Binding of Divalent Metal Ions to Synthetic Double-stranded Polyribonucleotides

Günther Strunk and Hartmut Diebler*
Max-Planck-Institut für Biophysikalische Chemie, 37077 Göttingen-Nikolausberg, Germany

The equilibria for the binding of Mg^{2+}, Ni^{2+} and Co^{2+} to the synthetic double-stranded polyribonucleotides poly(A)·poly(U), poly(I)·poly(C) and poly(G)·poly(C) have been investigated by UV spectrophotometry and (in part) by a metal-ion indicator technique. At ionic strength 0.1 mol dm^{-3} the apparent binding constants of Mg^{2+} are close to 2×10^3 dm^3 mol^{-1} in all cases; those for Ni^{2+} and Co^{2+} are 4–40 fold higher, depending on the type of purine base involved. The binding constants to poly(A)·poly(U) are similar to those for the extensively stacked single-stranded poly(A). Kinetic studies indicated that Mg^{2+} is bound mainly by electrostatic interactions (*i.e.* outer sphere) to the polynucleotides, whereas in the case of Ni^{2+} inner-sphere co-ordination (site binding) also occurs. The inner-sphere substitution at Ni^{2+}(aq) is reflected by reaction effects in the millisecond range, detected by stopped-flow techniques. The kinetic data can be rationalized only by assuming (at least) two successive inner-sphere binding steps, presumably due to co-ordination to N^7 of the purine bases and to a phosphate oxygen atom.

The interactions of divalent metal ions with mono- and polynucleotides are known to play an eminent role in a multitude of biological processes.[1] For a full understanding of these processes it is essential to know about the nature of the interactions between metal ions and nucleotides in aqueous solution. In previous investigations we have used spectrophotometric and kinetic techniques in order to learn about the strength and mode of binding of divalent metal ions to mononucleotides[2-4] and to single-stranded polyribonucleotides.[5-7] In the present paper we report on the interactions of Mg^{2+}, Ni^{2+} and Co^{2+} with the synthetic double-stranded polyribonucleotides poly(A)·poly(U), poly(I)·poly(C) and poly(G)·poly(C).†

Experimental

Materials.—All substances were of the highest quality commercially available and used without further purification. Stock solutions (1×10^{-3}–1×10^{-2} mol dm^{-3}) of poly(A)·poly(U), poly(I)·poly(C) (both Pharmacia) and poly(G)·poly(C) (Sigma) were prepared by dissolving the solid material in aqueous solutions of 0.1 mol dm^{-3} NaCl + 0.001 mol dm^{-3} cacodylate [=dimethylarsinate, $(CH_3)_2AsO_2^-$], pH 6.5–7.0. They were kept in the dark at −30 °C and checked for changes in absorption spectra prior to each experiment. The polynucleotide solutions were standardized spectrophotometrically, using literature absorption coefficients[8] or from the 'Certificate of Analysis' [poly(I)·poly(C)]. Stock solutions of Mg^{2+}, Ni^{2+} and Co^{2+} were standardized by complexometric titrations with ethylenediaminetetraacetate (edta),[9] using eriochrome black T as a metal-ion indicator for Mg^{2+}, and murexide [5-(hexahydro-2,4,6-trioxopyrimidin-5-ylimino)pyrimidine-2,4,6-trionate] for Ni^{2+} and Co^{2+}. The edta solution had been standardized by means of a Merck 'Titrisol' standard solution of Mg^{2+}. All solutions were prepared with water doubly distilled from a quartz apparatus.

Methods.—Most experiments were carried out at 25.0 ± 0.1 °C, ionic strength, I = 0.10 or 0.010 mol dm^{-3}, and pH 6.5–7.0 (during the titrations I increased by 1–4% in the cases of Ni^{2+} and Co^{2+}, and by 8–10% in the case of Mg^{2+}). Under these conditions the double-stranded forms of the polyribonucleotides considered here are stable[10] and are far away from any protolytic pK.[8] Measurements of pH were made using a Radiometer PHM 52 digital pH-meter equipped with a Metrohm EA 125 combined electrode. For measurements in solutions containing perchlorate the KCl solution in the reference compartment of the electrode was replaced by a NaCl solution. The pH-meter was calibrated using Merck 'Titrisol' buffer solutions. The pH of all solutions was stabilized by adding 0.001 mol dm^{-3} cacodylate buffer. At this concentration the buffer components do not interact noticeably with divalent metal ions.[2]

The binding equilibria were investigated spectrophotometrically using a Cary 118 or a computer-supported Perkin-Elmer Lambda 17 spectrophotometer. Since the spectral changes in the UV region (absorption by the nucleotide bases) upon the addition of divalent metal ions are generally small (1.5–6%), most of the spectrophotometric titrations were performed in the difference mode, *i.e.* with two cuvettes in each path, measuring (nucleotide + M^{2+}) (water) *vs.* (nucleotide + water) (M^{2+}) at a high sensitivity of the instrument (0.02–0.05 full absorption unit scale). This technique compensates for the dilution of the nucleotide solution and for the (small) absorption due to the metal ions. The extent of the dilution was kept low by adding μl amounts of a concentrated M^{2+} solution to a 2.5 cm^3 initial volume of the nucleotide solution, applying a micrometer syringe unit. When necessary, the small volume changes were corrected for in the evaluation.

