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The Interaction of the Ruthenium(III)–Chloride System with DNA

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Abstract

The interaction of $RuCl_3$ in aqueous solution with DNA has been studied at various r values. Electronic spectra, melting curves and sedimentation experiments indicate that Ru(III) is bound mainly to the phosphate moieties of DNA, causing stabilization of the double helix. For small values of r we observe renaturation upon cooling and possible interstrand cross-linking that persists at room temperature. During the second heating the melting temperature decreases, indicating substantial interaction of Ru(III) with the bases of the DNA. Ru(III) interacts with the nitrogen of the bases only when DNA is almost denaturated, and this cross-linking interaction seems to be quite strong.

Introduction

The effect of metal ions upon conformation of DNA was realized early, when it became apparent that metal ions are involved in the stabilization of the Watson–Crick double helix [1, 2]. Metal ions react with a variety of electron-donor sites on polynucleotides [3]. There are two main sites of interaction, the phosphate moieties of the ribose–phosphate backbone and the electron-donor groups of the bases. The two types of interaction carry with them quite different effects upon the structure of polynucleotides [4–7]. Reactions with the phosphate means stabilization of ordered structures but cleavage of phosphodiester bonds at high temperature [8–10].

Whether the binding is non-specific, *i.e.*, totally predictable on the basis of electrolyte theory, or whether specific bonds to the phosphate are produced, the result is to neutralize the array of negative charges on the double helix and thus to stabilize it. Such stabilization is accompanied by an increase in the 'melting temperature' of DNA [3, 11].

Reaction with the bases means destabilization of ordered structures, since metal ions can bind to the bases in such a way as to interfere with the hydrogen bonding and the base stacking interactions that hold together the two strands of DNA. Destabilization is accompanied by a decrease in the melting temperature, T_m of DNA [3, 11]. The differences in behavior of various metal ions with polynucleotides have made it apparent that some metal ions prefer the phosphate sites and other metal ions prefer the base sites. The difference was strikingly illustrated by the effect of magnesium(II) and cadmium(II) ions on the melting behavior of DNA [11]. However, metal ions cannot be placed into two categories of those that bind to phosphate and those that bind to the bases. Thus, copper(II) ions that are so effective in base binding also bind phosphate and are therefore capable of cleaving phosphodiester links in polyribonucleotides [12–17]. On the other hand, zinc(II) ions which are so effective in degrading phosphodiester links due to phosphate binding have been demonstrated to bring about a temperature-reversible unwinding of DNA through binding to the bases [18]. Also, DNA can be unwound at low ionic strength with copper(II) ions and subsequently rewound by cooling and then adding solid electrolyte. Evidence indicates that copper(II) forges intramolecular as well as intermolecular cross-links [17]. Mercury(II) and silver(I) ions bind to nucleoside bases in such a manner that the two chains become cross-linked; native double-stranded DNA can be regenerated from this structure by removal of these ions by complexing anions [19, 3].

The antitumor drug *cis*-$Pt(NH_3)_2Cl_2$ shows a wide variety of biological activity and seems to act by causing a primary lesion on cellular DNA by binding to the bases [20, 21]. The drug thus bound to native DNA causes both interstrand cross-linking and a partial destabilization of the DNA secondary structure [22, 23].

The preference for phosphate over base association decreases in the order Mg(II) > Co(II) > Ni(II) > Mn(II) > Zn(II) > Cu(II) [18]. Also trivalent rare earth ions [24, 9] and hard metals in general stabilize ordered structures by binding to phosphates. The above series could be extended to include softer heavy metal ions. In the presence of Pb(II) [25], Au(III) [26], Pt(II), Pt(IV) [27] or Rh(III) [28] the melting temperature of DNA is substantially lowered. Heavy metal ions may therefore be placed to the right of the above series.

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Several ruthenium complexes have been shown to inhibit cellular DNA synthesis *in vitro* at a level similar to that of *cis*-$Pt(NH_3)_2Cl_2$ and have shown antitumor activity in animal studies [30]. Compounds containing pentammine–ruthenium(II) and -(III) have been shown to inhibit DNA synthesis functioning, probably by binding to DNA [31].

The present investigation was undertaken in an effort to understand the interaction of the ruthenium(III)–chloride system with DNA.

