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The ability of cystamine to bind DNA

P. Allegra, E. Amodeo, S. Colombatto, and **S.P. Solinas**

Dipartimento di Medicina e Oncologia Sperimentale, Sezione di Biochimica, Università degli Studi di Torino, Torino, Italy

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Summary. The DNA-binding properties of cystamine compared with natural occurring polyamines have been studied *in vitro* by means of ethidium bromide displacement assays, studies of DNA thermal stability and analyses of DNA-B/DNA-A transition. While the first two methods did not put in evidence any peculiar property in the binding capability of cystamine, CD studies showed the interesting ability of cystamine to shift the equilibrium B/A-DNA towards the B-form. In the same experimental conditions spermine and spermidine induced the A form of DNA, instead putrescine and cadaverine did not show any particular activity. The ability of cystamine to bind DNA, as shown also by its DNA radioprotective capability, might be important in chromatin condensation and stabilization, and might be a cause of the antiviral activity observed by some authors.

Keywords: Amino acids – Cystamine – DNA interaction – Cysteine metabolism – Sulfur containing diamine – Polyamine

Introduction

Cystamine $NH_2—CH_2—CH_2—S—S—CH_2—CH_2—NH_2$ [2,2′-dithio-bis (ethylamine)] is a linear aliphatic diamine containing a disulfide bridge, coming from oxidation of the sulfhydryl groups of two cysteamine residues. In the cell, these compounds coexist in a redox equilibrium; consequently cystamine and cysteamine metabolisms are closely related, as their biological functions are. So far no information is available about their endocellular concentrations. These compounds are related to CoA metabolism, in particular cysteamine derives from 4′-phosphopanthotenyl-cysteine decarboxylation and from the subsequent hydrolysis of 4′-phosphopanthoteine to pantothenate and cysteamine. Up to now in mammals no enzyme is known to directly decarboxylate cysteine, unless the aminoacid is bound to phosphopanthotenic acid (Cooper, 1983; Stipanuk, 1986). Cystamine degradation and elimination pathways involve its enzymatic transformation into aldhymine (cyclic

aminoaldheyde) by diamineoxidase, a well known enzyme involved in the degradation of diamines. Then cystamine is metabolized in a variety of compounds, such as thiocysteamine, hypotaurine and taurine (De Marco et al., 1965; Sharma et al., 1995).

Apart from being involved in keeping redox equilibrium between thiols and disulfides [Gilbert, 1995], and from being the natural precursor of taurine, recent literature assigns to cystamine numerous different functions. It seems to be an inhibitor of transglutaminase, an enzyme that plays important roles in a number of biological systems (Birckbichler et al., 1981); it is accumulated and metabolized as taurine in lung cells, involving a thiol-disulphide exchange between cystamine and glutathion (Lewis et al., 1989).

It has been also well established that aminothiols exert a radioprotective action on DNA irradiation, both *in vitro* and *in vivo*, by two mechanisms: scavenging of hydroxyl radicals and chemical repair of DNA radicals (Shapiro et al., 1970; Vasilescu and Rix-Montel, 1980; Zheng et al., 1988; Singh et al., 1990). On this subject it is particularly puzzling that cystamine too can protect DNA by radiations, as well as other synthetic disulfides compounds (Newton et al., 1997; Smoluk et al. 1986; Zheng et al., 1988).

Besides recently it has been found that cystamine and some derivatives, at concentrations non toxic for host cells, show a significant antiviral activity against some DNA- and RNA-viruses, such as vaccinia virus, herpes simplex virus type 1, influenza A virus (Gutschow et al., 1995). Moreover it is of great interest that cystamine exerts a remarkable antiviral activity towards HIV-1 virus on different cell lines (Bergamini et al., 1994; Ho et al., 1995; Bergamini et al., 1996; Ho et al., 1996). Thus cystamine seems to be directly involved in DNA interaction, as aliphatic polyamines are (Feuerstein et al., 1988), to which actually this compound is structurally related. Because of these very different features cystamine and cysteamine still do not have a well defined and unique biological role.

It was for these reasons and for the above mentioned structurally similarities with polyamines, that we decided to examine *in vitro* the ability of cystamine to bind DNA.

