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THERMODYNAMIC AND KINETIC PARAMETERS OF ION CONDENSATION TO POLYNUCLEOTIDES. OUTER SPHERE COMPLEX FORMED BY Mg^{++} IONS

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The coupling of ion binding to the single strand helix–coil transition in poly (A) and poly (C) is used to obtain information about both processes by ion titration and field-jump relaxation methods. Characterisation of the field-jump relaxation in poly (C) at various concentrations of monovalent ions leads to the evaluation of a stability constant $K = 71\ M^{-1}$ for the ion binding to the polymer. The rate constant of helix formation is found to be $1.3 \times 10^7\ s^{-1}$, whereas the dissociation rate is $1.0 \times 10^6\ s^{-1}$. Similar data are presented for poly (A) and poly (dA).

The interaction of Mg^{++} and Ca^{++} with poly (A) and poly (C) is measured by a titration method using the polymer absorbance for the indication of binding. The data can be represented by a model with independent binding "sites". The stability constants increase with decreasing salt concentration from $2.7 \times 10^4\ M^{-1}$ at medium ionic strengths up to $2.7 \times 10^7\ M^{-1}$ at low ionic strength. The number of ions bound per nucleotide residue is in the range 0.2 to 0.3. Relaxation time constants associated with Mg^{++} binding are characterised over a broad range of Mg^{++} concentrations from 5 μM to 500 μM. The observed concentration dependence supports the conclusion on the number of binding places inferred from equilibrium titrations. The rate of Mg^{++} and Ca^{++} association to the polymer is close to the limit of diffusion control ($k_R = 1 \times 10^{10}$ to $2 \times 10^{10}\ M^{-1}\ s^{-1}$). This high rate demonstrates that Mg^{++} and Ca^{++} ions do not form inner-sphere complexes with the polynucleotides. Apparently the distance between two adjacent phosphates is too large for a simultaneous site binding of Mg^{++} or Ca^{++}, and inner sphere complexation at a single phosphate seems to be too weak. The data support the view that the ions like Mg^{++} and Ca^{++} surround the polynucleotides in the form of a mobile ion cloud without site binding.

1. Introduction

Many of the biological macromolecules are polyelectrolytes. Owing to their high charge density the conformation of these molecules is strongly influenced by the concentration and the type of counterions present in solution. A small change in the ion concentration may induce a great alteration in the conformation of a macromolecule. Thus the interactions between polyelectrolytes and their counterions have a regulating function upon the structure of the polymer.

The most detailed experimental data on these effects were collected for various polynucleotide systems [1–4]. Because of their well-defined, regular structure nucleic acids were also used as convenient models for theoretical work [5–8]. By various experimental investigations it was demonstrated that there is a strong association between counterions and polynucleotides [9–14]. However, it has not yet been possible to demonstrate convincingly which type of binding prevails. Two types of binding are under discussion: diffuse binding and site binding [15,16]. In the first case the ions are simply attracted by the electrostatic potential of the polyelectrolyte and form a highly mobile ion cloud around the polymer. In the case of site binding the ions go to specific binding places. The discussion on the types of ion binding presented in the literature makes apparent that it is very hard to distinguish these different types on the basis of the available equilibrium data.

In the present investigation the problem of ion binding to polynucleotides is approached by the measurement of both equilibrium and kinetic parameters. Single-stranded polynucleotides were used as model systems. The structure of the single strands is more flexible than that of double helices. However, in the case of the strongly

stacked polymers, like poly(A) and poly(C), the molecular dimensions are rather well defined [2,17]. The formation of a defined structure is due to the stacking interaction between adjacent nucleotide residues, which keeps them in a helical order with a distance of 3.4 Å between subsequent bases [18,19]. A particular advantage of these molecules as a model system is their ability to function as indicators for ion binding to themselves. Moreover the indication is fast enough to follow the kinetics of ion binding by spectroscopic techniques [20,21]. The field jump method [22] is used to displace the equilibrium and to measure the relaxation due to ion binding. The parameters obtained from these investigations provide new quantitative information about ion binding in polynucleotides.

2. Materials and methods

Polynucleotides: Poly(A) and poly(C) were obtained from Boehringer Mannheim, GmbH. Poly(dA) was purchased from PL Biochemicals, Inc. The polymers were subjected to an extensive dialysis against 10 mM Tris at pH 8.0, 1 mM EDTA, then 10 mM Tris, 0.5 mM Tris and finally for a period of a few hours against two changes of quarz-distilled water. The dialysis was performed (and the material subsequently stored) in vessels made from polypropylene, in order to avoid any ion contamination from glassware. The Mg^{++} and Ca^{++} content was checked using a Pye Unicam SP 90 B Serie 2 atomic absorption spectrophotometer. $LaCl_3$ was used as a masking agent. It was found that the dialysed polymers contained less than one Mg^{++} or Ca^{++} per 1000 nucleotide residues. $A(pA)_{13}$ was isolated as described previously [23]. Concentrations were determined on the basis of the following extinction coefficients valid at high monovalent ion concentration (0.2 M, 20°C): poly(A), $\epsilon_{257} = 10000\ cm^{-1}\ M^{-1}$; $A(pA)_{13}$, $\epsilon_{258} = 10200\ cm^{-1}\ M^{-1}$; poly(C), $\epsilon_{268} = 6300\ cm^{-1}\ M^{-1}$.

Photometric titrations were performed in a Zeiss PMQ III spectrophotometer using thermostated cuvette holders. Ions were added from a Hamilton syringe using an Agla micrometer syringe outfit. The pH of the polymer solutions was adjusted before the titrations to a value of 8.0 by addition of small amounts of NaOH. Titrations with buffer concentrations below 0.5 mM were performed under a nitrogen atmosphere, in order to avoid pH shifts by CO_2 absorption, which may result in the formation of double helices from poly(A) and poly(C). The relative accuracy of absorbance readings (within a given titration experiment) was ± 0.001. Absorbance spectra were taken by a Cary 118 spectrophotometer.