Additional spectrophotometric measurements were performed in the visible range, using murexide as an indicator of the concentration of free Ni^{2+} ions. Spectrophotometric titrations of murexide with Ni^{2+} produce very strong changes in the absorbancy near 520 nm,[6] and in the absence of polynucleotide enable the determination of the apparent equilibrium constant for the binding of Ni^{2+} to murexide. Analogous titrations performed in the presence of polynucleotide allow then the determination of the concentrations of free and murexide-bound Ni^{II}. The difference from the total amount of Ni^{2+} added yields the concentration of Ni^{2+} which is bound to the polynucleotide. The details of this technique have been described before.[5]

† Poly(A) = polyadenylate, poly(U) = polyuridylate, poly(I) = polyinosylate, poly(C) = polycytidylate and poly(G) = polyguanylate.

Kinetic studies of the M^{2+}–polynucleotide systems have been done by applying stopped-flow and temperature-jump relaxation methods (conventional and cable T-jump). For the stopped-flow experiments an apparatus was used which had been built at our institute and which is characterized by a dead-time of 1.3 ms. The conventional T-jump instrument[11] has a heating time of about 5 μs at $I = 0.1$ mol dm^{-3}, and for the cable T-jump apparatus[12] the lower time limit was about 100 ns under our conditions. In all cases the reaction was followed spectrophotometrically. The reaction signal was stored in a transient recorder (Biomation 1010 or Tektronix 7612D), displayed on a screen, and then transferred to an IBM 3090 computer for evaluation.

Results and Discussion

Equilibrium Studies.—Poly(A)·poly(U) + Ni^{2+}. Difference spectra of a titration of poly(A)·poly(U) with Ni^{2+} are shown in Fig. 1. The maximum dilution due to the addition of the Ni^{2+} solution was 2%. The largest changes in absorbancy are observed at 249 nm and amount to $\Delta A = 0.02$. The changes in absorbancy at 250 nm (read digitally) as a function of the concentration of total Ni^{2+} are plotted in Fig. 2. The shape of this curve is similar to one for a simple binding process, as has been observed before also with several other M^{2+} + polynucleotide systems.[6,7,13] Assuming a simple binding equilibrium of the type expressed by equation (1), where S

$$K = [NiS]/[Ni^{2+}][S] \quad (1)$$

denotes a binding site, the relationship between the changes in absorbancy and the metal-ion concentration is given by equation (2) if the Hildebrand–Benesi approximation[14] applies,

$$\frac{[Ni^{2+}]_{tot}}{\Delta A} = \frac{1}{K \cdot \Delta A_\infty} + \frac{[Ni^{2+}]_{tot}}{\Delta A_\infty} \quad (2)$$

i.e. $[NiS]^2 \ll [Ni^{2+}]_{tot}[S]_{tot}$ (the subscript tot denotes total concentration), and $[S]_{tot} \ll [Ni^{2+}]_{tot}$. At our concentrations these conditions are fulfilled to a good approximation ($[S]_{tot}$ is usually considerably smaller than the concentration of momomeric nucleotide units[5,7,13]) and a plot of the left-hand side of equation (2) *vs.* $[Ni^{2+}]_{tot}$ yields a good straight line (not shown) which gives (at 250 nm) $\Delta A_\infty = 0.0247$ (*i.e.* $\Delta A_\infty/A = 0.033$) and $K = 8.35 \times 10^3$ dm^3 mol^{-1} (best-fit values). Alternatively, equation (3) may be used for the evaluation.[6]

$$\frac{\Delta A_\infty}{\Delta A_\infty - \Delta A} = K\frac{\Delta A_\infty}{\Delta A}[Ni^{2+}]_{tot} - K[S]_{tot} \quad (3)$$

This equation does not depend on any approximation but requires knowledge of ΔA_∞. Accepting the value of ΔA_∞ given above, a plot according to equation (3) is shown in Fig. 3. From the slope, $K = 8.51 \times 10^3$ dm^3 mol^{-1}. The intercept is too small for a determination of $[S]_{tot}$. The mean value of six titrations was found to be $K = (8.6 \pm 0.8) \times 10^3$ dm^3 mol^{-1} at 25 °C, $I = 0.1$ mol dm^{-3}, pH 6.6. Another titration with a 1 mol dm^{-3} solution of Ni^{2+} confirmed that the absorbancy remains practically constant at high concentrations of Ni^{2+}, 4×10^{-3}–6×10^{-2} mol dm^{-3} (limiting value ΔA_∞).

Analogous measurements have been carried out at $I = 0.01$ mol dm^{-3} (0.009 mol dm^{-3} $NaClO_4$ + 0.001 mol dm^{-3} sodium cacodylate), pH 7.0, with [poly(A)·poly(U)] = 9.33×10^{-5} mol dm^{-3} and $[Ni^{2+}]_{tot} = 4 \times 10^{-6}$–$1.3 \times 10^{-4}$ mol dm^{-3}. Now the condition $[Ni^{2+}]_{tot} \gg [S]_{tot}$ is no longer fulfilled; therefore equation (3) had to be used to evaluate the data. Two titrations gave $K = (8 \pm 2) \times 10^4$ dm^3 mol^{-1} at 25 °C, $I = 0.01$ mol dm^{-3}, pH 7.0. At the low ionic strength the uncertainty in K is large since an evaluation according to equation (3) is rather sensitive to the value of ΔA_∞, which is difficult to establish exactly.