The interaction between a ligand and DNA can be investigated by different experimental approaches (Cain et al., 1978; Edwards et al., 1991; Hampel et al., 1991; Hsieh et al., 1994; Pallan and Ganesh, 1996). Among these, the displacement studies of the intercalating dye ethidium bromide from the DNA double strand, the studies of DNA melting temperature modification and those on B-DNA→A-DNA transition, are the most used (Mandel and Marmur, 1968; Stewart, 1988; Ivanov and Krylov, 1992; Gray et al., 1995). These methods give profoundly different information on ligand-DNA interaction. Indeed the ethidium bromide displacement studies point out the ability of the ligand to modify the DNA double helix, while the thermic denaturation studies give information about the ligand capability of stabilizing DNA; on the other hand neither method shows anything on the kind of modifications on the DNA structure. Contrary the ability of a ligand to modify the DNA structure is shown by its capability of shifting the equilibrium between B and A forms of DNA. This effect can be highlighted distinctly by means of circular

dichroism technique (Thomas et al., 1977; Ivanov and Krylov, 1992; Gray et al., 1995).

This work has been carried out investigating cystamine and some natural polyamines, as reference compounds (later referred to as RPA), in their interaction with DNA, by (i) studies of ethidium bromide displacement, (ii) analyses of thermal stability, (iii) interactions on B-DNA→A-DNA transition.

Materials and methods

Calf thymus DNA (CT-DNA, from Merck) was further purified to remove heavy metals and divalent cations. DNA was dissolved in ultra pure deionized water at 2 mg/ml concentration and left to gently stir at 4°C overnight. They day after 10 μM Na_2-EDTA and 0.1 M NaCl were added to the clear solution. After addition of the same volume of pure ethanol, the precipitated DNA was collected on a glass rod and resuspended in ultra pure deionized water at about 1 mg/ml concentration. The precise value of the DNA concentration was determined spectrophotometrically at 260 nm, knowing that an eukaryotic double strand DNA solution at 50 μg/ml has a value of $A_{260nm} = 1.0$. Double-distilled water and ethanol of spectroscopic grade were used. Cystamine, putrescine, cadaverine, spermidine, spermine (all in the hydrochloride form) and ethidium bromide (EtBr) were purchased from Sigma. All the materials utilized were of the highest purity commercially available and were used without further purification.

(i) Ethidium bromide displacement assay

The fluorescence (F) of intercalated ethidium was measured by a Perkin-Elmer LS 3B spectrofluorometer, with wavelength of excitation at 546 nm and of emission at 595 nm, according to Stewart (1988). The cuvette and the cell compartment were thermostated at 25°C. Working solution (WS) was prepared as the following: 70 μl of 514 μg/ml CT-DNA solution (= 36 μg), prepared as above, were added to 3.0 ml of 0.01 M SHE buffer (2 mM HEPES, 8 mM NaCl, 0.05 mM Na_2-EDTA) pH 7.0, containing 1.26 μM EtBr. The DNA concentration in WS was chosen to have an increased fluorescence value of approximately 7 times (F = 1.80) respect to the fluorescence value of buffered EtBr alone (F = 0.25). Cystamine or individual RPA solutions were added to WS in small aliquots until a maximum volume of 300 μl was reached and the fluorescence was recorded after each addition. To evaluate the aspecific fluorescence decrease due to dilution itself, appropriate blanks were prepared adding to WS the same aliquots of buffer alone. Since the scale is arbitrary, all the measurements, corrected for the respective blank value, have been normalized to a scale 0–100. Control experiments were also performed to show that the fluorescence was proportional to the amount of added duplex DNA, at least over the range of the DNA concentrations used.

(ii) Thermal denaturation of DNA

A Ultrospec 2000 spectrophotometer (Pharmacia Biotech), equipped with a Peltier heated cell holder for temperature control, was used for studying DNA thermal denaturation. The experimental system was controlled by Swift-Tm-software (Pharmacia Biotech) that allowed a constant and linear increase of temperature in the cuvette. According to Mandel and Marmur (1968), the working conditions were: wavelength 260 nm, temperature range 50–95°C, heating rate 0.5°C/minute, readings every 30 seconds. 140 μg of CT-DNA (prepared as above) were added to 2.5 ml of SHE buffer (2 mM HEPES, 8 mM

NaCl, 0.05 mM Na_2-EDTA) pH 7.0. The DNA concentration was chosen to have an initial A_{260nm} value of about 1.0. The incubate was preheated at 50°C for 10 minutes, in 1 cm pathlength cuvette. The melting profiles of DNA alone and in the presence of different concentrations of cystamine or alternatively of individual RPA were obtained. The Tm values were provided directly by the software from the melting data.

