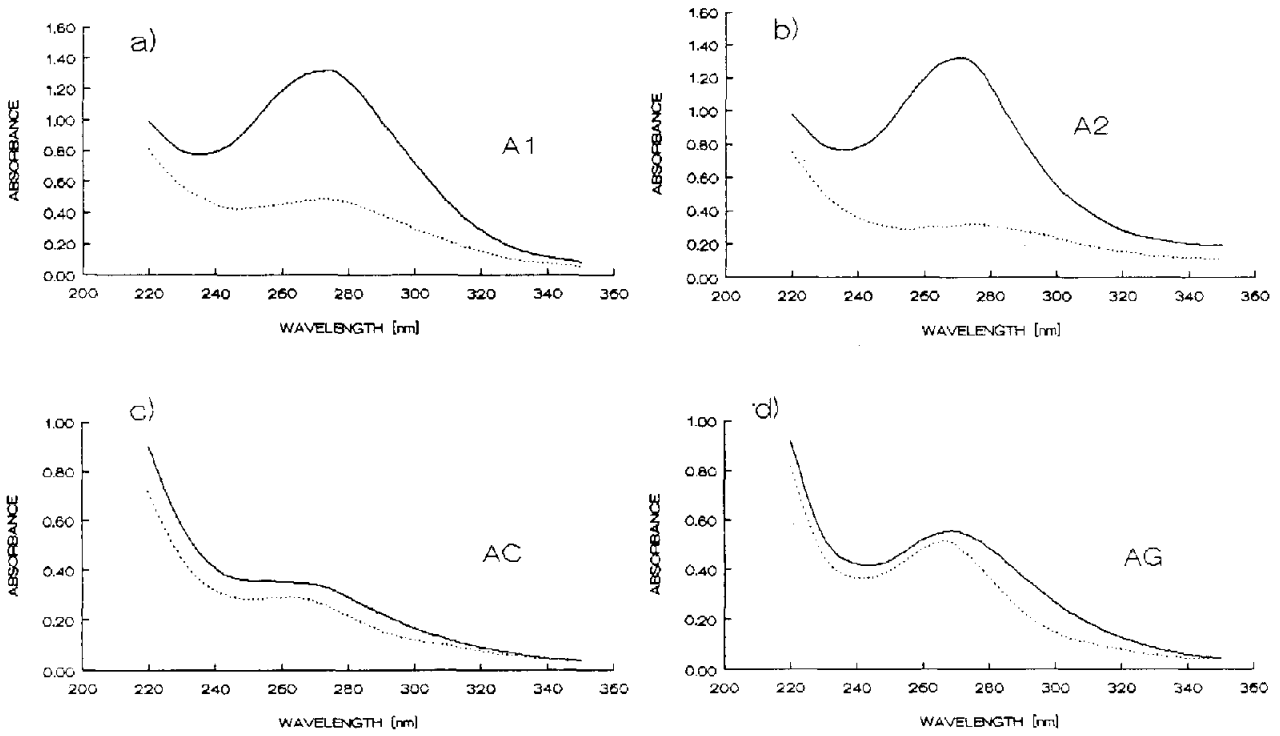

Figure 1. Absorption spectra of Cr(III) complexes (150×10^{-6} M) in 0.05 M sodium acetate buffer at pH 5.6: freshly dissolved (——) and after 24 h (.....). The reference sample was the 0.05 M sodium acetate buffer at pH 5.6. (**a**) A1, (**b**) A2, (**c**) AC and (**d**) AG.

process (Larkworthy & Nolan 1987). Both A1 and A2, having water molecules in the coordination sphere of Cr(III) ions, are more sensitive to the hydrolytic process which is based on deprotonation and substitution reactions (Spiccia & Marty 1991). Particularly A2 which possesses the nucleophile OH^- coordinated to chromium facilitates processes of substitution and thus formation of various polynuclear forms (Spiccia & Marty 1991). The mixed ligand complexes multidentately bonded to Cr(III) are evidently more resistant towards reaction with water.

Cr(III) complexes–DNA. The concurring reactions followed when DNA was added to the water solution of the complexes: the interaction of Cr(III) complexes with water and the interaction with DNA. Our previous studies (Gulanowski *et al.* 1992) showed that Cr(III) ions bound to DNA causing severe aggregations of DNA through the intermolecular DNA–DNA cross-linking.