The UV spectral changes which are observed during these titrations are in part due to changes in the conformation of the

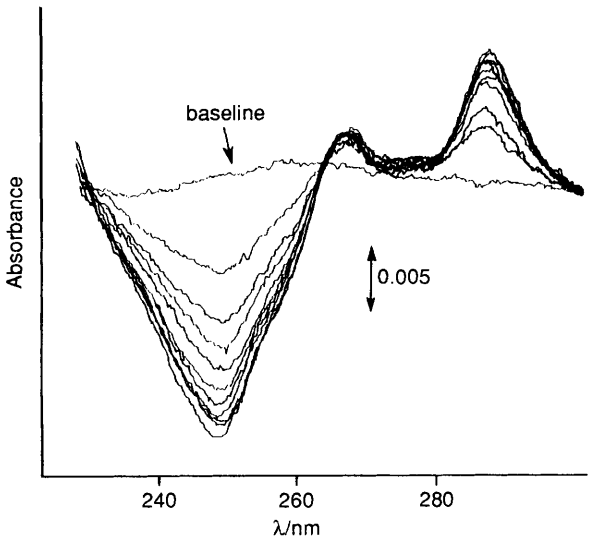

Fig. 1 Difference spectra of poly(A)·poly(U) *vs.* poly(A)·poly(U) + Ni^{2+}; [poly(A)·poly(U)] = 6.4×10^{-5} mol dm^{-3} (in base pairs), $[Ni^{2+}]_{tot} = 0.47 \times 10^{-4}$–$4.65 \times 10^{-4}$ mol dm^{-3}, 25 °C, 0.1 mol dm^{-3} $NaClO_4$, pH 6.6

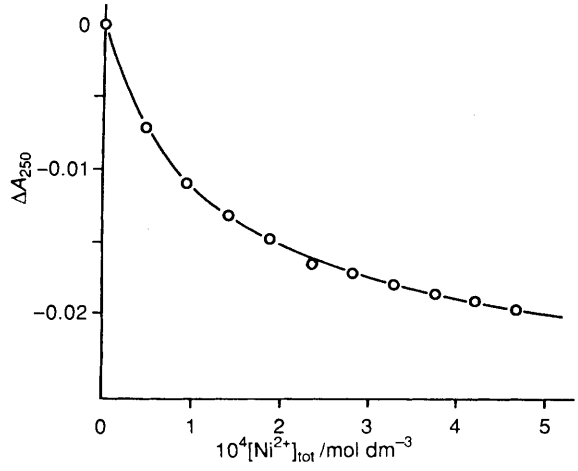

Fig. 2 Plot of the changes in absorbancy at 250 nm *vs.* the total nickel(II) concentration (same data as in Fig. 1)

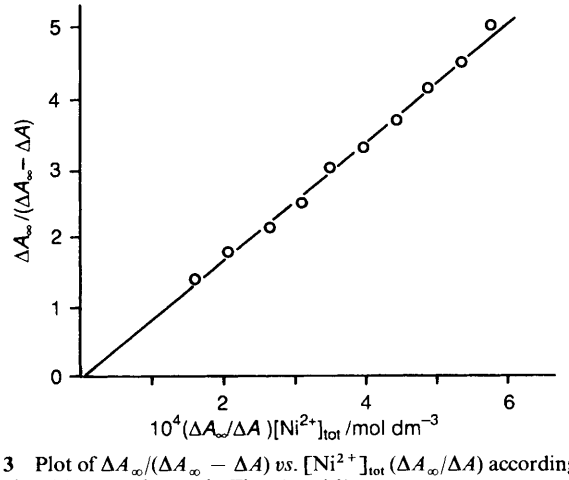

Fig. 3 Plot of $\Delta A_\infty/(\Delta A_\infty - \Delta A)$ *vs.* $[Ni^{2+}]_{tot}$ $(\Delta A_\infty/\Delta A)$ according to equation (3) (same data as in Figs. 1 and 2)

polynucleotide and in part to the binding of Ni^{2+} to the purine base. The UV spectrophotometric data can therefore be interpreted only in terms of an 'apparent' binding constant.[7] A second technique for a quantitative description of the binding process is based on making use of a metal-ion indicator, for instance murexide in the case of Ni^{2+}. The stability constant of the Ni^{2+}–murexide complex was taken from a recent study by O'Mara *et al.*[15] Using this indicator, spectrophotometric measurements in the visible range (near 520 nm) enable the determination of the concentrations of free and murexide-bound Ni^{2+}. The difference from the total amount of Ni^{2+} added is considered to be the concentration of Ni^{2+} which is bound to the polynucleotide. The results of these measurements have been evaluated in terms of the Scatchard equation[16] (4),

$$n/[Ni^{2+}]_{free} = Km - Kn \quad (4)$$

where n denotes the number of Ni^{2+} ions bound per monomeric unit (here per base pair) and m the number of binding sites per monomer (base pair). Plots of the left-hand side of equation (4) vs. n yielded reasonable straight lines, see Fig. 4. The linearity demonstrates that (within the concentration range considered) the process can be described as the binding of Ni^{2+} to one class of independent binding sites. From 10 titrations, with nucleotide concentrations (in base pairs) from 0.9×10^{-3} to 1.9×10^{-3} mol dm^{-3}, the following values have been obtained: $m = 0.22 \pm 0.04$ and $K = (1.1 \pm 0.3) \times 10^4$ dm^3 mol^{-1} at 25 °C, $I = 0.1$ mol dm^{-3}, pH 7.0.

Poly(A)·poly(U) + Mg^{2+}. The UV difference spectra which are observed when poly(A)·poly(U) is titrated with Mg^{2+} exhibit a negative maximum near 274 nm and are clearly different from those obtained with Ni^{2+}. At low ionic strength, $I = 0.01$ mol dm^{-3} $NaClO_4$, the spectrophotometric titration of 1.02×10^{-4} mol dm^{-3} poly(A)·poly(U) with Mg^{2+} (1.5×10^{-5}–2.8×10^{-4} mol dm^{-3}) yields ΔA_{273} values which are consistent with a simple binding equilibrium [equation (1)], *i.e.* plots according to equations (2) and (3) give straight lines ($\Delta A_\infty = 0.022$ and $\Delta A_\infty/A = 0.025$). Evaluation by means of equation (3) leads to $K = (1.3 \pm 0.1) \times 10^4$ dm^3 mol^{-1} at 25 °C, $I = 0.01$ mol dm^{-3}, pH 7.0 and $m \leqslant 0.2$.