Experimental

$RuCl_3 \cdot 3H_2O$ was purchased from Johnson-Matthey. Calf thymus DNA sodium salt was obtained from Sigma Chemicals Co. Stock solutions of DNA were prepared by placing 17 mg of solid DNA in 10 ml of aqueous buffered solution (0.15 M NaCl–0.015 M sodium citrate) followed by gentle shaking at room temperature. The solution was generally complete after two days. The stock solution was analyzed for DNA content spectrophotometrically at 260 nm as E(P) = 6600 and stored under refrigeration. The concentration was 5×10^{-5} M (DNA-P), 1.5×10^{-3} M NaCl and 1.5×10^{-4} M sodium citrate.

Buffered DNA solutions were mixed in all cases with aqueous solutions of ruthenium(III) chloride, and the pH of these solutions was between 6.5–6.7. The electronic spectra of $RuCl_3$ solutions in 0.01 M NaCl or with citrate buffer were identical at room temperature. However, at high concentrations of $RuCl_3$ (1×10^{-4} M, $r = 2$) at elevated temperatures the ultraviolet spectrum at 260 nm was different from that at room temperature. In the T_m experiments the absorbance due to $RuCl_3$ at 260 nm ($c = 1 \times 10^{-4}$ M which corresponds with $r = 2$) was subtracted from the corresponding absorbance values of Ru–DNA solutions at every temperature (see later Fig. 3).

Heating and cooling curves were obtained with the Gilford Model 2400 Recording Spectrophotometer equipped for automatically timed measurement of absorbance and temperature, a synchronous motor for regulated temperature change and a Haake Model F circulating water bath. The appropriate amounts of DNA and metal ion solutions were mixed immediately before placement into the glass-stoppered cuvets fitted with vacuum grease. A 1×10^{-2} M sodium chloride solution was used as a blank. The samples were heated and cooled at a constant rate of 1 °C/min. The melting curves were obtained by reading the absorbance and an appropriate blank at 260 nm at ambient temperature. Room temperature electronic spectra were obtained with a Cary 14 spectrophotometer. The pH of the solutions used in all melting and spectrophotometric experiments were obtained with a Radiometer Model 25 pH meter.

Radioactive thymidine (18 Ci/mmol ^3H) was added to the medium of the cultures, and the DNA of the cultured cells was isolated according to the method by Marmur [32].

Labeled DNA or labeled DNA-$RuCl_3$ solutions were transferred to the top of 5–20% alkaline sucrose gradients. Tubes containing these solutions were run at 48 000 rpm at 20 °C. After centrifugation the gradients were fractionated in seven drop fractions and were collected into Whatman 3MM paper strips. The DNA on the strips was fixed in 5% trichloroacetic acid (TCA), 0.01 M sodium pyrophosphate and subsequently washed twice in 96% ethanol, dried and put into scintillation vials. Five millilitres of toluene (+PPO + POPOP) solution was added to each vial and the radioactivity was measured in a Liquid Scintillation Counter. The specific radioactivity was estimated in cpm/μg of DNA with the help of a computer programme [33].

The average molecular weight was calculated from the formula [34],

$$M_r = A_{w,t} \frac{\Sigma_i [c_i (i - \frac{1}{2})^k]}{\Sigma_i c_i}$$

where: $A_{w,t}$ = constant depending on rpm and centrifugation time; $k = 2.5$; c_i = % molecules of DNA in i fraction; i = % cpm per total number of cpm in the tube.

Results and Discussion

Sedimentation Experiments

In Fig. 1 are presented the results of the sedimentation experiments. We observe for DNA solutions that DNA molecules with high molecular weight appear in the upper fractions of density gradients. On the contrary, DNA–$RuCl_3$ solutions indicate that the molecules with the higher molecular

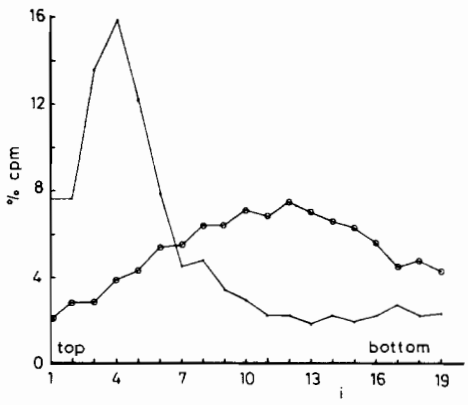

Fig. 1. Density gradient from ultracentrifugation experiments. • DNA solution, ○ DNA–$RuCl_3$ solution.

weight correspond to middle and lower fractions of the density gradients. Also, the ratio of the average molecular weight of DNA in $RuCl_3$ solution to the average molecular weight of DNA alone in solution is 2.2.

