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EXCLUDED-VOLUME EFFECT OF INERT MACROMOLECULES ON THE MELTING OF NUCLEIC ACIDS

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It is shown on the basis of the excluded-volume effect that inert macromolecules may be expected to suppress the dissociation of double-helical nucleic acids into single helices and thus to raise the melting point of the double helix. The rise in melting temperature of the ribonucleic acid [poly(I)·poly(C)] caused by dextran polymers and by poly(ethylene oxide) is described and compared with the theoretical prediction. Good agreement was found in respect of the extent of the rise in melting point and in respect of its dependence upon polymer length. An additional dependence upon the identify of the polymer was attributed to detailed effects of shape in solution.

1. Introduction

The excluded volume between molecules in solution [1] is formally analogous to the co-volume of a non-ideal gas [2], and its importance as a factor determining the behaviour of biological macromolecules is becoming increasingly clear. For example, by virtue of this effect, poly(ethylene oxide) (polyethylene glycol, PEG) stimulates the aggregation and precipitation of ribosomes [3] and of viruses [4], the association of the components of the multi-enzyme complex pyruvate dehydrogenase [5,6], the selective precipitation of antibody-antigen complexes [7], the fusion of whole cells [8] and the condensation of DNA into a tightly packed structure [9].

Quantitative treatment of these effects has so far been restricted to cases in which the geometry is simple or where good approximation is possible [6], and in particular to the self-exclusion of flexible, linear polymer molecules (ref. 10 and references therein). Excluded volumes have recently been exploited in the determination of axial ratios of globular protein molecules [11,12]. In this communication we show that inert polymers raise the melting temperature of a double-helical nucleic acid and we present a quantitative explanation of this in terms of the excluded-volume effect.

2. Theory

We consider a uniform double helix D of fixed length, which dissociates to give two distinct single helices S_1 and S_2; the dissociation is accompanied by molar enthalpy and entropy changes of ΔH and ΔS, respectively. In equilibrium at temperature T, the change in molar Gibbs free energy is zero, so that

$$0 = \Delta H - T\Delta S + RT \ln\frac{c_{S_1}c_{S_2}}{c_D} + RT \ln\frac{y_{S_1}y_{S_2}}{y_D} \quad (1)$$

where c denotes concentration and y activity coefficient. At the melting temperature T_m, we have $c_{S_1} \equiv c_{S_2} = c_D = \frac{1}{2}c_0$, where c_0 is the concentration of the polymer when it is all present in the

double-helical form. It follows that

$$T_m = \frac{\Delta H}{\Delta S - R \ln \frac{c_0}{2} - R \ln \frac{y_{S_1} y_{S_2}}{y_D}} \quad (2)$$

We now define the standard states such that all activity coefficients are unity in the absence of inert polymer. Under these conditions the melting temperature is T_m^0, given by

$$T_m^0 = \frac{\Delta H}{\Delta S - R \ln \frac{c_0}{2}} \quad (3)$$

whereby the second term in the denominator is negligible for very long chains. Comparing eqs. 2 and 3 we obtain for all chain lengths the relation

$$\frac{1}{T_m} = \frac{1}{T_m^0} - \frac{R}{\Delta H} \ln \frac{y_{S_1} y_{S_2}}{y_D} \quad (4)$$

It has been shown elsewhere [13] that the logarithm of the activity coefficient y_i is given to a good approximation by the product of the concentration c_P of the added inert polymer P and the so-called excluded volume α_{iP} of the polymer and the species i. The latter quantity is defined as the volume of the solution excluded to the molecules of i by the molecules of P, over and above the volume of the P molecules themselves, at unit concentration of P; it influences the activity coefficient in that, by virtue of the finite sizes of P and i, part of the solution is inaccessible to i, so that the effective concentration of i is raised by a factor $1/(1 - \alpha_{iP} c_P) \approx \exp(\alpha_{iP} c_P)$.

We can therefore rewrite eq. 4 as

$$T_m = T_m^0 + \frac{R T_m^{0^2}}{\Delta H} (\alpha_{S_1 P} + \alpha_{S_2 P} - \alpha_{DP}) c_P \quad (5)$$

where the approximation $T_m T_m^0 \approx T_m^{0^2}$ has been made. This is justified by the closeness of T_m and T_m^0 (cf. below).

3. Experimental

Buffers were prepared containing 100 mM NaCl, 2 mM EDTA, 20 mM sodium phosphate, pH 7.0, and in addition 0–10% (w/v) inert polymer and 0.05 g/l [poly(I)·poly(C)]. The inert polymers used were PEG 20000, PEG 4000, Dextran T70 and Dextran T10, with respective nominal molecular weights of 20000, 4000, 70000 and 10000. The RNA concentration had been chosen so as to give an absorption at 260 nm and room temperature of approx. 1.0. Before measurement, all solutions were co-dialysed for 4 h in low-M_r cut-off tubing against the same buffer without inert polymer or RNA, in order to eliminate any small variations in pH introduced by traces of acid or base contaminating the polymer.