Relaxation experiments were conducted using the field jump apparatus developed by Grünhagen [24]. The application of electric fields to solutions of single stranded polynucleotides caused both chemical and orientational effects. For the selective investigation of the chemical processes the relaxation was observed with polarised light with the polarisation plane inclined at 54.8° with respect to the vector of the electric field [25]. It has been shown previously that this technique allows the separation of the chemical and physical effects [26]. In the case of the polynucleotide–Mg^{++} systems orientational effects are much greater than those observed without addition of Mg^{++} (reduced dichroism $\Delta\epsilon/\epsilon$ for poly(A) + Mg^{++} up to −0.9). Thus the conditions for the selective observation of chemical effects had to be fulfilled very accurately. An air spaced Glan-prism (from B. Halle, Berlin) served as polariser. The optical parts had to be adjusted very carefully. The main technical problem came from shock waves induced by the field jumps. These shock waves caused strain in the observation windows of the measuring cell, which in turn resulted in birefringence and rotation of the polarisation plane. In order to avoid these perturbations the quarz windows of the measuring cells had to be selected and inserted extremely carefully. The cell contained a sample volume of about 1 ml. Platinum electrodes covered the sample volume at a distance of 6.7 mm. The light path was 8.5 mm. Unless indicated otherwise, the relaxation was observed at $\lambda = 248$ nm. Time constants were evaluated from the relaxation process observed, when the field was turned off, i.e., at zero field strength. A simulator for exponential curves developed by C.R. Rabl (publication in preparation) was used for data fitting. The time constants reported are average values obtained from 5 to 10 jump experiments.

Temperature-jump measurements at high ionic strengths were performed with an improved version of the cable temperature jump apparatus [27]. Details about these measurements will be reported separately (D. Pörschke, in preparation).

Computer calculations were performed on the Univac 1108 of the Gesellschaft f. wiss. Datenverar-

beitung, m.b.H., Göttingen. Relaxation spectra were calculated using a program developed by G. Ilgenfritz (publication in preparation).

3. Results

3.1. Monovalent ion binding

3.1.1. Equilibrium titration curves

In a previous investigation [26] it has been shown, that the helix coil equilibrium of single stranded poly(A) is influenced by ion binding. Low ionic strengths lead to an increase in the UV absorbance around 260 nm indicating a decrease in the number of stacked residues. A similar phenomenon is observed in single stranded poly(C) (cf. fig. 1). The ionic strength dependence can be readily explained by the fact that the single strand helix is characterised by a higher charge density than the extended coil form of the polynucleotide. Thus an ion depletion will lead to the dissociation of stacks and the formation of an extended coil form. In contrast to poly(A) and poly(C), equilibrium titration curves performed with poly(dA) do not show similar changes of UV absorbance, although it has been demonstrated that there are strong stacking interactions in poly(dA) [28]. The difference may be due to a peculiar spatial arrangement of the nucleotide residues in poly(dA). There is independent evidence for a specific poly(dA) structure [28,29].

3.1.2. Relaxation experiments

More quantitative information about the coupling of ion binding to the helix–coil equilibrium can be obtained from relaxation experiments. By a short pulse of electric field the ion–helix–coil equilibrium is shifted [26] and, when the pulse is terminated, the relaxation towards equilibrium is observed. Both poly(C) and poly(A) exhibit a single relaxation process, when the polymer solution contains monovalent ions such as sodium or tris ions. The relaxation process is strongly dependent upon the ionic strength (cf. fig. 2). The time constant of this process is not affected when tris ions are exchanged against sodium ions provided that the ionic strength is kept at the same value. At low ionic strengths there is a linear dependence of the reciprocal relaxation time upon the ionic strength. When the ionic strength is increased

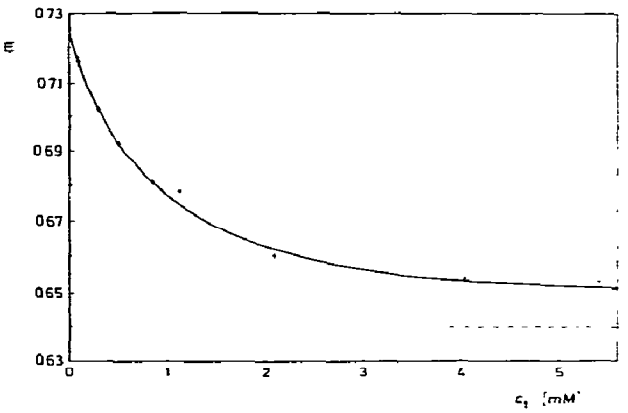


Fig. 1. Absorbance of poly(C) at 280 nm as a function of the ionic strength (tris ions, pH 8, 20°C, c_p = 0.15 mM). The line represents a least-squares fit according to a model with independent ion binding sites on the polymer (interrupted line ≡ limit absorbance at high ionic strength).

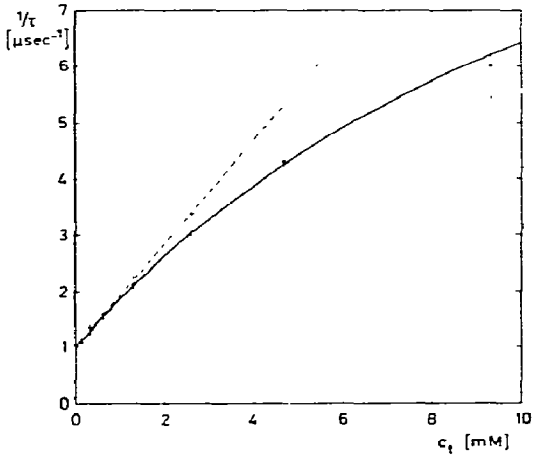


Fig. 2. Reciprocal relaxation time $1/\tau$ as a function of the ionic strength (tris ions, pH 8, 20°C, c_p = 0.08 mM). The line is calculated according to eq. (2) with the parameters given in table 1 [interrupted line ≡ limit slope according to eq. (3)]. The limit $1/\tau$ value at high ionic strength (1.05 M) is 14.1 μs^{-1} (measured by temperature-jump method).

to medium values a deviation from the linear dependence is observed. Finally at high ionic strengths the relaxation times attain a constant level.