At $I = 0.1$ mol dm^{-3} ($NaClO_4$) the situation is more complicated. The addition of Mg^{2+} gives rise to an initial decrease in absorbancy (273 nm) but does not lead to a limiting value. Instead, the absorbancy continues to fall gradually, until about $[Mg^{2+}] = 0.02$ mol dm^{-3}, and then it starts to increase drastically, until about $[Mg^{2+}] = 0.05$ mol dm^{-3}. After standing overnight, a precipitate formed. The initial section of the ΔA *vs.* $[Mg^{2+}]$ curve does not permit one to evaluate an accurate value of the binding constant, since it cannot be established when the other processes start to interfere with the binding reaction, *i.e.* ΔA_∞ cannot be determined. Limiting-case considerations lead to the conclusion that at $I = 0.1$ mol dm^{-3} the value of K lies between 1×10^{-3} and 3×10^{-3} dm^3 mol^{-1}, say $(2 \pm 1) \times 10^3$ dm^3 mol^{-1}.

Analogous measurements were done also with the other polynucleotides. These systems may be characterized by the following short remarks.

Poly(I)·poly(C) + Mg^{2+}. The difference spectra exhibit negative maxima at 277 and 252 nm. The spectral changes are small; for instance, $\Delta A_\infty/A = 0.017$ at 277 nm, $I = 0.1$ mol dm^{-3}.

Poly(I)·poly(C) + Ni^{2+}. The spectral changes are appreciably larger than in the titrations with Mg^{2+}, see Fig. 5. Now $\Delta A_\infty/A = 0.05$ at 277 nm, $I = 0.1$ mol dm^{-3}. At high concentrations of Ni^{2+} about 0.004–0.04 mol dm^{-3}, ΔA remains essentially constant ($= \Delta A_\infty$). At low ionic strength, 0.01 mol dm^{-3}, the experimental data do not fit well to equation (3). Apparently the assumption of a simple binding equilibrium is not really correct under these conditions. Thus only approximate figures for the binding constants with Ni^{2+} and Co^{2+} can be given.

Poly(G)·poly(C) + Mg^{2+}. The difference spectra show a negative maximum at 252 nm and a small positive maximum at 294 nm. The spectral changes are small, $\Delta A_\infty/A = 0.016$ at 252 nm, $I = 0.1$ mol dm^{-3}. At high concentrations of Mg^{2+}, 0.01–0.09 mol dm^{-3}, ΔA remained constant ($= \Delta A_\infty$).

Poly(G)·poly(C) + Ni^{2+}. The spectral changes are much more pronounced than with Mg^{2+}, now $\Delta A_\infty/A = 0.065$ at 252 nm, $I = 0.1$ mol dm^{-3}.

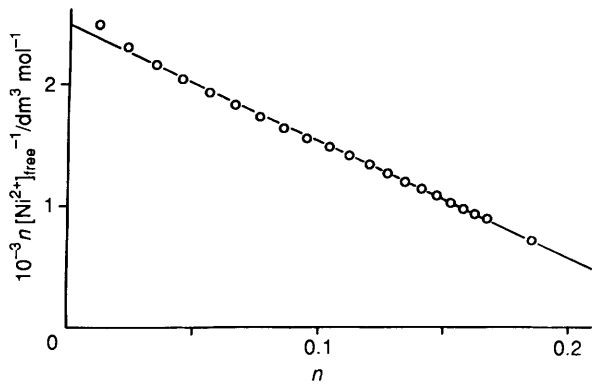

Fig. 4 Plot of $n/[Ni^{2+}]_{free}$ *vs.* n according to equation (4); [poly(A)·poly(U)] = 0.91×10^{-3} mol dm^{-3}, $[Ni^{2+}]_{tot} = 2 \times 10^{-5}$–$4.7 \times 10^{-4}$ mol dm^{-3}, 25 °C, 0.1 mol dm^{-3} KNO_3, pH 7.0

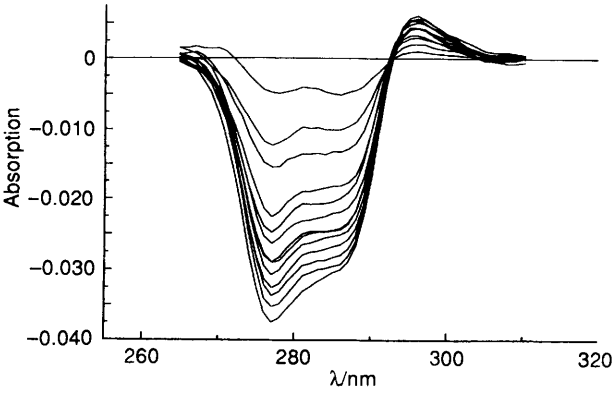

Fig. 5 Difference spectra of poly(I)·poly(C) *vs.* poly(I)·poly(C) + Ni^{2+}; [poly(I)·poly(C)] = 6.2×10^{-5} mol dm^{-3} (in base pairs), $[Ni^{2+}]_{tot} = 1.1 \times 10^{-5}$–$3.8 \times 10^{-4}$ mol dm^{-3}, 25 °C, 0.1 mol dm^{-3} $NaClO_4$, pH 7.0

It is remarkable that nearly all of the experimental data, obtained by the UV or the murexide technique, can be fitted well by equations which have been derived assuming that there is only one class of non-interacting binding sites, see Figs. 3 and 4. Such a simple two-state model would not be expected *a priori* to be applicable to a polyanion, but was found to be a suitable description also for several other metal ion–polynucleotide systems.[5–7,13,17] It does not appear to be reasonable that there should be $[S]_{tot} = m[poly(N)]_{tot}$ binding sites, with $m = 0.2$–0.3 at the beginning of the binding process (as follows from the Scatchard evaluation and also from our evaluation of the UV data). A value of m close to '1' when the first metal ions are being bound, as obtained by the refined binding model of McGhee and von Hippel,[18] would be more realistic. However, this treatment cannot be applied to our UV data (the binding density is not known) and so we will stay with the 'apparent' binding constants[7] which result from equations (2)–(4). A summary of the constants which have been derived during this study is presented in Table 1.