In this work we have observed the shift of the 'ascorbate' band position and its intensity, which were most significant in A1 and A2 (see Figure 2). After 24 h the intensity of the band returned to its previous value and even slightly exceeded it (A1). Evidently DNA counteracts the hydrolysis processes by exchanging water molecules into ligating atoms of nucleotides. These atoms most probably stem from the phosphate groups of DNA (Snow 1991, Gulanowski *et al.* 1992). However, coordination of the nitrogen atoms of the bases cannot be excluded (Wolf *et al.* 1989).

Similar to the results above, the changes in the mixed ligand AC and AG complexes are the slightest (see Figure 2). Evidently it is easier to remove water molecules than to break even a single bond of the multidentate coordinated ligand.

Interesting results have been obtained for the $[Cr(asc)(cys)]^-$–H_2O–DNA, i.e. the AC–DNA system. The decrease of its intensity follows upon addition of DNA (see Figure 2). Although there seems not to be a significant change, in A1, A2 and AG we have observed the opposite effect: the absorption increased upon adding DNA. In the AC–DNA system the addition of DNA influences the molecular levels of the ascorbic part of the complex.

In conclusion we must state that the simple complexes A1 and A2 interact more strongly with DNA than the mixed complexes. It also can be caused by steric effects. The importance of the

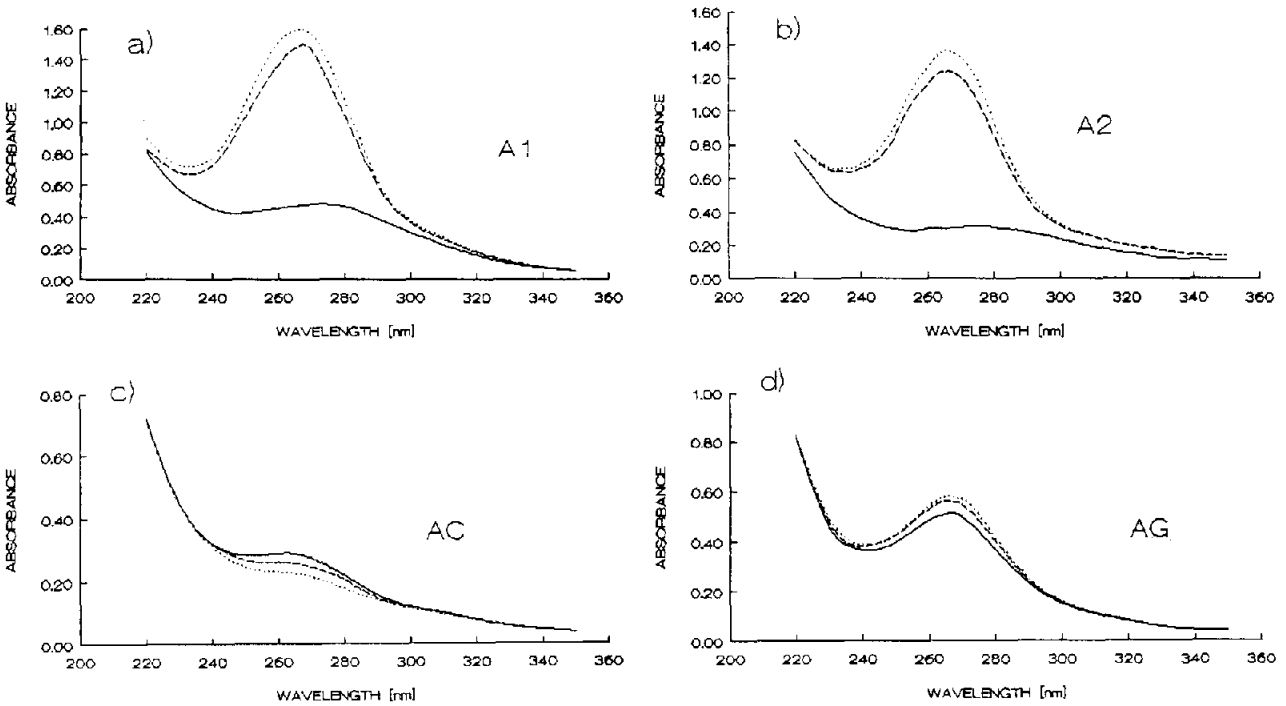

Figure 2. Absorption spectra of Cr(III) complexes (150×10^{-6} M) after 24 h incubation with DNA in 0.05 M sodium acetate buffer at pH 5.6. The reference sample was the DNA in sodium acetate buffer at the same concentration as in the studied sample. (**a**) A1, DNA concentration was 0, 5 and 50×10^{-6} g ml^{-1} (from bottom to top). (**b**) A2, DNA concentration was 0, 5 and 50×10^{-6} g ml^{-1} (from bottom to top). (**c**) AC, DNA concentration was 0, 50 and 100×10^{-6} g ml^{-1} (from top to bottom). (**d**) AG, DNA concentration was 0, 5 and 50×10^{-6} g ml^{-1} (from bottom to top).