A kinetic scheme consistent with these relaxation data is given by

$$I + C \underset{k_{10}}{\overset{k_{01}}{\rightleftharpoons}} I \cdot C \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} I \cdot H \qquad (1)$$

where the bimolecular step represents the binding of the ions I to the polynucleotide C (in the coil form) followed by an intramolecular step representing the stacking or helix–coil reaction. This reaction scheme is characterised by two relaxation processes. If one of the reaction steps is fast compared with the other, a simplified expression can be obtained for the time constant of the slow process. When ion binding is assumed to be fast compared with the stacking reaction, the relaxation time for the stacking reaction is given by [30]

$$\tau = [k_{21} + k_{12}k'_{01}/(k'_{01} + k_{10})]^{-1}, \qquad (2)$$

with $k'_{01} = k_{01}(c_i + c_c)$, where c_i and c_c are the free concentrations of ions and binding sites (coil form) respectively. At low concentration ($k'_{01} \ll k_{10}$) eq. (2) may be simplified further to

$$\tau = [k_{21} + k_{12}(k_{01}/k_{10})(c_i + c_c)]^{-1}. \qquad (3)$$

This relation is used to derive the terms k_{21} and $k_{12}k_{01}/k_{10}$ from the first linear part of the $1/\tau$ versus $(c_i + c_c)$ plot represented in fig. 2. At high ion concentrations, where $k'_{01} \gg k_{10}$, the relaxation time is given by

$$\tau = [k_{21} + k_{12}]^{-1}. \qquad (4)$$

The relation (4) is used to derive the rate constant k_{12}. According to this procedure the rate constants k_{12} and k_{21} as well as the pre-equilibrium constant k_{01}/k_{10} can be obtained directly from relaxation measurements.

It has to be demonstrated, however, that the assumption made about the rate of ion binding is justified. Complex formation involving univalent ions is known to be a very fast process [31]. Since in the present case there is hardly any chance for chelate formation, the rate will be diffusion controlled; i.e., $k_{01} \approx 1 \times 10^{10}$ M^{-1} s^{-1}. Using an estimated value $k_{01}/k_{10} \approx 100$ M^{-1} the rate constant k_{10} is about 10^8 s^{-1}. Thus the relaxation time due to the ion binding

$$\tau_I = [k_{01}(c_i + c_c) + k_{10}] \qquad (5)$$

will be in the nanosecond time range with $\tau_I \leq 10$ ns and the assumption made above clearly is justified.

Relaxation data were obtained for three different polynucleotides: poly(C), poly(A) and poly(dA). It was possible to measure field-jump relaxation in poly(dA), although this polymer did not exhibit effects in ion-titration experiments under equilibrium conditions. This result demonstrates that high electric fields cause a substantial depletion of the ionic atmosphere around the polymer, resulting in a strongly increased electrostatic repulsion between the nucleotide residues.

The data evaluated from the relaxation experiments are summarised in table 1. The pre-equilibrium constants for the ion binding are of the same order of magnitude for all the polymers. Some dependence upon the nature of the polymer is observed in the rates of the helix–coil transition. However, the parameters given for poly(A) and poly(dA) can only be taken as purely empirical and their interpretation as preliminary. In both polymers a second relaxation process [20] is observed in the long wavelength range ($\lambda > 280$ nm). The nature of this process is not yet fully elucidated. A discussion of this aspect on the basis of more data will be given elsewhere (D. Pörschke, to be published).

A simple test of the data given in table 1 is possible by comparison with the equilibrium titration curves. The curve presented in fig. 1 was analysed using the following model: (1) Ions bind to independent binding sites at the polymer according to the law of mass action. (2) Each binding step makes the same contribution to the absorbance change. The number of binding sites cannot be determined in the present case, since the ligands are present at much higher concentrations than the polymer binding sites. The equilibrium constant is defined according to

$$K = c_h/c_i c_c,$$

Table 1
Rate parameters of the single strand helix coil transition in the presence of monovalent ions at 20°C. [Definition of parameters corresponding to eq. (1)]

Polymer	k_{01}/k_{10}	k_{12}	k_{21}
poly(C)	71	1.3×10^7	1.0×10^6
poly(A) [a]	61	4.6×10^6	6×10^5
poly(dA) [a]	57	2.1×10^7	4.9×10^5

[a] Preliminary data, cf. text.

where c_h is the concentration of the occupied (mainly helical) sites. Least squares fitting of the titration curve given in fig. 1 led to a value $K = 1.3 \times 10^3\ M^{-1}$, which is in reasonable agreement with the product $K = (k_{01}/k_{10})(1 + k_{12}/k_{21}) = 990\ M^{-1}$.

3.2. Divalent ions

3.2.1. Equilibrium titration curves

When divalent ions like Mg^{++} or Ca^{++} are added to desalted poly(C) or poly(A), substantial changes of the polynucleotide absorbance are observed (cf. fig. 3). These changes are attributed to an increase in the number of stacked bases due to a decrease of the electrostatic repulsion. Such effects were observed previously [11,32], but have never been used for any quantitative analysis of ion binding. This is probably due to the fact that a direct quantitative analysis has to be based upon an assumption about the relation between the absorbance change and the degree of ion binding. In the following it will be assumed that the absorbance change is a linear function of the degree of ion binding. The titration curves may then be analysed according to models of varying complexity. It was found, however, that the experimental data can be represented very accurately by a simple model. According to this model there is a number of binding sites n per nucleotide residue (with n usually smaller than 1). The binding of the ions to these sites is described by the law of mass action with a stability constant

$$K = c_h/c_{Me}c_c, \tag{6}$$

where c_c, c_h and c_{Me} are the equilibrium concentrations of the free sites, occupied sites and the ions respectively. The relations of mass conservation are

$$c^0_{Me} = c_{Me} + c_h, \tag{7}$$

$$nc_p = c_h + c_c, \tag{8}$$

with $c^0_{Me} \hat{=}$ total ion concentration and $c_p \hat{=}$ total concentration of nucleotide residues. The information available from the titration curve is the ratio α measured at various ion concentrations

$$\alpha = c_h/c_c = (E_0 - E)/(E - E_f), \tag{9}$$

where E is the absorbance at a given ion concentration, E_0 the absorbance before the addition of ions and E_f the limiting absorbance approached at high ion concentration. A combination of the equations (6) to (9)