(iii) Circular dichroism studies

We have studied the effects of the cystamine bond to DNA evaluating structural modifications induced by the ligand on the DNA structure when it is in equilibrium between B and A forms. As is known the equilibrium between B and A DNA forms can be reached by dehydrating DNA with ethanol, at concentration near to 70% (v/v). For this purpose circular dichroism (CD) analysis has been demonstrated to be a very efficient technique (Ivanov and Krylov, 1992; Gray et al., 1995).

CD studies were carried out according to Ivanov and Krylov (1992) with some minor modifications. CD spectra were recorded with a Jasco J-600 spectropolarimeter, equipped with a data processor, which was previously standardized, using a 10 mm light-path cell thermostated at 25°C. First of all, the ethanol concentration at which CT-DNA is 50% in B form and 50% in A form was experimentally determined. For this purpose different 3 ml mixtures, containing 50 μg/ml CT-DNA, 1 mM NaCl, and increasing ethanol amounts were carefully prepared, to have a final ethanol concentration varying from 0% (prevalence of B-DNA form) to 80% (prevalence of A-DNA form). DNA precipitates over 80% ethanol concentration. Spectra were recorded in the range 220–300 nm and the $\Delta\varepsilon$ (in mdeg $cm^{-1}M^{-1}$) at 270 nm was determined. The θ (theta) value $[\theta = (\Delta\varepsilon - \Delta\varepsilon_B)/(\Delta\varepsilon_A - \Delta\varepsilon_B)]$ at 270 nm was calculated, considering $\Delta\varepsilon_A$ the value obtained at 80% (v/v) ethanol concentration and $\Delta\varepsilon_B$ the value obtained in absence of ethanol and plotted against the alcohol volume percent. 50% transition A-DNA/B-DNA in 72% ethanol was obtained.

In these equilibrium conditions, 2.5 μl of 2.5 mM cystamine (or of RPA individual solutions) were added stepwise to obtain a 2 μM increase in concentration, until 20.6 μM final was reached. After each single addition the CD spectrum in the range 220–300 nm was recorded. From the spectrum data the variation in CD magnitude ($\Delta\varepsilon$) was calculated at 270 nm ($\Delta\varepsilon$ ligand − $\Delta\varepsilon$ 72% ethanol) and plotted against the ligand concentration.

Results

(i) Ethidium bromide displacement from DNA

By means of this technique the DNA binding properties of cystamine and RPA were investigated. Polyamines are not intercalating agents, as ethidium is, but because of their high positive charge, they bind the DNA double helix. In this way, when ethidium bromide has been previously intercalated into DNA, the subsequent bond of polyamines induces such a modification into DNA that the intercalated ethidium is displaced and the fluorescence (F) decreases (Cain et al., 1978; Edwards et al., 1991). Figure 1 shows the F% values as a function of cystamine or RPA concentrations utilized in tests, all in the same experimental conditions. From these results the highest fluorescence decrease, i.e. the largest ethidium displacement, was obtained with spermine and spermidine concentrations in the range of 2×10^{-7}–1×10^{-5} M and 1×10^{-5}–4×10^{-4} M, respectively. As expected, data obtained with RPA confirmed those of literature (Mandel and Marmur, 1968; Cain et al., 1978;