non-covalent interaction for the selectivity in the biological systems is now generally accepted (Frieden 1975). The interaction can be due to the formation of the bond between the chromium atom and the oxygen of the phosphate or nitrogen of the nucleoside part of the DNA. As polydentate ligands are generally more strongly bound than monodentate ones, DNA coordination to chromium causes the removal of water molecule(s) in preference to those of ascorbic acid.

Chromatographic studies

Intermolecular cross-linking studies. In our earlier study we found that Cr(III) aqua ions $[Cr(H_2O)_6]^{3+}$ interact with DNA causing, for example, DNA–DNA cross-linking (Gulanowski *et al.* 1992). This conclusion has been drawn from the observation of the decrease in the elution yield from a hydroxyapatite column of the DNA incubated with the water solution of chromium(III) nitrate $[Cr(NO_3)_3]$. The present paper reports the same experiment carried out for the system of Cr(III) complexes with the main cellular reductants–DNA.

Among the complexes under study, only A1 showed a significant decrease in the elution yield (47%) although much smaller in comparison to $[Cr(H_2O)_6]^{3+}$ (0%). For other compounds we have found the following yield reduction: 65, 64 and 74% of the DNA loaded for A2, AC and AG, respectively (see Figure 3). For the control DNA sample the elution yield was 65%.

Figure 4 presents the elution profile of DNA, and DNA after incubation with A1. The decrease of the elution yield of the DNA after incubation with A1 has been observed.

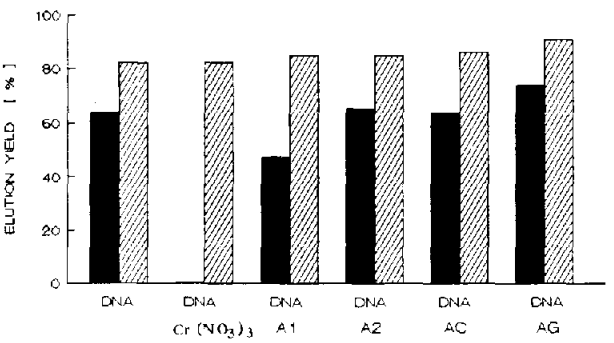

Figure 3. The elution yield of DNA from hydroxyapatite columns after 24 h incubation with or without Cr(III) compounds (150×10^{-6} M) in 0.05 M sodium acetate buffer at pH 5.6. The DNA concentration was 50×10^{-6} g ml^{-1}. Dark bars, no sonication; dashed bars, after sonication before loading the sample on the column.

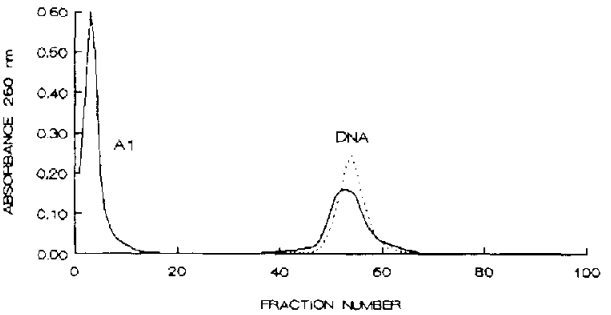

Figure 4. The elution profile of the DNA from hydroxyapatite columns after 24 h incubation with A1 (—-) or without A1 (.....) in 0.05 M sodium acetate buffer at pH 5.6. The concentration of DNA was 50×10^{-6} g ml^{-1} and of A1 was 150×10^{-6} M.

For A2 and AC the same elution yield as for DNA in the control sample has been observed.

The fact that the elution yield for DNA (control DNA sample) was not complete could be explained by the irreversible adsorption of DNA on the crystals of the hydroxyapatite. It can be also explained by the over large molecular weight of the molecule and by the formation of DNA–DNA aggregates (Bernardi 1971). Sonication leads to the increase of the elution yield up to 82, 85, 86 and 91% for DNA in the control sample, $[Cr(H_2O)_6]^{3+}$–DNA, and A1–DNA, AC–DNA and AG–DNA, respectively (see Figure 3).