Melting was followed by continuous monitoring of the hyperchromic shift of the RNA at 260 nm: analogue temperature and absorbance outputs from a modified Pye Unicam SP8-150 spectrophotometer were registered simultaneously so as to give a direct absorbance/temperature plot on an X-Y plotter. The temperature sensor was in direct contact with the solution sample and the temperature was raised at 1 degree/min; lower rates led to identical melting curves, so artefacts due to thermal lag were concluded to be absent.

4. Results

A typical absorption/temperature curve is shown in fig. 1. The melting temperature was determined in two ways, as indicated in the figure: (i) by the half-way point in the rise in absorbance, corresponding to T_m as defined above; and (ii) by the temperature T_m' at which the melting was complete. Strictly speaking, T_m is the correct melting temperature, but T_m' can be read off more accurately than T_m and, as the curves all have the same general form, T_m and T_m' should both depend upon polymer concentration in the way expressed by eq. 5.

Measured values of T_m and T_m', plotted as functions of the concentration of each polymer, are shown in fig. 2. Each measurement was made in duplicate with two separate aliquots of RNA solution prepared as described above. Although the effects are small, the reproducibility was satisfactory.

The T_m and T_m' values were subjected to a linear least-squares regression analysis. The inter-

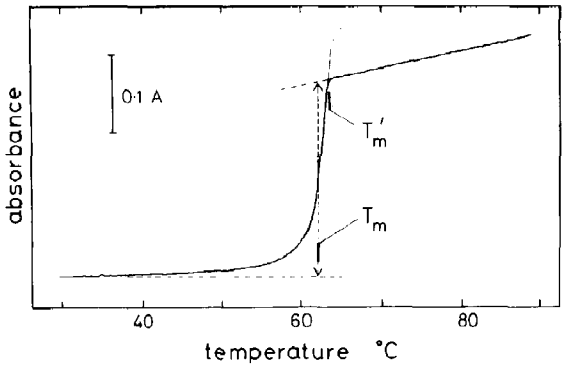

Fig. 1. Absorption/temperature curve of [poly(I)·poly(C)]. The curve shown, with absorbance zero suppression of approx. 1 unit, is for rising temperature (see text). Curves for falling temperature were very nearly superimposable, with a small error due to hydrolysis; these were not evaluated. The melting temperatures T_m and T'_m are indicated by thick bars: T_m is the temperature at which the hyperchromic shift due to melting is bisected by the melting curve, in accordance with the definition of T_m (see section 2); T'_m is the point of intersection of the extrapolated melting and post-melting branches of the curve.

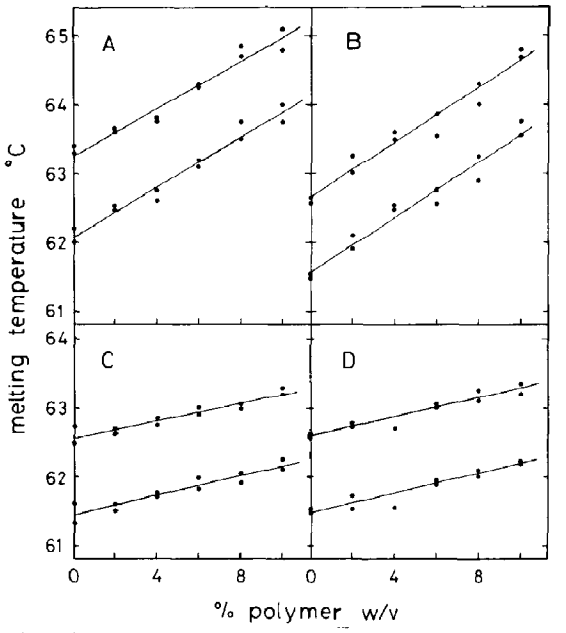

Fig. 2. Plots of T_m and T'_m vs. polymer concentration. A, PEG 20000; B, PEG 4000; C, Dextran T70; D, Dextran T10. In each case the upper line denotes T'_m and the lower T_m (cf. fig. 1). Values from duplicate measurements are indicated separately. The lines are those of the best fit (cf. table 1).

cepts T_m^0 or T'^0_m and the gradients β giving the best fit are shown in table 1. For PEG 4000, Dextran T70 and Dextran T10 the melting temperatures agree exactly ($T_m^0 = 61.6$, 61.4, 61.5°C; $T'^0_m = 62.6$, 62.6. 62.6°C, respectively). In the case of PEG 20000, the temperatures are all raised by approx. 0.6 degrees, which we attribute to a pH error as discussed above. This error has no noticeable effect on the gradient or on the subsequent analysis.