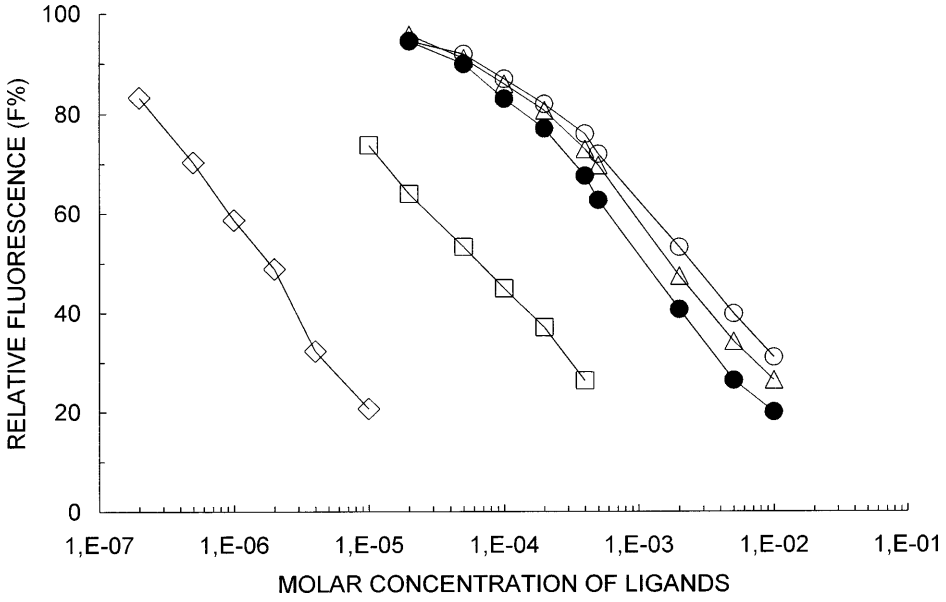

Fig. 1. Ethidium bromide displacement assay. Displacement of ethidium from CT-DNA following addition of increasing amounts of ligands is displayed. To CT-DNA were added different amounts of cystamine (●) spermine (◇), spermidine (□), cadaverine (△) and putrescine (○). The fluorescence values are provided as percentage of the maximum. The IC_{50} values are directly available from such plots. For other details see "Materials and methods"

Edwards et al., 1991; Hampel et al., 1991; Hsieh et al., 1994; Pallan and Ganesh, 1996). Instead cystamine showed the same displacement capacity only in a much higher concentration range, i.e. between 2×10^{-5} M and 1×10^{-2} M, similarly to putrescine and cadaverine. From the fluorescence data, we have drawn the corresponding IC_{50} value (Table 1), which indicates for each compound the concentration required to decrease the fluorescence intensity by 50%. A high IC_{50} value indicates a very low capacity to displace EtBr from DNA. As shown in Table 1, the cystamine IC_{50} value (1.2 mM) is 600 times higher than that for spermine (2×10^{-3} mM), it is 20 times higher than for spermidine (6.5×10^{-2} mM), while it seems to be very similar to that for cardaverine (1.65 mM) and only slightly lower than that for putrescine (2.5 mM). This EtBr displacement effect seems to be related not only to the molecule length, but also to the positive charges belonging to the displacing agent. In fact it has been shown (Cain et al., 1978) that the binding constant of EtBr to DNA depends on DNA flexibility, that can be modified by altering the cation concentration in the reaction medium.

(ii) Effect of cystamine and RPA on thermal denaturation of DNA

We analyzed the ability of cystamine and RPA to stabilize the double helix while DNA was subjected to an increase in temperature. From the DNA melting profiles obtained recording the variation of 260 nm absorbance as a

function of increasing temperature from 50°C to 95°C, we calculated the Tm values, i.e. the temperature value at which 50% of DNA had been denaturated. Figure 2 shows the Tm values calculated as function of molar concentration of different ligands used. At any concentration, spermine and spermidine have a much greater stabilization effect than that of other diamines on the double helix. In particular, the Tm variations as a function of 3×10^{-5}M concentration of each ligand are of 20°C for spermine, of 15°C for spermidine and finally of only 3°C for cystamine, putrescine and cadaverine. In these experimental conditions higher concentrations of cystamine, i.e. in the millimolar range, lead to DNA denaturation and precipitation, as well as putrescine and cadaverine do. From our data it is evident that cystamine possesses weaker stabilization properties than spermine and spermidine, but very similar to putrescine and cadaverine, the short chain diamines. We can therefore affirm that the effect on the Tm becomes greater as ligand chain, containing positively charged secondary amino group(s), gets longer. In fact it is evident that spermidine and spermine, tying on the double helix of the DNA, form some crosslinks that make the DNA more stable to heat denaturing effect.