Thus from the distribution of molecular fractions in the sedimentation experiments and the increase of the average molecular weight of DNA in $RuCl_3$ solution, we conclude that the DNA is interacting with the Ru(III) ions.

Electronic Spectra

$RuCl_3$ in the presence of chloride ions (1×10^{-2} M NaCl) exhibits an absorption spectrum in the region 350 to 560 nm with maxima at 379 nm, 460 nm and 552 nm characteristic of Ru(III) ions in water solution at concentrations between 5×10^{-4} and 1×10^{-3} M in the pH region 6.5 to 6.7. Several chlorohydroxy derivatives must exist in equilibrium in this pH region [35]. Spectra of unbuffered solutions or buffered citrate solutions exhibit no significant differences at these concentrations. The respective spectra in the presence of DNA at 25 °C showed a decrease in absorption maxima and a small shift of the bands in the visible spectrum (Table I). The observed changes are not very significant and indicate that Ru(III) does not interact with the bases at room temperature. The decrease in absorbance observed could be interpreted as a decrease in effective metal ion concentration due to interaction with the phosphates.

TABLE I. Electronic Spectra of DNA–$RuCl_3$ Solutions (nm)

Solution	l (cm)	A_1	λ_1	A_2	λ_2	A_3	λ_3
Ru^{3+} 5.0×10^{-4}	1.0	0.83	552	1.18	460	1.94	379
Ru^{3+} 1.0×10^{-3}	0.5	1.14	552	1.58	460	2.42	379
r = 5.0	1.0	0.85	556	0.96	460	1.69	372
r = 10.0	0.5	0.70	560	1.24	460	2.26	374
r = 0.0	1.0	0.32	258				
r = 0.2	1.0	0.78	258				
r = 0.5	1.0	0.88	258				
r = 2.0	1.0	1.38	259				
r^a = 0.1	1.0	0.34	259				
r^a = 0.5	1.0	0.44	260				
r^a = 1.5	1.0	0.62	262				

[a]Spectra of solutions after heating to denaturation and cooling.

The conclusion that Ru(III) interacts with the phosphates at room temperature is further supported from the ultraviolet spectra of Ru(III)–DNA solutions. Ultraviolet spectra of Ru(III)–DNA solutions that were taken using in the reference cell concentration of the metal ion the same as in the sample cell do not show any change in the 258 nm DNA band. The absorbance of these solutions is greater than in the respective DNA–NaCl solutions and is increasing with the r value.

The ultraviolet spectrum of DNA, as is well known, shows a maximum at 258 nm. The position of this maximum remains unchanged by the addition of Cu(II) ions at 25 °C, but on increasing the temperature there was a small shift in the maximum towards longer wavelengths and an increase in the absorption due to decrease in hypochromicity. It was concluded that Cu(II) ions at room temperature bind to phosphate sites only, but at higher temperatures when some relative motion of the two strands, in the DNA helix is possible, such as occurs at the 'annealing temperature', penetration of the helix by the Cu(II) ions can occur, which results in binding of the Cu(II) ions to nitrogen atoms of the bases [12]. This was further evidenced by the increased absorption and the blue shifting of the bands in the visible region of the spectrum caused by the stronger ligand field of the nitrogen derivatives on coordination to Cu(II) [13]. Similar effects were observed in the ultraviolet and visible part of the spectrum with Au(III) [26] and *cis*-$Pt(NH_3)_2Cl_2$ [23], and these changes were interpreted as being due to reduction of stacking interactions of the bases, disruption of hydrogen bonds between base pairs and interaction of the metal ions with the nitrogen of the bases.

The ultraviolet spectra of Ru(III)–DNA solutions that were heated up to 37 °C remained unaltered, indicating that no observable reaction with the bases takes place up to this temperature. However, the ultraviolet spectra of dilute Ru(III)–DNA solutions that had been previously heated to 75 °C and then cooled to room temperature exhibit an increased absorption and a shifting to longer wavelengths (Table I). Thus during thermal denaturation close to or at the 'annealing temperature', some interaction takes place between Ru(III) ions and the bases of the DNA. Steric hindrance is most probably the reason that this interaction does not take place at an earlier stage of the denaturation process. This type of interaction, however, seems to be quite strong since it remains even after the solution is cooled. Ru(III) ions most probably form interstrand cross-links with the DNA at room temperature in a manner similar to that observed for *cis*-Pt(II) [23].