At $I = 0.1$ mol dm^{-3} the constant for the binding of Mg^{2+} to each of the three polynucleotides is near 2×10^3 dm^3 mol^{-1}, with considerable uncertainty (experimental problems, aggregation and precipitation) in the case of poly(A)·poly(U). Since kinetic effects in the time range expected for inner-sphere substitution at Mg^{II} could not be detected (see below), we

Table 1 Apparent binding constants K (in $dm^3\ mol^{-1}$) and number m of binding sites per base pair for the binding of Mg^{2+}, Ni^{2+} and (in part) Co^{2+} to various double-stranded polynucleotides (25 °C, pH *ca.* 7.0)

Polynucleotide	M^{2+}	K	m*	I/mol dm^{-3}	Method
Poly(A)·poly(U)	Mg^{2+}	*ca.* $(2 \pm 1) \times 10^3$		0.10	UV
	Mg^{2+}	$(1.3 \pm 0.1) \times 10^4$	≤0.2	0.010	UV
	Ni^{2+}	$(8.6 \pm 0.8) \times 10^3$		0.10	UV
	Ni^{2+}	$(8 \pm 2) \times 10^4$		0.010	UV
	Ni^{2+}	$(1.1 \pm 0.3) \times 10^4$	0.22	0.10	Murexide
Poly(I)·poly(C)	Mg^{2+}	$(2.0 \pm 0.3) \times 10^3$		0.10	UV
	Mg^{2+}	$(3.5 \pm 1) \times 10^4$	0.18	0.010	UV
	Ni^{2+}	$(2.2 \pm 0.3) \times 10^4$		0.10	UV
	Ni^{2+}	*ca.* 1×10^5		0.010	UV
	Co^{2+}	*ca.* 1×10^5		0.010	UV
	Ni^{2+}	$(4.6 \pm 0.3) \times 10^4$	0.28	0.10	Murexide
Poly(G)·poly(C)	Mg^{2+}	$(2.1 \pm 0.2) \times 10^3$		0.10	UV
	Mg^{2+}	$(1.9 \pm 0.2) \times 10^4$		0.010	UV
	Ni^{2+}	$(8.8 \pm 1.2) \times 10^4$	0.18	0.10	UV
	Ni^{2+}	*ca.* 2×10^5		0.010	UV
	Co^{2+}	*ca.* $(2–3) \times 10^5$		0.010	UV
	Ni^{2+}	$(1.8 \pm 0.3) \times 10^5$	0.30	0.10	Murexide

* Uncertainty about ±15%.

conclude that most of the Mg^{2+} is bound by electrostatic interactions only (mobile cloud, outer-sphere binding), as has been found also with double-stranded poly(dG-dC)[17] and with single-stranded polyribonucleotides.[6,13] The conformations of the three double strands are similar, and so the binding constants (for Mg^{2+}) are almost the same, contrary to the single-stranded polynucleotides.[6] The ions Ni^{2+} and Co^{2+} bind more strongly than Mg^{2+} to all three double strands, indicating partial inner-sphere binding. This binding mode presumably involves co-ordination to the purine bases, with N^7 of A, I and G being the preferred binding site.[19] The base-bound transition-metal ion may in addition co-ordinate to a second site, *e.g.* a phosphate O atom.[20] It has been demonstrated that the N^7 atoms of I and G are better metal-ion binding sites than that of A.[4,21] Also, the metal-ion binding ability of the N^7 atom is directly related to the pK for its protonation which increases from A to G, *i.e.* $pK_{(A)} < pK_{(I)} < pK_{(G)}$ (evaluated for the nucleotides), according to Sigel and co-workers.[22] These differences are reflected also in our binding constant for Ni^{2+} (Table 1) which rises from poly(A)·poly(U) to poly(I)·poly(C) to poly(G)·poly(C) by a factor of 10 at $I = 0.1$ mol dm^{-3}. At $I = 0.01$ mol dm^{-3} the binding constants (according to the UV method) are higher than at $I = 0.1$ mol dm^{-3}, often by about one order of magnitude, sometimes somewhat less. Of particular interest is a comparison of the constants for binding of Ni^{2+} which have been obtained by UV spectrophotometry and by the metal-ion indicator (murexide) technique. For poly(A)·poly(U) the two constants are fairly similar [this was the case also with single-stranded poly(A)[6]]. For poly(I)·poly(C) and poly(G)·poly(C) the two methods yield values which differ by about a factor of 2. These differences are outside the experimental uncertainties; presumably they reflect deficiencies in the methodology. Consider the murexide technique: here we determine the amount of bound metal as a function of the free metal-ion concentration. The value of the binding constant depends on what we consider to be a binding site. The UV spectrophotometric data, too, are not easily rationalized. The changes in absorbancy may in part be due to direct co-ordination of the metal ion to the nucleotide base (if inner-sphere binding occurs) and in part to conformational changes of the polynucleotide, induced by its interactions with the metal ions. Both contributions may not show the same dependence on the metal-ion concentration.