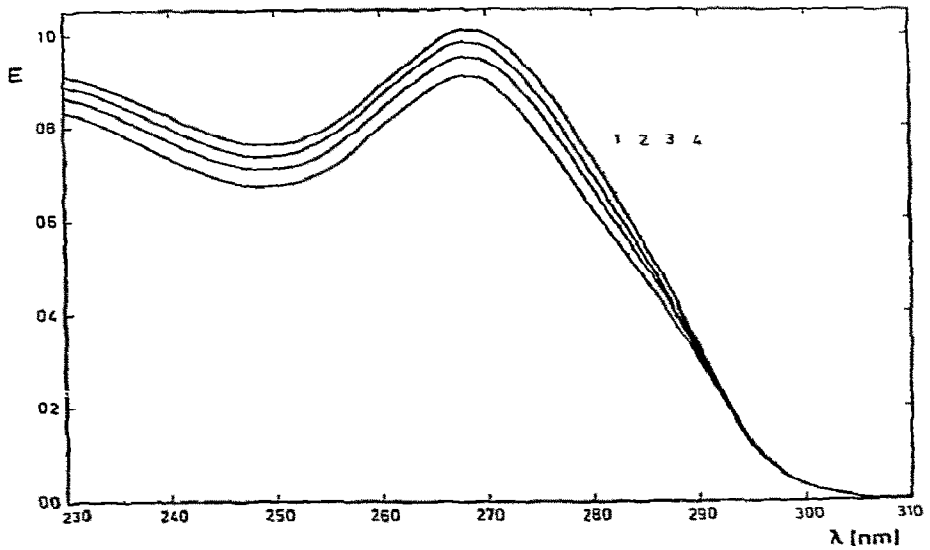


Fig. 3. Spectral change of poly(C) upon addition of Mg^{++} ions in 0.5 mM tris, pH 8, c = 0.147 mM. Total Mg^{++} concentrations: (1) < 0.5 μM; (2) 8.2 μM; (3) 19.1 μM; (4) 46.3 μM.

yields by rearrangement

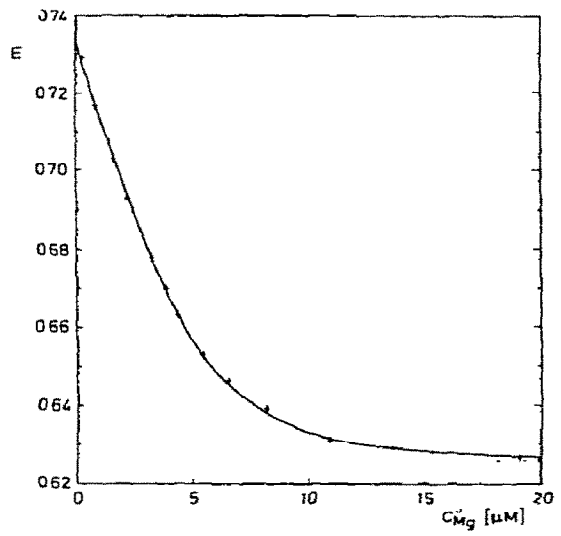
$$\alpha = K c^0_{Me}\left(\frac{1}{\alpha}+1\right) - (Kn c_p + 1). \qquad (10)$$

According to this equation a plot of α versus $c^0_{Me}(1/\alpha + 1)$ should result in a straight line. An example is given in fig. 4b, using the data of the titration curve represented in fig. 4a. Within experimental accuracy a linear relationship is obtained. The slope is equal to the stability constant K, and the number of binding places n can be calculated from the point of intersection at the abscissa. The graphical evaluation according to eq. (10) yields parameters ($K = 1.8 \times 10^6$ M^{-1}; $n = 0.18$) which are in close agreement with parameters obtained from a least squares computer evaluation of the titration curve ($K = 2.2 \times 10^6$ M^{-1}; $n = 0.19$). The validity of these parameters was tested by independent titrations at a nucleotide concentration higher by a factor of 5. Both parameters, K and n, were equal to those given above within experimental accuracy. Titration data were collected for Mg^{++} and Ca^{++} binding to poly(A) and Mg^{++} binding to poly(C). The resulting parameters are compiled in table 2.

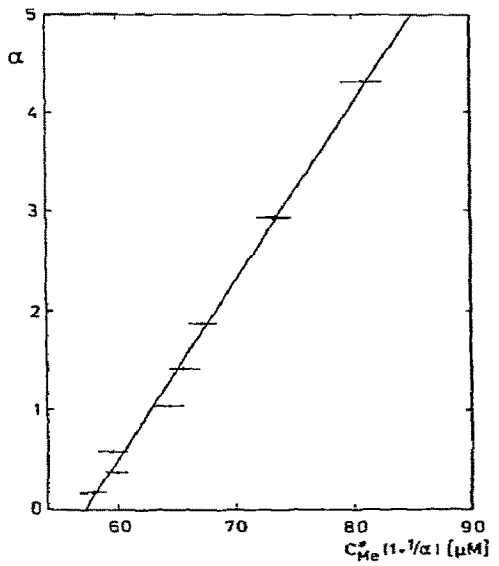
Table 2
Stability constants K and number of binding places n obtained from titration curves. (Average values obtained from several titrations; estimated accuracy ± 20%)

System	Tris-buffer concentration (mM)	K (M^{-1})	n
poly(A) + Mg	–	2.7×10^7	0.29
	0.1	1.8×10^7	0.30
	0.2	1.6×10^7	0.31
	0.5	3.8×10^6	0.22
	2.0	1.1×10^6	0.22
	10.0	1.9×10^5	0.19
	20.0	2.7×10^4	–
poly(A) + Ca	0.5	3.6×10^6	0.23
poly(C) + Mg	0.5	2.0×10^6	0.20

The deoxyribopolynucleotide poly(dA) showed rather small changes of absorbance when Mg^{++} or Ca^{++} ions were added. A least squares analysis of these data indicated a slightly lower binding affinity of Mg^{++} to poly(dA) than of Mg^{++} to poly(A). However, because of the rather high error involved in the poly(dA) evalua-



(a)



(b)

Fig. 4. (a) Poly(C) absorbance at 280 nm as a function of the total Mg^{++} concentration (20°C, $c_p = 2.94 \times 10^{-5}$ M). The line represents a least squares fit according to the model described in the text (interrupted line ≡ limit absorbance at high Mg concentration). (b) Titration data shown in fig. 4a plotted according to eq. (10).

tion, these data are not included in table 2.