Heating and Cooling Curves

Figure 2a represents the well-known heating and cooling curves of DNA at low ionic strength (0.01 M NaCl). The melting temperature, T_m, is 67 °C and the transition is relatively noncooperative. Cooling of the denatured DNA produces a slight decrease in absorbance due to randomized restacking. Figures 2b–2f represent the heating, cooling and reheating curves of DNA at different metal ion concentrations, and in Table II are summarized the melting temperatures at various r values. We observe that the melting

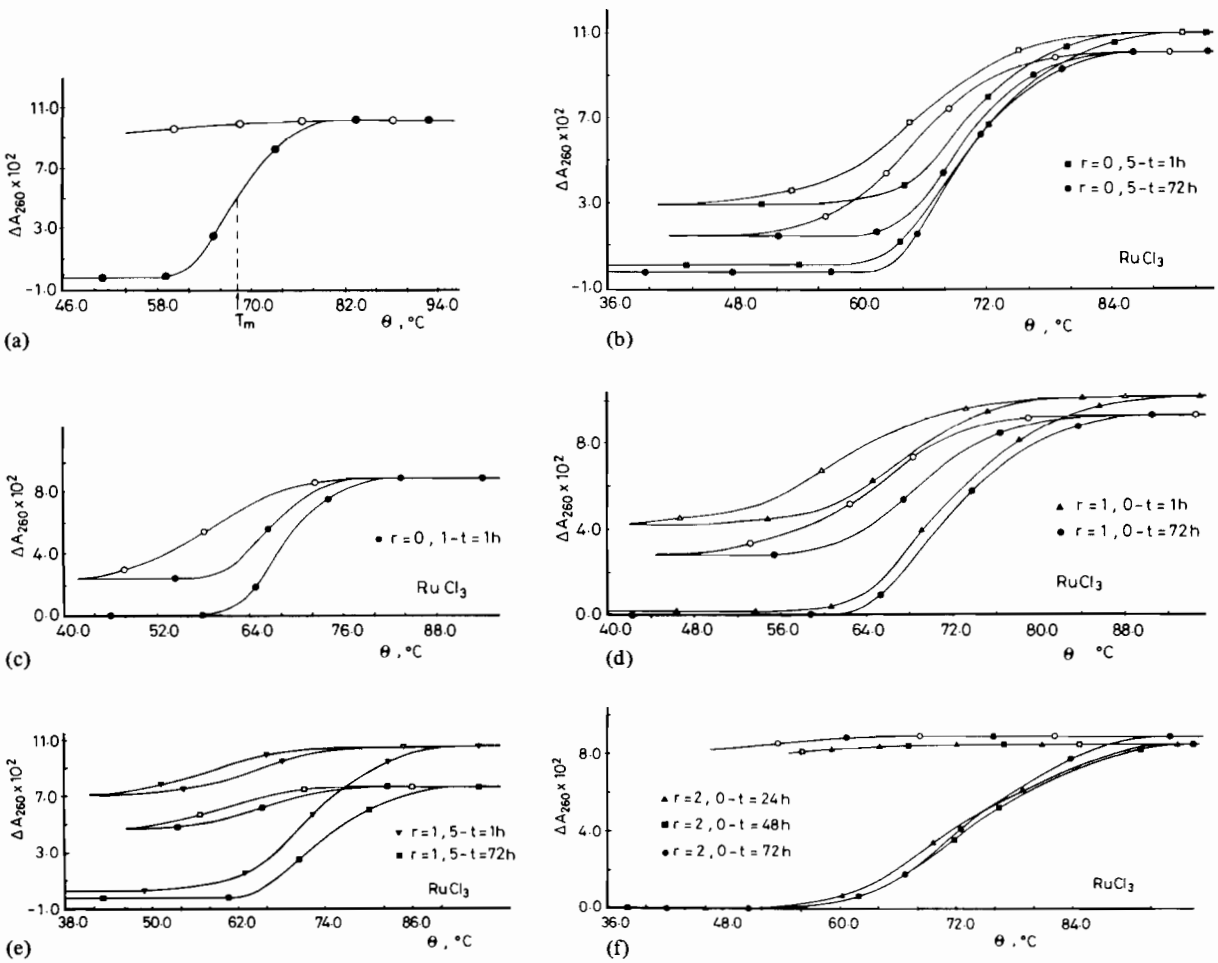

Fig. 2. Melting behavior of Ru(III)–DNA solutions. All solutions contained 5×10^{-5} M(P) DNA, 1×10^{-2} M NaCl and Ru(III) in mole ratios to DNA shown. Absorbances measured at 260 mμ. ●, Closed symbols are found on heating curves; ○, open symbols are found on cooling curves.