We have observed the decrease of the DNA elution yield: complete for $[Cr(H_2O)_6]^{3+}$, partial for $[Cr(asc)_2(H_2O_2)]^-$ and none for the other complexes. The decrease of the elution yield may possibly be attributed to the formation of DNA–DNA aggregates. The aggregates are probably formed by interaction between Cr(III) species and DNA through the removal of water molecules from the chromium coordination sphere. These results are in line with those obtained from the spectral studies showing the strongest DNA impact on A1. As Cr(III) in A1 has two sites to bind DNA, it is possible that two DNA molecules can be coordinated. Generally such coordination leads to intermolecular cross-linking and the formation of DNA–DNA aggregates. The control experiment showed that DNA fragmentation under the sonication process had caused an increase of the amount of eluted DNA. Thus we exclude irreversible adsorption of Cr(III) compounds coordinated to DNA on the hydroxyapatite crystals as a reason for the DNA, elution decrease.

It is difficult to explain the about 10% increase in the elution yield for the DNA incubated with AG (see Figure 3).

Figure 5 presents the dependence of the elution yield of DNA molecules on the concentrations of $[Cr(H_2O)_6]^{3+}$ and $[Cr(asc)_2(H_2O)_2]^-$. These data indicate that the latter is several times weaker in DNA–DNA cross-linking than the former.

Figure 6 shows that equilibrium in the system $[Cr(H_2O)_6]^{3+}$–DNA was reached after 24 h, whereas for the A1 complex it took 72 h. Evidently the interaction of the Cr(III) hexaaqua complex with DNA is more effective than A1.

Stability of the double-stranded DNA molecule. The binding of the metal ion complexes to DNA may have various impacts on the stablility of the hydrogen bondings which link the complementary DNA strands. The decrease of the stability of these bondings, their weakening or breaking may be revealed through the increase of the amount of single-stranded (deseparated) DNA fragments. On the other hand, increase of the stability of the DNA molecule and its greater packing is manifested through a decrease of single-stranded fragments (Pawłowski *et al.* 1989, Świątek & Gulanowski 1990). To evaluate these effects, column chromatography can be used where the sonicated single- and double-stranded DNA binds differently to the hydroxyapatite. Through elution with phosphate buffer of increasing concentration the single-stranded DNA, being more weakly bound, is eluted first followed by the more strongly bound double-stranded molecule (Bernardi 1971).

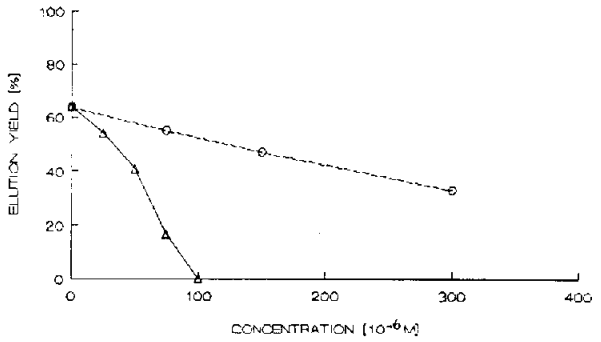

Figure 5. Influence of the Cr(III) compounds concentration on the elution yield of DNA from hydroxyapatite column after 24 h incubation in 0.05 M sodium acetate buffer at pH 5.6. The DNA concentration was 50×10^{-6} g ml^{-1}. ○, DNA with A1; △, DNA with $Cr(NO_3)_3$.

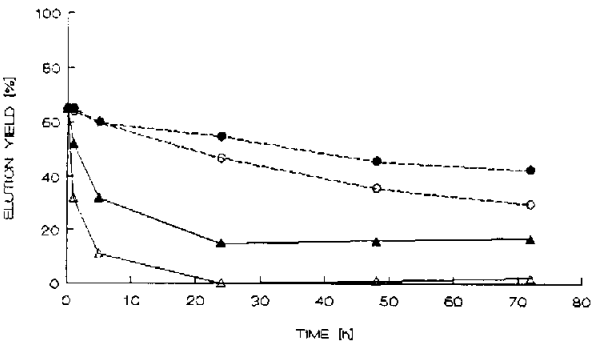

Figure 6. Influence of the incubation time of the DNA with $Cr(NO_3)_3$ and A1 complex in 0.05 M sodium acetate buffer at pH 5.6 on the elution yield of DNA from hydroxyapatite columns. The $Cr(NO_3)_3$ concentrations were 75×10^{-6} M (▲) and 150×10^{-6} M (△). The A1 concentrations were 75×10^{-6} M (●) and 150×10^{-6} M (○).