The gradients β of the T_m and T'_m plots are identical, within the stated error, in each case

Table 1

Analysis of the dependence of T_m upon polymer concentration

Data are taken from fig. 2. Average values of the gradients were used in calculating $\Delta\alpha$. The units of $\Delta\alpha$ are 1 mol^{-1} (a) and Å^3 per molecule (b); 'mole' and 'molecule' both refer to one [I·C] base-pair.

Polymer	Temperature	Intercept (°C)	Gradient (°C/1% polymer)	$\Delta\alpha$	
				a	b
PEG 20000	T_m	62.1	0.182±0.011	8.2	14000
	T'_m	63.3	0.171±0.011		
PEG 4000	T_m	61.6	0.202±0.013	1.8	3000
	T'_m	62.6	0.200±0.014		
Dextran T70	T_m	61.4	0.072±0.008	10.5	18000
	T'_m	62.6	0.064±0.007		
Dextran T10	T_m	61.5	0.071±0.005	1.6	2600
	T'_m	62.6	0.070±0.005		

(table 1); for subsequent calculations their averages were used. Differentiation and rearrangement of eq. 5 lead to the relation

$$\alpha_{S_1P} + \alpha_{S_2P} - \alpha_{DP} = \Delta\alpha = \frac{\Delta H}{RT_m^{0^2}} \cdot \frac{M_P}{10} \cdot \beta \qquad (6)$$

in which $\Delta\alpha$ is the change in molar excluded volume accompanying the melting and M_P is the molecular weight of the inert polymer (the factor $M_P/10$ converts the concentration units from % (w/v) to mol/l). To evaluate $\Delta\alpha$, ΔH was taken as 5 kcal/mol, a typical value [14], and R and T_m^0 were taken as 2 cal mol^{-1} K^{-1} and 336 K, respectively. The effective molecular weights of PEG 20000 and Dextran T70 have been found by Georgalis [11] to be $(21 \pm 2) \times 10^3$ and $(70 \pm 2) \times 10^3$, and the nominal molecular weights of 4000 and 10000 for PEG 4000 and Dextran T10, respectively, were assumed to be correct.

5. Discussion

The raising of the melting temperature of [poly(I)·poly(C)] by the inert polymers PEG and dextran is clear. This phenomenon cannot be due to electrostatic interaction between the RNA and the polymers, since the latter are neutral and do not contain strongly dipolar groups; 'hydrophobic' association and charge destabilisation by reduction of the bulk dielectric constant can also be ruled out, since both of these factors would favour the single strands and the addition of polymers would thus lower T_m rather than raise it. An explanation in terms of specific chemical interaction may also be ruled out, on account of the considerable difference between the chemical natures of the polyether PEG and the polyhydroxylic dextran.

It remains to be seen how far the rise in T_m can be explained by the excluded-volume effect. Qualitatively, the trend of the $\Delta\alpha$ values calculated on the basis of the thermodynamic reasoning presented above is consistent with the excluded-volume effect, since (i) the addition of inert polymer raises rather than lowers T_m, in accordance with the reasonable assumption of a positive value for $\Delta\alpha$ (eq. 6) and with the general tendency of inert polymers to enhance association (cf. section 1), and (ii) both for PEG and for dextran the higher-M_r species results in the greater observed value for $\Delta\alpha$. Furthermore, the values of $\Delta\alpha$ (table 1) lie within the range of macromolecular volumes and are therefore of the expected order of magnitude.

Having established that the excluded-volume effect is the qualitative cause of the rise in T_m on addition of inert polymer, we proceed to investigate this more quantitatively. To do this we require considerable simplifiying approximations. First, we regard the single and double helices as very long cylinders with radii r_S and r_D, respectively, while the polymer molecules are represented by equivalent spheres [15] of radius R. It is standard practice to make the latter assumption, while the former is reasonable, since both forms of RNA will be much stiffer than PEG or dextran, owing to their electrostatic charge and to the stacking of their bases.

Secondly, we need an expression for the exclusion volume. For a spherical macromolecule i (radius r_i) and a spherical inert polymer P this is a simple calculation: the centre of the macromolecule is excluded from a shell of radius $(r_i + R)$ around the surface of P, so that according to the definition (above and ref. 13), the excluded volume is given by

$$\alpha_{iP} = \tfrac{4}{3}\pi\left[(r_i + R)^3 - R^3\right] \qquad (7)$$

For a cylindrical macromolecule this may be reworked in two ways; however, neither of these is entirely satisfactory.