(iii) Effect of cystamine and RPA on B-DNA/A-DNA equilibrium

By means of circular dichroism, we were able to evaluate the specific cystamine and RPA effects on the equilibrium B-DNA/A-DNA. Experimentally it is possible to induce DNA from B to A conformation by adding to DNA in NaCl solution an increasing amount of pure ethanol that decreases

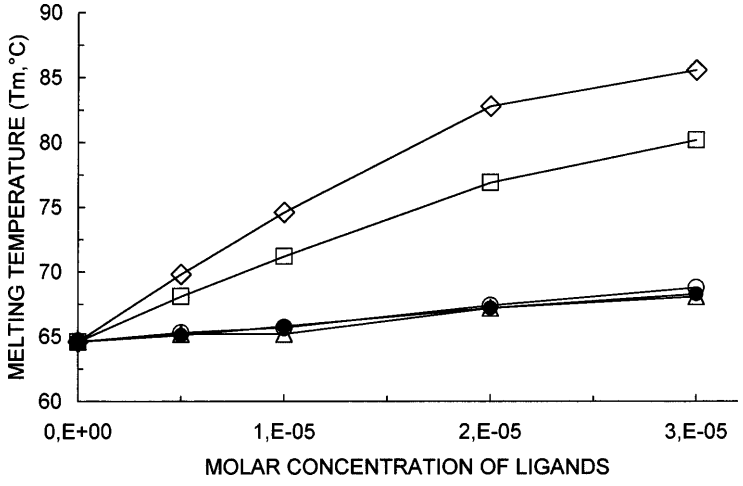

Fig. 2. Effect of cystamine and RPA on thermal denaturation of CT-DNA. Tm values, calculated from the thermal denaturation curves of DNA as the midpoint of each transition, are reported as a function of increasing molar concentration of ligands: cystamine (●) spermine (◇), spermidine (□), cadaverine (△) and putrescine (○). For other details see "Materials and methods"

the relative humidity (Ivanov and Krylov, 1992). From the magnitude values at 270 nm of CD spectra of DNA in NaCl solution containing variable ethanol concentrations (from 0% to 80%, see materials and methods for more details), we drew the conditions of B-DNA/A-DNA equilibrium. At 72% ethanol concentration we obtained a θ value of 0.5, that corresponds to the transition equilibrium between the two DNA forms. Figure 3(A) shows this