TABLE II. Melting Temperatures of DNA–$RuCl_3$ Solutions

r = 0.1		r = 0.5		r = 1.0		r = 1.5		r = 2.0	
t (h)	T_m (°C)	t (h)	T_m (°C)	t (h)	T_m (°C)	t (h)	T_m (°C)	t (h)	T_m (°C)
1	1st 68.0	1	1st 70.3	1	1st 71.4	1	1st 72.0	1	–
	2nd 68.0		2nd 70.3		2nd 67.7		2nd 64.6		
		24	1st 69.9	24	1st 72.2	24	1st 73.1	24	1st 73.0
			2nd 69.2		2nd 68.8		2nd 65.8		
		48	1st 70.2	48	1st 70.7	48	1st 72.4	48	1st 73.7
			2nd 69.8		2nd 67.6		2nd 65.0		
		72	1st 69.6	72	1st 71.9	72	1st 73.5	72	1st 73.9
			2nd 69.8		2nd 69.0		2nd 65.2		

temperatures, T_m, of Ru(III)–DNA solutions are increasing during the first heating with increasing concentration of the metal ion and in all cases are higher than the T_m of DNA alone. Thus, ruthenium-(III) ions behave in the manner to be expected for metal ions that bind exclusively to phosphate [7]. The negative charges on the phosphate groups of native DNA repel each other and tend to unwind the molecule unless counterions are present. The more such counterions there are, the lower the

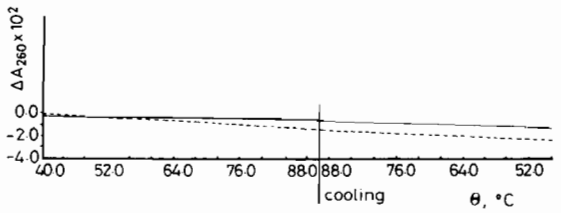

Fig. 3. Effect of temperature on $RuCl_3$ solutions at 260 nm. ———, Ru^{3+} 2.5×10^{-5} M, NaCl 1.0×10^{-2} M; ------, Ru^{3+} 1.0×10^{-4} M, NaCl 1.0×10^{-2} M.

tendency to unwind. Thus, the higher the ruthenium(III) concentration, the higher the melting temperature of DNA.

Figure 4 shows the change in T_m with increasing concentration of Ru(III) during the first heating at various times. We observe that in all cases equilibrium is attained rather quickly. This conclusion is also supported from the electronic spectra of these solutions at room temperature.

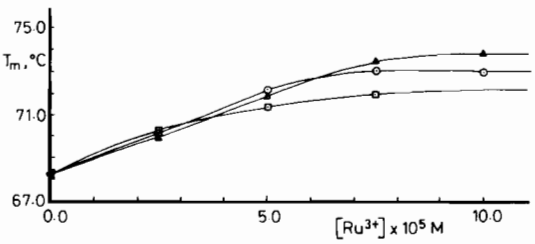

Fig. 4. T_m dependence as a function of metal ion concentration at various times, during the first heating. ▫ t = 1 h, ○ t = 24 h, △ t = 72 h.

From the cooling curves of Figs. 2b–2f we observe a decrease in absorbance depending on the r value. The decrease in absorbance is greater for small values of r. At high r values the decrease in absorbance of the cooling curves is small, and for $r = 2$ the decrease becomes minimal.

Eichorn *et al.* [11] observed that Co(II) and Ni(II) increase the T_m with increasing metal ion concentration, and the absorbance decreases for high values of r on cooling. It was suggested that Co(II) and Ni(II) interact with the phosphates for small values of r; however, at high r values Co(II) and Ni(II) also interact with the bases. Thus, the addition of sufficient Co(II) or Ni(II) allows some of the metal to hold complementary bases in reserve during heating, so that rewinding can occur on cooling. The melting behavior of Zn(II) and Mn(II) suggested that these ions bind to DNA both from the phosphate and from the bases. Thus, Zn(II) and Mn(II) are capable of base interaction to such an extent that a large proportion of double-stranded DNA appears to be regenerated on cooling.