We have examined the influence of Cr(III) aqua as well as A1, A2, AC and AG complexes on the stability of the double-stranded DNA molecule. It was found that $[Cr(H_2O)_6]^{3+}$ ions have a strong impact on the stability of the DNA structure. While DNA without Cr(III) complexes exhibits 9% of single-stranded fragments, after incubation with $[Cr(H_2O)_6]^{3+}$ ions, no such fragments were found. This is in agreement with findings that the presence of Cr(III) ions stabilized the DNA molecule, as manifested by an increase of the melting temperature of the DNA and double-stranded polynucleotides (Wolf *et al.* 1989). Complexes with ascorbic acid, glutathione and cysteine, although bound to DNA, did not have any influence on the amount of the deseparated fragments.

The significant influence of the Cr(III) aqua complexes on the stability of the DNA molecule can be explained through the coordination properties of the ion. The Cr(III) ions bind to phosphates as well as to nitrogen bases of the nucleotides (Wolf *et al.* 1989). Under the conditions of our experiment one $[Cr(H_2O)_6]^{3+}$ ion is bound to four nucleotides, as was calculated from electrochemical analysis (Gulanowski *et al.* 1992).

Interstrand cross-linking studies. The binding of chromium ions to the existing single-stranded DNA molecules or those formed under experimental conditions at the phosphates and/or bases may connect two DNA single strands via chromium. Chromium ion binding to the nitrogen bases of the nucleotides in the double-stranded DNA molecule is another possible mechanism which could explain DNA interstrand cross-linking by Cr(III). It was found that the Cr(III) aqua ion is bound preferentially to polynucleotides with GC pairs (Wolf *et al.* 1989) and that this ion is able to cause interstrand DNA cross-linking (Gulanowski *et al.* 1992).

The hydroxyapatite chromatography experiments indicated that, both in the control sample and in the samples where DNA was incubated with A1, A2 and AG, no double-stranded fractions were found. The whole amount of DNA was denaturated resulting in single-stranded molecule formation. Only after incubation with AC did some (4%) double-stranded DNA remain. Under the same experimental conditions, incubation of the DNA with Cr(III) hexaaqua ions led to about 6% of double-stranded DNA remaining after the denaturation process (Gulanowski *et al.* 1992). When the concentration of AC increased 2-fold and the incubation temperature was 37 °C, 7% of double-stranded DNA was found. It is possible that AC connects two opposite strands and counteracts their breaking under thermal denaturation. The spectrophotometric data showed that AC does indeed interact with DNA in a different manner than other complexes. Because this complex does not show DNA–DNA intermolecular cross-linking, at this stage, it is impossible to give more details on the nature of the AC–DNA interaction.

Toxicity and genotoxicity of Cr(III) complexes

All complexes tested in the studied range of concentrations appeared to be non-toxic to both rec^- and rec^+ strains. The Cr(III) complexes did not induce genotoxic effects in the bacterial cells, because there was no difference in the diameter of the killing zones in the M45 and H17 plates.

It can be explained that, similar to the majority of Cr(III) complexes (Warren *et al.* 1981), these complexes are not able to cross the membranes of the bacterial cells. However, it is also possible that they are taken up, but not in sufficient amounts to cause DNA damage.

Conclusions

(i) Cr(III) complexes with ascorbic acid, glutathione and cysteine (obtained from the reduction of Cr(VI) with these cellular reductants) bind to DNA

(ii) $K[Cr(asc)_2]$, (A1), similar to the previously studied $Cr(NO_3)_3$, caused the formation of DNA–DNA intermolecular bonds.

(iii) K[Cr(asc)(cys)] (AC), like $Cr(NO_3)_3$, revealed the DNA interstrand cross-linking effect. Its nature, however, seems to be different from the latter.

(iv) The Cr(III) complexes studied were non-toxic and non-genotoxic in the bacterial test.

The general conclusion is that Cr(III) complexes with cellular agents obtained from Cr(VI) reduction interact with DNA. This interaction depends on the nature of the complex and is weaker in comparison with the Cr(III) hexaaqua ions.

As various chemical processes for dissolved Cr(III) compounds depend on pH, concentration and environment, it should be interesting to study the interactions of Cr(III) complexes with DNA under physiological conditions.

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