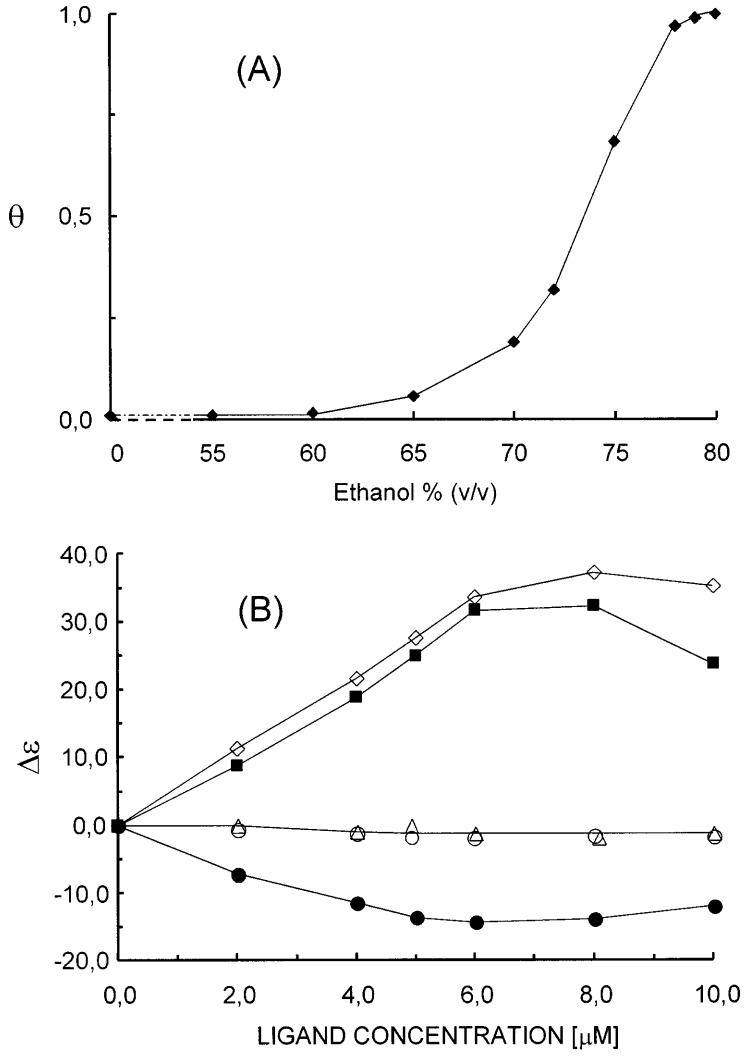

Fig. 3. **A** Curve of the DNA-B→DNA-A transition. The B-A transition curve was built by plotting the value of the A-form fraction [A]/[A + B] ($\theta = (\Delta\varepsilon - \Delta\varepsilon_B)/(\Delta\varepsilon_A - \Delta\varepsilon_B)$), as a function of % (v/v) ethanol. The curve is characterized by a transition point at 72% ethanol concentration that corresponds to the transition equilibrium between the B-DNA/A-DNA forms. **B** Effect of cystamine and RPA on DNA conformation. In equilibrium conditions [72% (v/v) ethanol concentration], cystamine (●) spermine (◇) spermidine (□), cadaverine (△) and putrescine (○) were added stepwise to get a 2μM increase of concentration until final 20.6μM was reached. The $\Delta\varepsilon$, i.e. the variation in CD magnitude (in degrees), at 270 nm was calculated considering 0 (zero) the value obtained in absence of ligands. For other details see "Materials and methods"

Table 1. Interation of cystamine and polyamines with CT-DNA

Ligand	Effect on Et-Br displacement Ic_{50} (mM)	Effect on thermal denaturation of DNA Tm (°C)	Effect on DNA conformation $\pm\Delta\varepsilon$
Spermine	2×10^{-3}	82.8	+33.6
Spermidine	6.5×10^{-2}	76.9	+31.6
Cystamine	1.20	67.2	−14.4
Cadaverine	1.65	67.2	−1.5
Putrescine	2.50	67.4	−1.2

The table sums up the most significant results of our studies. The IC_{50} value represents the millimolar concentration of compound needed to decrease the CT-DNA bound ethidium fluorescence by 50%; the Tm value represents the melting temperature of CT-DNA relative to 2×10^{-5} molar concentration of ligands; $\Delta\varepsilon$ is the variation in CD magnitude (in degrees) at 6×10^{-6} M concentration of ligands; is calculated considering 0 (zero) the $\Delta\varepsilon$ value obtained in absence of ligands. Values reported are the average of at least of three independent experiments and are ±10%.

experiment. Under the B-A transition point conditions, we tested the cystamine and RPA effect on DNA. Figure 3(B) correlates the variation in CD magnitude at 270 nm to ligand concentrations, while the equilibrium value is zero without any ligand addition. The value will rise if a ligand is A-DNA specific, and it will drop if the ligand is B-DNA specific. This method is extremely sensitive, since at the transition point the free energies of the B and A forms are equal, even very small preferences of a ligand for the A or B form will be seen immediately. It is evident that in the range 4.0–8.0 μM, spermine and spermidine favoured the A-DNA form, while cystamine was B-DNA specific. Moreover putrescine and cadaverine were almost completely ineffective in shifting the B-A/DNA transition, at least at the used concentrations.

Table 1 sums up the more significant results of ours experiments.

Discussion

The analyses of experimental data obtained either by ethidium bromide displacement assays, or by thermal denaturation, show that cystamine has DNA binding properties more similar to shorter chain polyamines putrescine and cadaverine than spermidine and spermine. The effect is in agreement with the small structure of cystamine. In particular, according to the data from the ethidium bromide displacement studies, cystamine interacts with DNA at much higher concentrations respect to spermine (600 times) and to spermidine (about 200 times). On the other hand, from the data obtained from the thermic denaturation profiles, in the concentration range utilized, cystamine does not show any advantage in DNA stabilization over spermine and spermidine. These two investigation methods simply point out that cystamine has

weaker DNA binding properties than spermine and spermidine, but they do not explain anything on the nature of these interactions or on the DNA molecular backbone modifications involved.