An oligonucleotide, $A(pA)_{13}$, was also tested for its Mg^{++}-binding properties. The titration curve obtained was the only one which could not be fitted according to the model described above. A best least-squares fit was obtained for $K = 1.2 \times 10^5\ M^{-1}$ and $n = 0.07$ (corresponding to one binding site per oligomer; titration at 0.2 mM Tris). However, the fitted curves deviated clearly from the experimental points, mainly at high Mg^{++} concentrations. These deviations are probably due to "end effects", which are expected to be more apparent in an oligomer than in polymers.

3.2.2. Relaxation experiments

The dynamics of bivalent ion binding to polynucleotides were studied by the field jump method [22, 26]. All experiments were performed in the presence of tris buffer, in order to ensure constant pH values. An example of the relaxation observed in the poly(C)–Mg^{++} system is represented in fig. 5. Two relaxation processes are clearly separated. The slower one is associated with an amplitude of about 30% of the total amplitude. The nature of the two processes could clearly be demonstrated by the characterisation of their concentration dependence. When the tris concentration was increased at constant Mg^{++} concentration, the time constant of the "fast" process τ_f was decreased, whereas the time constant of the slow process τ_s remained almost unchanged. Furthermore the time constant obtained for the "fast" process at a given tris concentration agreed well with that measured in the absence of Mg^{++} (except at high Mg^{++} levels). Thus the "fast" process is associated with the binding of tris ions to the polymer. The "slow" process, associated with the Mg^{++} binding, showed a very characteristic concentration dependence (cf. table 3). At a fixed polymer concentration the time constant of the "slow process" shows rather little dependence upon the Mg^{++} concentration, as long as the Mg^{++} concentration remains below a certain limit. At higher Mg^{++} concentrations a rather strong concentration dependence is observed. Finally at high Mg^{++} levels the "slow" and the "fast" relaxations merge into a relaxation curve which is represented by a single exponential within experimental accuracy.

The qualitative explanation of these data is fairly simple. Due to the high binding constant of Mg^{++} to the polynucleotide almost all Mg^{++} ions are bound to the polymer as long as free binding sites are available. In this range an increase in the Mg^{++} concentration does not lead to an increase in the free reactant concentration and thus is not reflected by a decrease in the relaxation time of the slow process. However, when the Mg^{++} concentration exceeds the concentration of binding sites, a strong decrease in the relaxation time is observed at increasing Mg^{++} concentration. Thus the concentration dependence of the Mg^{++} relaxation gives a clear indication of the number of binding sites present on the polymer.

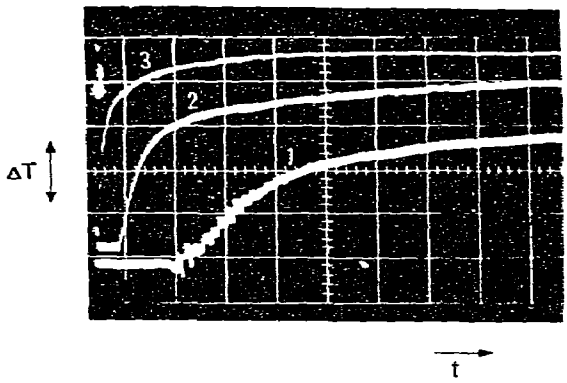


Fig. 5. Field-jump relaxation observed at 248 nm (cf. methods) for poly(C) (c_p = 59 μM) at 10 μM $MgCl_2$ and 0.5 mM tris buffer after termination of the field pulse (53.7 kV/cm). The time units (t) are: (1) 0.5 μs; (2) 2 μs, and (3) 10 μs (each per big scale unit). The amplitude units (ΔT) are 2% transmission change per big scale unit. (Starting points are shifted for each experiment).

Table 3
Experimental (exp) and calculated (calc) relaxation time constants τ_f and τ_s for poly(C) as a function of the total Mg^{++} concentration c^0_{Mg} (at 0.5 mM tris pH 8, 20°C; $c^0_p \triangleq$ total nucleotide concentration)

c^0_{Mg} (μM)	c^0_p (μM)	τ_f (exp)	τ_f (calc)	τ_s (exp)	τ_s (calc)
5	65.1	0.65	0.70	11.3	12.9
10	59.1	0.67	0.70	10.9	20.7
25	37.8	0.83	0.67	8.1	8.6
25	60.7	0.68	0.66	7.3	7.0
50	59.9	0.64	0.62	3.7	3.6
100	58.6	0.47	0.49	–	–
200	59.2	0.26	0.32	–	–
500	57.1	0.16	0.15	–	–