Nevertheless, the data of Table 1, obtained by UV spectrophotometry, are in reasonable agreement with several other literature values, which have been determined by applying other techniques and which refer to a different ionic strength: potentiometric measurements yielded for the binding of Mg^{2+} to poly(A)·poly(U) a constant $K = 6.3 \times 10^3$ $dm^3\ mol^{-1}$ in 0.005 mol dm^{-3} sodium phosphate + 0.01 mol dm^{-3} Na^+ as counter ion; practically the same value was obtained also for Mg^{2+} + poly(I)·poly(C).[23] Again for Mg^{2+} + poly(A)·poly(U), using eriochrome black T as a metal-ion indicator for Mg^{2+},[24] a binding constant $K = 4 \times 10^3$ $dm^3\ mol^{-1}$ in 0.029 mol dm^{-3} Na^+ was derived.[25] Furthermore, the constants for Ni^{2+} and Co^{2+} given in Table 1 are of a similar magnitude like many of those which have been reported for the binding of divalent first-row transition-metal ions (and Zn^{2+}) to double-stranded polynucleotides, see the compilation in ref. 26.

Finally, it should be noted that the binding constants ($I = 0.1$ mol dm^{-3}) for Mg^{2+} to the three double-stranded polynucleotides considered here are practically the same as that for Mg^{2+} + single-stranded (but strongly stacked) poly(A); those with poly(C), weaker stacking, and poly(U), unstacked, are considerably lower.[6] Nickel(II), too, binds to poly(A)·poly(U) and poly(A) with the same constant. Previously it had been reported that the constants for the binding of Mg^{2+} to poly(A), poly(A)·poly(U) and calf thymus DNA are nearly the same, (6–6.3) $\times 10^3$ $dm^3\ mol^{-1}$ (at a lower ionic strength).[23] Another source gave $K = 3 \times 10^3$ $dm^3\ mol^{-1}$ for Mg^{2+} + poly(A) and 4×10^3 $dm^3\ mol^{-1}$ for Mg^{2+} + poly(A)·poly(U) in 0.029 mol dm^{-3} Na^+.[25] Thus it is apparent that the electrostatic binding of divalent metal ions to the single-stranded but extensively stacked poly(A) is just as strong as to double-stranded polyribonucleotides.

Kinetic Studies.—Measurements of the kinetics of the metal-ion binding reactions have been carried out initially by temperature-jump relaxation techniques (conventional and cable T-jump). The experimental conditions were similar to those in the equilibrium studies: [polynucleotide] = 4×10^{-5}–15×10^{-5} mol dm^{-3}, $I = 0.1$ mol dm^{-3} $NaClO_4$, 25 °C, pH ≈ 7.0. Preliminary experiments in the absence of divalent metal ions revealed that the polynucleotides alone display relaxation effects. Following T-jumps of $\Delta T = 3.0$ °C, small changes in the transmitted light intensity (< 1%) were observed between 265 and 275 nm. Their temporal variation could be fitted by two time constants, $\tau_1 = 16 \pm 3$, 20 ± 3 and < 50 μs for poly(A)·poly(U), poly(I)·poly(C) and poly(G)·poly(C), respectively, and $\tau_2 = 150 \pm 30$ μs (very small amplitude) for all three polynucleotides. These time constants do not depend on the polynucleotide concentration and are observed also in 0.1 mol dm^{-3} NaCl. Apparently they reflect conformational changes of the double-stranded polynucleotides. A 19 μs effect was found also with poly(dG-dC).[17]

In the experiments done in the presence of divalent metal ions the metal-ion concentrations were adjusted such as to span the transition range for binding of M^{2+}, and the reactions were monitored at wavelengths where the difference spectra showed reasonable amplitudes. For instance, with Mg^{2+} + poly(A)·poly(U) the concentration of Mg^{2+} was varied from 2.5×10^{-4} to 27×10^{-4} mol dm^{-3}, and the reaction was monitored at 276 nm. No additional reaction effects (beyond those mentioned above) were found for the three polynucleotides in the presence of Mg^{2+}. This is not unexpected since previous studies had indicated that Mg^{2+} ions apparently do not co-ordinate in an inner-sphere manner to polynucleotides.[6,13,17] However, also with Ni^{2+}, specific reaction effects which can be assigned to inner-sphere substitution processes at Ni^{2+}(aq) and which are sufficiently pronounced to be evaluated reliably have not been observed, although the equilibrium data (Table 1) and earlier studies with several other polynucleotides[5–7,17] demonstrate inner-sphere binding of transition-metal ions to purine polynucleotides. It has been observed before (*e.g.* ref. 7) that the T-jump relaxation amplitudes of such binding processes may be rather small, possibly too small in some cases to demonstrate

site binding by this technique. Indeed, by determining the equilibrium constant for the binding of Ni^{2+} to poly(I)·poly(C) at 10 and 40 °C (I = 0.1 mol dm^{-3}), we found values K = 2.5 × 10^4 and 2.3 × 10^4 dm^3 mol^{-1}, respectively, *i.e.* there is hardly any temperature dependence of this binding equilibrium.