For this reason it was important to test, by means of circular dichroism, the DNA binding properties of the chosen ligands in the experimental conditions of the B/A transition point. In fact in an aqueous solution probably most specific ligands may not be able to make DNA acquire A or B form; rather in these equilibrium conditions they may only shift the B/A-DNA to the side of A-form or B-form, thereby facilitating or inhibiting the transition induced by alcohol. This equilibrium shift may occur even in presence of weak interactions between ligand and DNA.

Thus the study on DNA B/A transition emphasizes an interesting and unexpected behaviour of cystamine. In fact at the same concentration values, while spermine and spermidine induce the transition-state-DNA towards the A-DNA form, cystamine drives the DNA transition from the equilibrium B/A towards the B-DNA form. It is extremely important to point out that, in the same experimental conditions, cadaverine and putrescine have no influence on DNA B/A transition. In particular, unlike the EtBr-displacement and the thermic denaturation studies, this effect on DNA transition occurs at equivalent concentrations of cystamine, spermine or spermidine.

We think that this intriguing effect could result via affecting the hydration properties of DNA in an indirect way. Because the main structural difference among the cystamine and the tested RPA molecules (apart from the chain length) is due to the presence of the disulfide bridge instead of —NH— group(s), we suppose a specific role of this disulfide group in altering the equilibrium in the DNA transition experiments.

On the other hand spermine and spermidine are polycationic compounds (containing in their structures both —NH_2^+— and —NH_3^+ groups) and consequently they strongly interact with the negatively charged phosphates of nucleic acids. These electrostatic interactions are able of provoking a conformational transition in DNA from its usual B-form to A-form, and in poly(dG-dC) from B- to Z-form at physiologically relevant cationic concentrations (Tomita et al., 1989; Yuky et al., 1996). Instead cystamine, as putrescine and cadaverine, binds DNA by means of weaker electrostatic interactions between the terminal positively charged amino nitrogen atoms (—NH_3^+) and the negatively charged phosphates of DNA. This difference is well showed in the ethydium bromide displacement and in the thermal denaturation experiments.

Moreover cystamine unlikely polyamines contains two sulfur atoms in a disulfide bridge that may interact with a greater number of water molecules being acceptors of hydrogen bonds. This likely increased number of water molecules surrounding the cystamine molecule may lead to a greater DNA hydration, inducing the observed DNA transition toward the B form.

Nevertheless we can not exclude the possibility that, in the experimental conditions used, the cystamine molecule, because of disulfide bond, may directly interact with the DNA sugar puckering leading to the observed transition.

In the light of our results it is reasonable to suppose that the radioprotection exerted by cystamine on nucleic acids, explained by various authors as high concentration of cations near the DNA (Newton et al., 1997; Smoluk et al., 1986; Smoluk et al., 1988; Zheng et al., 1988), can be also due to an increased number of water molecules surrounding the disulfide bridge of cystamine bound to DNA phosphates by electrostatic interactions.

Besides we suppose that this direct interaction might be a cause of the antiviral activity observed by many authors (Bergamini et al., 1994; Gutschow et al., 1995; Ho et al., 1995; Bergamini et al., 1996; Ho et al., 1996).

Furthermore, since the disulfide bridge can be structurally modified in the presence of naturally occurring reducing agents (i.e. reduced glutathion), the cystamine-DNA interaction may be directly controlled *in vivo* by cell redox potential, only regulating cystamine-cysteamine equilibrium.

In conclusion the most significant feature of our results is a new evidence that cystamine affects the conformation of DNA. This is also in agreement to previous papers in which the authors report that low molecular weight amines induce a conformational shift of DNA towards the C-form (Kondakova et al., 1975; Tselikova et al., 1976).

Our data have encouraged us to evaluate if cystamine was able to produce this effect on DNA *in vivo*. Nowadays we are verifying the interaction of cystamine with DNA/protein complexes (work in progress), to investigate its potential role in chromatin condensation and/or stabilization.

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Authors' address: Prof. Sandro P. Solinas, Dipartimento di Medicina e Oncologia Sperimentale, Università di Torino, Via Michelangelo Buonarroti, 27, I-10126 Torino, Italy

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