The rate parameters of Mg^{++} binding were evaluated by computer simulation of the measured relaxation time constants. Several models were tested. The data given below were obtained by simulation according to the following reaction scheme:

$$C + Mg \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C \cdot Mg \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} H \cdot Mg \qquad (11a)$$

$$C + 2I \underset{k_{10}}{\overset{2k_{01}}{\rightleftharpoons}} C \cdot I + I \underset{2k_{10}}{\overset{k_{01}}{\rightleftharpoons}} C \cdot I_2 \qquad (11b)$$

$$k_{12} \downarrow\uparrow k_{21} \qquad k_{12} \downarrow\uparrow k_{21}$$

$$H' \cdot I \qquad H \cdot I_2$$

The first part of reaction scheme (11a) reflects the binding of Mg^{++} with a bimolecular step followed by the intramolecular stacking reaction. The second part shows the tris binding reaction under the assumption that the binding site for one Mg^{++} can be occupied by two tris ions. The rate constants involved in the tris binding were taken from the evaluation described in section 3.1.2. A statistical factor of 2 was employed (cf. eq. 11b). It may be expected for a first approximation that the rate parameters of the stacking reaction reflect mainly the properties of the polynucleotide and do not depend very much upon the type of ion bound. Thus the rate constants k_2 and k_{-2} used in the simulation were equal to k_{12} and k_{21} respectively. Finally there is information about the overall equilibrium binding constant K obtained from the titration curves. Since $K = (k_1/k_{-1})(1 + k_2/k_{-2})$ there is only one parameter left, that can be adjusted for simulation of the relaxation data. In the case of Mg^{++} binding to poly(C) a satisfactory fit is obtained with a rate constant $k_1 = 1.3 \times 10^{10}\ M^{-1}\ s^{-1}$.

An example of a relaxation spectrum calculated by a computer is given in table 4. The nature of the six different relaxation processes will not be discussed in detail. From simulations at various concentrations and from the amplitudes (cf. discussion below) it can be shown that the slow process ($N = 1$) is associated with the Mg^{++} binding, whereas the process $N = 3$ is associated with the tris binding. The other processes can be neglected because of their low relative amplitude. At Mg^{++} concentrations above 50 μM the simulations yield a rather strong decrease of the amplitude A_1, whereas the amplitude A_3 is increased, accompanied by a decrease of τ_3.

Table 4
Relaxation time constants τ and relative amplitudes A (%) calculated according to reaction scheme (11). ($c^0_{Me} = 10\ \mu M$, $c^0_T = 0.3\ \mu M$, $nc_p = 12\ \mu M$, rate parameters according to t. 1 and 5, cf. text)

N	τ_N (μs)	A_N
1	20.7	40.3
2	1.05	1.1
3	0.703	59.5
4	0.07	−0.4
5	0.006	−0.5
6	0.003	$< 10^{-6}$

As shown in table 3 the relaxation was measured over a very broad range of Mg^{++} concentrations. The rather close agreement of the experimental and the calculated relaxation time constants over this broad range demonstrates that the model and the rate parameters used for the calculation are essentially correct. There is one disagreement between experimental and calculated time constants at $c^0_{Mg} = 10\ \mu M$, which is probably not due to experimental error. According to the calculations the value of τ_s should have a maximum around $c^0_{Mg} = 10\ \mu M$. It may be that this maximum is not found in the experiments, because there is some exchange reaction of Mg^{++} ions by direct transfer from occupied to free binding sites.

All the calculations presented above were performed with $n = 0.2$ as the number of Mg^{++} binding sites per nucleotide residue. Some relaxation data were calculated with different values of n. It could be shown that the measured set of time constants are not represented as accurately when the value of n is significantly lower or higher than 0.2. In particular it was shown that a value of $n = 0.5$ (expected for binding with complete charge compensation) is not appropriate for an accurate representation of the experimental set of time constants.

The amplitudes observed in the field jump experiments contain valuable information abut the equilibrium shift in the presence of the electric field. Model calculations indicate that the relative change of the equilibrium constant for Mg^{++} binding depends upon the Mg^{++}-to-polymer concentration ratio in a different way than does the corresponding change for the binding of tris. This may be due to a different response of the Mg^{++} and tris field effects to changes in the charge density of the polymer. At present there is no satisfactory theory of the

Table 5
Rate constants of ion binding to polynucleotides (definition of k_1 and k_{-1} according to scheme (11); estimated accuracy: poly(C) ± 20%, poly(A) and $A(pA)_{13}$ ± 30%)

Polymer	Tris-buffer concentration (mM)	Ion	k_1 ($M^{-1}s^{-1}$)	k_{-1} (s^{-1})
poly C	0.5	Mg^{++}	1.3×10^{10}	8.5×10^4
poly A	0.5	Mg^{++}	2×10^{10}	4×10^4
poly A	0.5	Ca^{++}	1×10^{10}	2×10^4
$A(pA)_{13}$	0.2	Mg^{++}	5×10^{10}	8×10^5

dissociation field effects [33] in polyelectrolytes. Due to these difficulties the amplitudes are not discussed in further detail. The relative amplitudes given in table 4 are calculated with the assumption that the species H·Mg and H·2I give the same contribution ΔA to the absorbance change, whereas the species H·I contributes only $\Delta A/2$.

A compilation of the rate parameters obtained according to the procedure described above is given in table 5. The parameters for Mg^{++} binding to $A(pA)_{13}$ were evaluated according to the reaction scheme (11a) without consideration of (11b), since the amplitude due to the tris binding was very small in $A(pA)_{13}$.

3.2.3. Temperature jump relaxation

The field jump method can only be applied at low or medium ionic strength. Thus the kinetics of Mg^{++} and Ca^{++} binding at high ion concentrations had to be studied by the temperature-jump method [27,30]. Again the stacking of the nucleotide bases was used for the indication of binding. It has been reported previously that bivalent ions can exert a specific influence upon the kinetics of the single strand helix–coil transition in poly(A) [20]. In the present investigation, using an improved cable temperature-jump apparatus, it was possible to resolve the relaxation curves observed previously into further detail. Two different relaxation processes were found for poly(A) in the presence of 5 mM $MgCl_2$ and 0.1 M sodium cacodylate pH 6.9: $\tau_1 = 0.44$ μs, $\tau_2 = 8$ μs, amplitude of the slow process about 30% at 248 nm. Experiments performed with Ca^{++} ions under corresponding conditions also showed the existence of two different relaxation processes: $\tau_1 = 0.3$ μs, $\tau_2 = 4$ μs, amplitude of the slow process about 40% at 248 nm. A control experiment using poly(C) in the presence of 5 mM $MgCl_2$ and 0.1 M sodium cacodylate pH 6.9 did not show any slow relaxation process ($\tau < 0.1$ μs). All the relaxation time constants reported in the present section were determined by temperature jumps from 20 to 24°C (observation at $\lambda = 248$ nm).