(i) We consider a stiff cylinder of radius r_i and length L moving in a fixed orientation (which stands for all other orientations) around a spherical inert polymer P. The volume unavailable for the axis of the cylinder is then, with end effects neglected, given by $[\pi(r_i + R)^2 L - \frac{4}{3}\pi R^3]$, or $\pi(r_i + R)^2$ per unit length, since $L \gg R$. Thus, for the melting of the double helix, we have per unit length

$$\Delta\alpha = 2\pi(r_S + R)^2 - \pi(r_D + R)^2 \qquad (8)$$

However, $R \gg r_D$, r_S, so that to a good approximation

$$\Delta\alpha = \pi R^2 t \qquad (9)$$

per base-pair, where t is the thickness of a purine or pyrimidine ring.

(ii) Alternatively, we may choose to regard the cylinder as being semi-flexible and composed of notionally spherical segments that we treat independently of one another. Since $R \gg r_D$, r_S, the formula for spheres (eq. 7) can be simplified to give $\alpha_{iP} \approx 4\pi r_i R^2$. Therefore we have, per base-pair,

$$\Delta\alpha = 4\pi R^2(2r_S - r_D)/n \qquad (10)$$

where n is the number of base-pairs in a segment.

The data do not allow a choice between the models. A plot of $\log_{10}(\Delta\alpha)$ vs. $\log_{10}(R)$ has a slope of 1.9 ± 0.9 (fig. 3), superficially confirming the square law expressed in eqs. 9 and 10. However, the fit is poor and the points for PEG lie systematically above the regression line and those for dextran below it. This is probably because the 'effective sphere' approximation is less applicable to the interaction between these polymers and the filamentous RNA than to the interaction between the polymers and proteins. It is also possible that the effective radii, measured at 20°C, may be different at 60°C.

The plot in fig. 3 yields $\Delta\alpha = (2 \pm 1)\pi R^2$ (radii in Å). This is compatible with eq. 9, in which the value of t expected is 3.4 Å. An exact comparison with eq. 10 cannot be performed, since r_S, r_D and n are not known; however, if we posit that the assumption of notionally spherical segments makes n of the order $n \approx r_D/t$, then we can take the experimental value of 2 and equate it with $\frac{4}{n}(2r_S - r_D)$ in eq. 10. This leads to the expression

$$\frac{r_D}{2r_S} \approx 1 - \frac{n}{4r_S} \qquad (11)$$

which with reasonable values of n (3–5) and r_S (10) gives $r_D/2r_S \approx 90\%$, which is of the right order of magnitude.

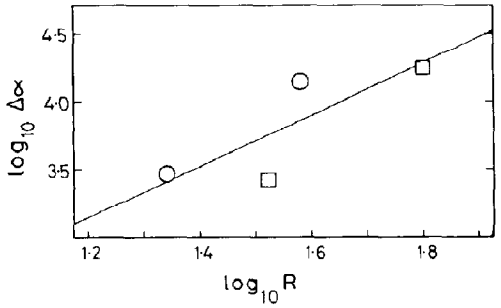

Fig. 3. Log-log plot of $\Delta\alpha$ vs. R. Circles, PEG; squares, dextran. For details, see text. R is the effective radius of the hydrated polymer, determined by Georgalis [11] for PEG 20000 (38 ± 2 Å) and for Dextran T70 (63 ± 2 Å) at 20°C; values for the smaller polymers are here assumed in proportion to the cube roots of their respective molecular weights (for PEG 4000, 22 Å and for Dextran T10, 33Å).

To recapitulate, close agreement between theory and experiment cannot at present be expected, since apart from the numerical approximations made in assuming the value of ΔH and in ignoring the inhomogeneity of the polymers in respect of their size, better prediction of $\Delta\alpha$ is made impossible by three factors: (i) the irregular geometry of the RNA, making r_S and r_D difficult to estimate and the difference $(2r_S - r_D)$ even more so, (ii) the unknown degree of penetration of the hydrated polymers by the filamentous RNA, worsening the effective-sphere assumption, and (iii) the interdependence of adjacent segments of the RNA molecule.

We conclude that the rise in melting temperature of [poly(I)·poly(C)] is most likely to be a result of the difference in the excluded volume between the inert polymer and double vis-à-vis single strands. Since the concentration of macromolecular material in the biological cell is frequently around 15–20% (w/w) [16], this effect may also be expected to influence significantly the association and dissociation of double helices of DNA in vivo, and to raise their melting point by some 2 degrees or more. Interestingly, it has been shown that the oriC enzyme system (DNA replication) is active in vitro only in the presence of approx. 6% (w/v) of inert polymer (PEG, poly (vinyl alcohol) or methylcellulose) [17]. If the normal functioning of complex, co-ordinated biological systems requires a certain concentration of non-participant macromolecules, then it is clear that excluded-volume effects in vivo are of more than academic importance.

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