Satisfactory reaction curves (which demonstrate inner-sphere binding!) were obtained, however, when Ni^{2+} was mixed with poly(I)·poly(C) or poly(G)·poly(C) in a stopped-flow apparatus, see Fig. 6. For instance, with 9 × 10^{-5} mol dm^{-3} (in base pairs) of poly(I)·poly(C) and $[Ni^{2+}]_{tot}$ = 1 × 10^{-5}–1.1 × 10^{-3} mol dm^{-3}, measurements at 280 nm gave reaction effects with amplitudes from 20 to 60 mV (*i.e.* $\Delta I_c/I_c$ = 4 × 10^{-3}–12 × 10^{-3} where I_c = light intensity). The curves could be fitted well by a single exponential, the time constant of which decreased only slightly with increasing concentration of Ni^{2+}, from τ = 5.7 ms at $[Ni^{2+}]_{tot}$ = 1 × 10^{-5} mol dm^{-3} to 3.1 ms at 1.1 × 10^{-3} mol dm^{-3} (20 °C, 0.1 mol dm^{-3} NaCl, pH 7.0).

With 4.8 × 10^{-5} mol dm^{-3} poly(G)·poly(C) and $[Ni^{2+}]_{tot}$ = 5 × 10^{-6}–2.5 × 10^{-4} mol dm^{-3}, stopped-flow experiments at λ = 250 nm yielded effects of amplitudes 40–125 mV ($\Delta I_c/I_c$ = 8 × 10^{-3}–25 × 10^{-3}), with time constants between 5.2 and 3.6 ms.

Several mechanisms have been considered for an interpretation of the kinetic data. Clearly, a simple two-step mechanism of the form shown in equation (5) is not able to account for the

$$Ni + S \overset{K_o}{\rightleftharpoons} Ni,S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} NiS \qquad (5)$$

experimental findings (Ni,S = outer-sphere state, NiS = inner-sphere complex; charges neglected). If $[Ni^{2+}]_{tot} \geqslant [S]_{tot}$ (fulfilled for $[Ni^{2+}] \geqslant 1 \times 10^{-4}$ mol dm^{-3}), the reciprocal time constant is given by equation (6). The value of K_1 ($=k_1/k_{-1}$)

$$1/\tau = k_{-1}\{1 + K_1K_o[Ni]/(1 + K_o[Ni])\} \qquad (6)$$

can be determined from $K = K_o(1 + K_1)$ if the outer-sphere constant K_o is assumed to be the same as for Mg^{2+} (K_o = 2 × 10^3 dm^3 mol^{-1}). According to equation (6), $1/\tau$ should vary much more over the concentration range considered than is actually observed. As mentioned above, transition-metal ions obviously co-ordinate to the nucleotide bases (N^7 of the purines) *and* to phosphate oxygens of nucleotides. Therefore, a more likely mechanism includes two inner-sphere substitution steps after the fast outer-sphere association, equation (7), where

$$Ni + S \overset{K_o}{\rightleftharpoons} Ni,S \overset{K_1}{\rightleftharpoons} Ni\text{–}S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} Ni{=}S \qquad (7)$$

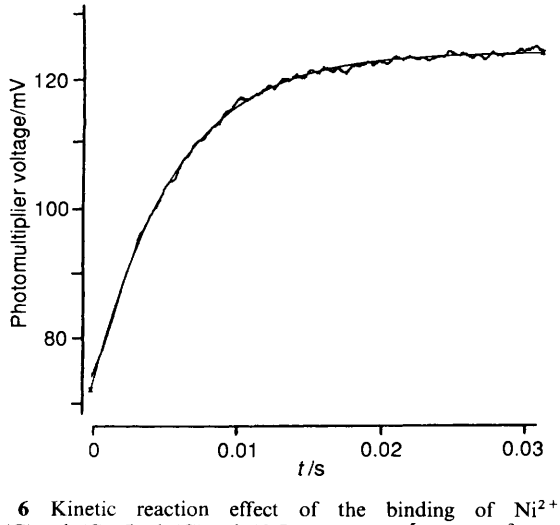

Fig. 6 Kinetic reaction effect of the binding of Ni^{2+} to poly(G)·poly(C); [poly(G)·poly(C)] = 4.8 × 10^{-5} mol dm^{-3} (in base pairs), $[Ni^{2+}]_{tot}$ = 1.0 × 10^{-5} mol dm^{-3}, λ = 250 nm, 20 °C, 0.1 mol dm^{-3} NaCl, pH 7.0

Ni–S and Ni=S denote the mono- and bi-dentate species respectively. The experimental data can be rationalized now by assuming that the last step is rate determining. Then, we obtain equation (8) where $K_2 = k_2/k_{-2}$. If, for Ni^{2+} + poly(G)·poly(C), $K_2 \leqslant 1$ and therefore $K_1 \geqslant 20.5$ {K_1 and K_2 are related by $K = K_o[1 + K_1(1 + K_2)]$}, then the variation of $1/\tau$ with [Ni] according to equation (8) is indeed as low as observed experimentally. Similarly, for Ni^{2+} + poly(I)·poly(C) we conclude that $K_2 \leqslant 2$ and $K_1 \geqslant 3.3$. The value of k_{-2} for both systems is near 180 s^{-1}. Consequently, $k_2 \leqslant 180$ s^{-1} in the case of poly(G)·poly(C) and $\leqslant 360$ s^{-1} for poly(I)·poly(C). These values of k_2 are much lower than those of 'normal' first-order inner-sphere substitution rates at hydrated Ni^{2+}, which are close to 1 × 10^4 s^{-1}.[27] Presumably the polynucleotide has to undergo some change in conformation after the first substitution step before the second site can attach to the Ni^{2+} ion, thus slowing down the second binding step. According to the estimates for the stepwise equilibrium constants given above, the monodentate intermediate is not a steady-state species. Therefore, its formation should also be observable. The first binding step is expected to occur without hindrance, *i.e.* k_1 is near 10^4 s^{-1}, and then the time constant of this step will be below 1 ms, too short to be detected by our stopped-flow apparatus. The conclusion that the recorded reaction is preceded by another step is supported also by the observation that the measured reaction amplitudes are only 25–50% of those calculated from the absorption changes which have been recorded during the equilibrium studies.