4. Discussion

4.1. Thermodynamics

The titration method used in the present investigation provides a very convenient procedure for the characterisation of Mg^{++} and Ca^{++} binding in single stranded polynucleotides. Although the evaluation of quantitative thermodynamic parameters has to be based upon an assumption about the relation between absorbance change and degree of ion binding, it seems, that the assumption of a linear relation (cf. section 3.2.1) is quite realistic. The results obtained from the titration experiments can be subjected to various tests. First of all it has been shown that titrations at various nucleotide concentrations yield the same result. The most convincing evidence in favour of the thermodynamic data is the fact that they can be used for the simulation of the relaxation data without any constraint. Finally the data can be compared to some results, which were obtained previously by different methods. The comparison will be arranged according to three different aspects:

(a) *Independence of binding sites*, which was found in the present investigation, was reported previously for the binding of Mn^{++} to single stranded polynuceotides [9,10,34]. The binding of this specific ion was studied by NMR techniques. Other techniques had to be used for the characterisation of Mg^{++} binding. Some data obtained by the application of specific dyes [11,12] and also by potentiometry [13] showed electrostatic interaction between the binding sites with a decrease of the binding constant as the degree of binding increases. Thus the result obtained on interactions between binding sites depends upon the method used for characterisation.

(b) *The number of binding sites* obtained in the present investigation is clearly lower than expected for a simple stochiometry based upon complete charge compensation. The experimental results obtained previously on the number of binding sites are somewhat contradictory. Conduc-

tometric titrations performed by Felsenfeld and Huang [35] showed a strong point of inflection corresponding to $n = 0.29$ and a second, somewhat weaker, corresponding to $n = 0.5$. The authors concluded that there is a stochiometry of one bivalent ion to two phosphate groups, but did not explain the strong point of inflection corresponding to $n = 0.29$. The stochiometry in the case of Mn^{++} binding to single stranded polynucleotides could be studied by the NMR method. The results vary from $n = 0.4$ to 0.5 reported by Eisinger et al. [9] and $n = 0.38$ to 0.43 reported by Cohn et al. [10].

(c) *Binding constants* are obtained for a broad range of ionic strengths in the present investigation. The parameters evaluated for high ionic strengths are compatible with those reported previously [9–13]. A considerable increase in the binding constants is observed with decreasing ionic strength. This effect can partly be explained by an increase of the effective charge density of the polymer at low ionic strength. In addition there is a contribution from the stacking equilibrium in the polymer. The titration curves demonstrate (cf. fig. 1), that the number of stacked residues is reduced at low ionic strengths. When bivalent ions are bound to the polymer under these conditions, a relatively high contribution of free energy is obtained from the incorporation of base residues into the single strand helix.

4.2. Kinetics

Relaxation data are presented for the binding of both monovalent and bivalent ions to single-stranded polynucleotides. In the case of monovalent ions it has been possible to determine a pre-equilibrium constant for ion binding as well as the rate parameters of the single-strand helix–coil transition. The latter parameters are of particular interest, since the evaluation was based exclusively on measured relaxation time constants. It was not necessary to use any equilibrium parameter obtained from optical melting curves. The results obtained for poly(C) are in satisfactory agreement with those evaluated in a recent investigation [21]. For poly(A) and poly(dA) more data are required to elucidate the nature of the second relaxation process observed.

The rate constants obtained for the binding of Mg^{++} and Ca^{++} ions to polynucleotides clearly demonstrate that the binding of these ions is a diffusion-controlled process. There are no comparable data on bivalent ion binding to polynucleotides. However, many data were collected for the reaction of more simple ligands with Mg^{++} and Ca^{++}. All these data showed a characteristic low substitution rate ($k_R \approx 10^5\ s^{-1}$) for Mg^{++} complexes, being almost independent of the ligand [36]. It was demonstrated that the low rates are due to the process of H_2O dissociation from the hydration sphere of the Mg^{++} ion, which precedes the formation of an "inner sphere" complex in aqueous solution. With this information it can be concluded immediately that Mg^{++} does not form an "inner sphere" complex with single stranded polynucleotides. The measured rate constants can only be explained by the formation of an "outer sphere" complex. This means that the inner hydration sphere with the first layer of water of molecules around the Mg^{++} ion remains unaffected when the Mg^{++} is bound to the polynucleotide.

In the case of Ca^{++} ions the formation of inner sphere complexes is a much faster process [31,36] with $k_R = 10^8$ to $10^9\ s^{-1}$. Thus there is not as much difference between the "inner sphere" rate constants and the rate constants observed for polynucleotide binding. However, it is very likely that Ca^{++} also forms an "outer sphere" complex with polynucleotides.

At a first glance it may be surprising that the Mg^{++} and Ca^{++} polynucleotide complexes, characterised by very high binding constants (up to $3 \times 10^7\ M^{-1}$), do not effect the inner hydration sphere of the metal ions. A satisfactory explanation seems to be possible however, when the structure of the polynucleotides is considered. Models of single-strang helices show that there is a distance of 7 to 8 Å between adjacent phosphate residues. Thus Mg^{++} and Ca^{++} could form an inner sphere complex at one phosphate binding site only, but not simultaneously at two adjacent sites, because the distance is too large. The formation of an inner sphere complex at one phosphate site would leave a local positive charge, which seems to be unfavored. It may be that the Mg^{++} and Ca^{++} ions are preferentially located between two adjacent phosphates providing a minimum of electrostatic energy. It may also be that this minimum is rather shallow and that the ions move more or less freely in the ion cloud around the polymer. At any rate there is no evidence for a binding involving a direct attachment of the ions to a specific group.