From the melting curves, Figs. 2b–2f, we conclude that Ru(III) is interacting with the phosphate at room temperatures; however, at elevated temperatures it is also interacting with the bases of the DNA. The phosphate interaction is dominant in all cases since the T_m is increasing for all values of r. However, for small values of r a portion of the metal ion during denaturation holds the two chains in close proximity so that the double helix is regenerated on cooling. This phenomenon is similar to that observed for Co(II) and Ni(II), although there it was observed for high r values as opposed to our case in which this was observed for small values of r. Also, for Cu(II) and *cis*-Pt(II) the interaction of these metals with the bases of DNA starts at about 37 °C; for Ru(III), however, this interaction becomes apparent at much higher temperatures, most probably when most of the DNA is close to melting. These conclusions were also supported from the electronic spectra of Ru(III)–DNA solutions, which did not show any drastic changes when they were heated up to 40 °C. Thus, it seems to be a preferential binding of Ru(III) to the bases of single-stranded DNA *versus* double-stranded DNA; steric reasons most probably induce this preferential binding. For small values of r the metal ion concentration is enough to hold the two strands in close proximity and leave enough positions of the bases free to cause rewinding on cooling. Ru(III) ions interact strongly with the bases so that the bonds remain intact on cooling. These conclusions are strongly supported from the electronic spectra of Ru(III)–DNA solutions that had been previously melted and then cooled to room temperature. Thus, in these cases interstrand crosslinking is proposed to be primarily responsible for the renaturation procedure. Also, an amount of the metal ion that is bound to phosphate is acting cooperatively on cooling in a manner similar to the addition of solid electrolyte observed for the rewinding of DNA in the presence of Cu(II) and Cd(II) [3].

In Table III is presented the percentage of renaturation of DNA as was calculated from the relative hypochromicity of the solutions on cooling [23]. We observe that the percentage of renaturation is increasing with time for a certain r value. This may be due to increased interaction of the metal ion

TABLE III. Percentage Renaturation of DNA–$RuCl_3$ Solutions

t (h)	r				
	0.1	0.5	1.0	1.5	2.0
1	78	73	60	33	
24		76	63	34	
48		85	65	35	
72		86	70	37	

with the phosphate groups, thus leaving a greater number of free bases for renaturation. This is in accordance with our previous statement that Ru(III) ions are preferentially bound to phosphates.

From Figs. 2b–2f we observe that at high metal ion concentrations ($r = 1$, 1.5, 2) renaturation is reduced for steric reasons since quite a few positions of the bases are now blocked from ruthenium(III) ions, forming intrastrand cross-links. Pascoe and Roberts [36] demonstrated that only 1 out of every 400 reactions of *cis*-Pt(II) to HeLa cell DNA was a cross-link, and only 1 out of every 4000 reactions with *trans* Pt(II) was a cross-link. Thus, it is reasonable to assume that the single-stranded reactions of Ru(III) with the DNA bases dominate over cross-linking reactions. Renaturation at high r values is now inhibited because of the increased probability that the reaction with the opposite strand would be blocked due to the presence of another monofunctionally-bound ruthenium(III) molecule at that site.

In Table II and in Figs. 2b–2f we observe that the T_m values during the second heating coincide with those of the first heating only for small values of r. In Fig. 5 is presented the change in T_m with increasing metal ion concentration during the second heating. The T_m is decreasing with increasing r value. This can be explained by the fact that during the second heating the cross-linked Ru(III)–DNA facilitates the interaction of Ru(III) ions with the bases of the DNA, thus decreasing the T_m with increasing r values.

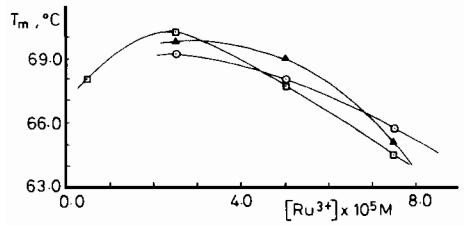

Fig. 5. T_m dependence as a functin of metal ion concentration at various times, during the second heating. □ $t = 1$ h, ○ $t = 24$ h, △ $t = 72$ h.

In conclusion, Ru(III) is interacting with the phosphate moieties of DNA and only at denaturation temperatures starts to interact with the nitrogen of the bases forming interstrand cross-links responsible for the renaturation process. However, this occurs only for small values of r. Steric hindrance probably inhibits the renaturation procedure for high r values. The interstrand cross-links persist even at room temperature and are primarily responsible for the decrease in melting temperatures during the second heating.

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