$$1/\tau = k_{-2}\{1 + K_2K_1K_o[Ni]/(1 + K_o[Ni] + K_1K_o[Ni])\} \qquad (8)$$

References

1 T. G. Spiro, in *Inorganic Biochemistry*, ed. G. L. Eichhorn, Elsevier, Amsterdam, 1973, vol. 1, p. 549; W. J. O'Sullivan, in *Inorganic Biochemistry*, ed. G. L. Eichhorn, Elsevier, Amsterdam, 1973, vol. 1, p. 582; G. L. Eichhorn, in *Inorganic Biochemistry*, ed. G. L. Eichhorn, Elsevier, Amsterdam, 1973, vol. 2, p. 1210; *Nucleic Acid–Metal Ion Interactions*, ed. T. G. Spiro, Wiley, New York, 1980.
2 R. S. Taylor and H. Diebler, *Bioinorg. Chem.*, 1976, **6**, 247.
3 A. Peguy and H. Diebler, *J. Phys. Chem.*, 1977, **81**, 1355.
4 A. Nagasawa and H. Diebler, *J. Phys. Chem.*, 1981, **85**, 3523.
5 M. J. Hynes and H. Diebler, *Biophys. Chem.*, 1982, **16**, 79.
6 H. Diebler, F. Secco and M. Venturini, *Biophys. Chem.*, 1987, **26**, 197.
7 K. S. Dhathathreyan and H. Diebler, *Ber. Bunsenges. Phys. Chem.*, 1991, **95**, 880.
8 B. Janik, *Physicochemical Characteristics of Oligonucleotides and Polynucleotides*, IFI/Plenum, New York, 1971.
9 *Vogel's Textbook of Quantitative Inorganic Analysis*, 4th edn., Longman, New York, 1978, p. 322.
10 *CRC Handbook of Biochemistry and Molecular Biology*, 3rd edn., CRC Press, Cleveland, OH, 1975, Nucleic Acids, vol. 1, p. 575ff.
11 M. Eigen and L. De Maeyer, in *Technique of Organic Chemistry*, 2nd edn., ed. A. Weissberger, Interscience, New York, 1963, vol. VIII/2, p. 895.
12 G. W. Hoffman, *Rev. Sci. Instrum.*, 1971, **42**, 1643.
13 D. Poerschke, *Biophys. Chem.*, 1976, **4**, 383.
14 See F. J. C. Rossotti and H. Rossotti, *The Determination of Stability Constants*, McGraw-Hill, New York, 1961, p. 276.
15 C. O'Mara, J. Walsh and M. J. Hynes, *Inorg. Chim. Acta*, 1984, **92**, L1.
16 G. Scatchard, *Ann. N.Y. Acad. Sci.*, 1949, **51**, 660.
17 Th. Schoenknecht and H. Diebler, *J. Inorg. Biochem.*, 1993, **50**, 283.
18 J. D. McGhee and P. H. von Hippel, *J. Mol. Biol.*, 1974, **86**, 469.
19 L. G. Marzilli, T. J. Kistenmacher and G. L. Eichhorn, in *Nucleic Acid–Metal Ion Interactions*, ed. T. G. Spiro, Wiley, 1980; D.-K. Chang and D. R. Kearns, *Biopolymers*, 1986, **25**, 1283; S. Adam, P. Bourtayre, J. Liquier and E. Taillandier, *Nucl. Acids Res.*, 1986, **14**, 3501; X. Jia, G. Zon and L. G. Marzilli, *Inorg. Chem.*, 1991, **30**, 228.
20 A. Yamada, K. Akasaka and H. Hatano, *Biopolymers*, 1976, **15**, 1315; Ch. Zimmer, G. Luck and H. Triebel, *Biopolymers*, 1974, **13**, 425; R. van Steenwinkel, F. Campagnari and M. Merlini, *Biopolymers*,

1981, **20**, 915; M. D. Reily and L. G. Marzilli, *J. Am. Chem. Soc.*, 1986, **108**, 8299; L. M. Torres and L. G. Marzilli, *J. Am. Chem. Soc.*, 1991, **113**, 4678.
21 R. B. Martin and Y. H. Mariam, in *Metal Ions in Biological Systems*, ed. H. Sigel, Marcel Dekker, New York, 1979, vol. 8.
22 H. Sigel, N. A. Corfu, L.-N. Ji and R. B. Martin, *Comments Inorg. Chem.*, 1992, **13**, 35; N. A. Corfu and H. Sigel, *Eur. J. Biochem.*, 1991, **199**, 659.
23 C. Sander and P. O. P. Ts'o, *J. Mol. Biol.*, 1971, **65**, 1.
24 H. Krakauer, *Biopolymers*, 1971, **10**, 2459; *Biochemistry*, 1974, **13**, 2579.
25 M. T. Record, jun., T. M. Lohman and P. de Haseth, *J. Mol. Biol.*, 1976, **107**, 145.
26 J. J. Butzow, G. L. Eichhorn and Y. A. Shin, in *Landolt-Börnstein*, New Series, ed. W. Saenger, Springer, Berlin, Heidelberg, 1990, vol. VII/1c, Table 4.4.2.1.1.
27 H. Diebler, M. Eigen, G. Ilgenfritz, G. Maass and R. Winkler, *Pure Appl. Chem.*, 1969, **20**, 93; R. G. Wilkins, *Acc. Chem. Res.*, 1970, **3**, 408.

Received 14*th February* 1994; *Paper* 4/00890A