It may be expected that inner-sphere complexes are

formed at high concentrations of metal ions. The "slow" process observed for poly(A) at 5 mM Mg^{++} (cf. section 3.2.3) may be considered as an indication of inner-sphere complexation. Since a similar process is observed in the presence of Ca^{++}, however, it is more likely that this process is due to some specific poly(A) structure formed in the presence of bivalent ions. Moreover, poly(C) at 5 mM Mg^{++} did not show any "slow" relaxation process. This observation confirms the conclusion that the "slow" effect observed in poly(A) is an indication of the formation of a specific poly(A) structure and not an indication of inner-sphere-complexation.

In summary, the kinetic data obtained in the present investigation support the view that ions like Mg^{++} and Ca^{++} surround the polymer in the form of a mobile ion cloud. The association of these ions with polynucleotides seems to be designated properly by the term "ion condensation".

At the end of this discussion it should be mentioned that the reaction schemes used for the evaluation of thermodynamic and kinetic information are not expected to reflect the real processes correctly in every aspect. In general, reaction schemes are selected which allow the representation of experimental data in the most simple way. Thus the evaluation is based upon a reaction scheme assuming the existence of binding sites, although the kinetic data do not give any evidence for the existence of such binding sites. At the present stage of experimental accuracy it would be useless to apply sophisticated models, which consider the specific type of interactions expected for ion atmosphere binding. Most of the relevant information can already be obtained by application of simple models.

4.3. Biochemical implications

The dynamic properties of the Mg^{++}–polynucleotide complex are quite remarkable. Until now this Mg^{++} complex is the only one which exhibits both high stability and high rate constants. It may well be that these specific properties are not accidental, but are required for the biological function of the polynucleotides. As has been discussed previously, one requirement for the function of polynucleotides is a high dynamic flexibility, in order to assure high rates of information transfer. It is apparent that in the case of Mg^{++} binding to polynucleotides this flexibility can be achieved in a much better way by outer-sphere complexation, characterised by high rates, than by inner sphere complexation, with its relatively low rates. In this respect it seems to be an advantage that ubiquitous ions like Mg^{++} do not form specific coordination complexes with polynucleotides. More specific interactions with an adapted spacing of charged groups may be reserved for protein–polynucleotide interactions.

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References

[1] K. Hamaguchi and E.P. Geiduschek, J. Amer. Chem. Soc. 84 (1962) 1329–1338.
[2] G. Felsenfeld and H.T. Miles, Ann. Rev. Biochem. 36 (1967) 407–448.
[3] C. Zimmer, Zeitschrift f. Chem. 11 (1971) 441–458.
[4] H. Eisenberg, in: Basic Principles in Nucleic Acid Chemistry, Vol. II, ed. P.O.P. Ts'O (Academic, New York, 1974) p. 232–240.
[5] L. Kotin, J. Mol. Biol. 7 (1963) 309–311.
[6] C. Schildkraut and S. Lifson, Biopolymers 3 (1965) 195–208.
[7] M.T. Record, Biopolymers 5 (1967) 975–992.
[8] G.S. Manning, Biopolymers 11 (1972) 937–949.
[9] J. Eisinger, F. Fawaz-Estrup and R.G. Shulman, J. Chem. Phys. 42 (1965) 43–53.
[10] M. Cohn, A. Danchin and M. Grunberg–Manago, J. Mol. Biol. 39 (1969) 199–217.
[11] A.M. Willemsen and G.A.J. Van Os, Biopolymers 10 (1971) 945–960.
[12] H. Krakauer, Biopolymers 10 (1971) 2459–2490.
[13] C. Sander and P.O.P. Ts'O, J. Mol. Biol. 55 (1971) 1–21.
[14] A.R. Mathieson and J.Y. Olayemi, Arch. Biochem. Biophys. 169 (1975) 237–243.
[15] J.W. Lyons and L. Kotin, J. Amer. Chem. Soc. 87, (1965) 1670–1678.
[16] J. Skerjanc and U.P. Strauss, J. Amer. Chem. Soc. 90 (1968) 3081–3085.
[17] J. Witz and V. Luzzati, J. Mol. Biol. 11 (1965) 620–630.
[18] P.O.P. Ts'O, in: Basic Principles in Nucleic Acid Chemistry, Vol. II, ed. P.O.P. Ts'O (Academic, New York, 1974) p.305–469.
[19] V.A. Bloomfield, D.M. Crothers and I. Tinoco, Jr., Physical Chemistry of Nucleic Acids (Harper and Row, New York, 1974).

[20] D. Pörschke, Eur. J. Biochem. 39 (1973) 117–126.
[21] D. Pörschke, Biochemistry 15 (1976) 1495–1499.
[22] L. DeMaeyer and A. Persoons, in: Techniques of Chemistry, Vol. 6 part 2, eds. A. Weissberger and G. Hammes, (Wiley, New York, 1973) p. 211–235.
[23] D. Pörschke, Biopolymers 10 (1971) 1989–2013.
[24] H.H. Grünhagen, Messtechnik 1974, 19–23.
[25] H. Labhart, Chimia 15 (1961) 20–26.
[26] D. Pörschke, Nucl. Ac. Res. 1 (1974) 1601–1618.
[27] G.W. Hoffman, Rev. Sci. Instrum. 42 (1971) 1643–1647.
[28] A. Adler, L. Grossman and G.D. Fasman, Biochemistry 8 (1969) 3846–3859.
[29] D. Pörschke, Proc. Nat. Acad. Sci. US. 70 (1973) 2683–2686.
[30] M. Eigen and L. DeMaeyer, in: Techniques of Organic Chemistry, Vol. 8 part 2, eds. S.L. Friess, E.S. Lewis and A. Weissberger (Interscience, New York, 1963).
[31] M. Eigen and G. Maass, Z. f. Phys. Chem. N. F. 49 (1966) 163–177.
[32] G. Felsenfeld and S. Huang, Bio. Bio. Acta 37 (1960) 425–433.
[33] L. Onsager, J. Chem. Phys. 2 (1934) 599–615.
[34] A. Danchin, Biopolymers 11 (1972) 1317–1333.
[35] G. Felsenfeld and S. Huang, Bio. Bio. Acta 34 (1959) 234–242.
[36] M. Eigen, Pure Appl. Chem. 6 (1963) 97–115.