Topography of Nucleic Acid Helices in Solutions

THE INTERACTION SPECIFICITIES OF OPTICALLY ACTIVE AMINO ACID DERIVATIVES

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The synthesis and interactions of the D- and L-enantiomers of the amino acid amide derivatives $\text{NH}_3^+ \cdot \text{CHR} \cdot \text{CO} \cdot \text{NH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{NH}(\text{CH}_3)_2 2\text{Br}^-$ (I) and lysyl dipeptides $\text{NH}_3^+ \cdot [\text{CH}_2]_4 \cdot \text{CH}(\text{NH}_3^+) \cdot \text{CO} \cdot \text{NH} \cdot \text{CHR} \cdot \text{CO}_2\text{H} 2\text{Br}^-$ (II) with poly rI, poly rC, poly rA, poly rU and calf thymus DNA is reported. The following results were found. (1) The degree of stabilization of the helices as measured by the T_m ('melting' temperature) of the helix-coil transition was dependent on the nature of the amino acid. (2) For the poly rI-poly rC helix, the L-enantiomers of salts (I) and (II) stabilized more than the D-enantiomers. The same was true for calf thymus DNA in the presence of salts (II) and for poly rA-poly rU in the presence of salts (II) and the proline derivatives of salts (I). (3) As R increased in size and became more apolar, the amount of stabilization of the poly rI-poly rC helix in the presence of salts (I) decreased. On the other hand, the amount of stabilization increased with more polar substituents. An attempt was then made to determine whether the difference in stabilization of the double-stranded helices at the T_m in the presence of the L- and D-enantiomers of salts (I) is due to the interaction with the helix, the random coil or both. A new method was developed for determining the binding of small ions to polyions that involves a competition between an insoluble polystyrene ion-exchange resin and the soluble polyion for the counterion. Dissociation constants are obtained for the complexes of single- and double-stranded helices with the salts (I). The results are illuminating and indicate that with certain helices, i.e. poly rA-poly rU, the interactions of salts (I) with the single strands may not be ignored. It is concluded that the high optical specificity found in Nature, i.e. D-ribose in nucleic acids and L-amino acids in proteins, cannot be attributed solely to monomer-polymer interactions described by Gabbay (1968).

The interaction of diamines

and substituted polyamines with nucleic acids has been the subject of numerous publications in the past few years. Gabbay & Kleinman (1967), Gabbay, Kleinman & Shimshak (1968) and Gabbay (1968) have briefly reported on their studies utilizing optically active polycationic amino acid derivatives of the general structure

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is observed for the helix-coil transition. Apolar R groups decreased the degree of stabilization of the helices by the salts (I). It should be noted that, at the T_m , the helix and the random coils are in equilibrium and that the salts (I) and (II) are expected to cause relative stabilization of one with respect to the other. To help clarify the factors involved, the investigation of the interactions of amino acid amide derivatives (I) with polynucleotides was undertaken. The effect of varying the side chain R and the effect of L- and D-enantiomers of salts (I) as well as the enantiomers of lysyl dipeptides (II) on the T_m of the helix-coil transition of poly rA-poly rU, poly rI-poly rC and calf thymus DNA were investigated. Moreover, the dissociation constants of the complexes of the salts (I) with poly rI, poly rC, poly rI-poly rC, poly rA, poly rU and poly rA-poly rU at two temperatures are reported in order to elucidate the interactions as the T_m is approached. A modified method utilizing an ion-exchange resin to determine binding affini-

ties of oppositely charged ions, i.e. dications to polyanions, is described. The technique promises to be useful and is shown to have several advantages over equilibrium-dialysis methods.

RESULTS AND DISCUSSION

Synthetic methods

The general reactions for the synthesis of the aminoacylethylenediamine derivatives (I) and the

lysyl dipeptides (II) are shown in Scheme 1. The condensation reaction was accomplished via the mixed anhydride at -5°C in dry toluene and/or tetrahydrofuran solvent. Under these conditions little racemization is observed (Greenstein & Winitz, 1961). The final products were characterized by melting point, C and H analysis, i.r., p.m.r. (proton magnetic resonance) and optical rotation measurements. Table 1 summarizes the physical properties of salts (I) and (II).

Scheme 1. General reactions for the synthesis of aminoacylethylenediamine derivatives (I) and the lysyl dipeptides (II). Cbz, $\text{C}_6\text{H}_5 \cdot \text{CH}_2 \cdot \text{O} \cdot \text{CO}$.

Table 1. *Physical characterization of the amino acid amides (I) and lysyl dipeptides (II)*

Compound	Derivative of (I) or (II)	m.p. ($^{\circ}\text{C}$)	$[\alpha]_D^{25}$ in water	Calc.		Found	
				C	H	C	H
1	Glycine	185–186	—	23.47	5.58	23.61	5.59
2	L-Alanine*	178–180	+14.6 $^{\circ}$	25.42	6.11	25.16	5.85
3	D-Alanine*	178–179	–15.8	25.42	6.11	25.35	5.90
4	L- α -Aminobutyric acid	153–156	+30.4	28.97	6.32	28.84	6.38
5	D- α -Aminobutyric acid	156–157	–32.6	28.97	6.32	29.04	6.41
6	L-Proline	174	–14.6	31.21	6.08	31.08	6.04
7	D-Proline	174	+15.5	31.21	6.08	31.20	6.14
8	L-Lysine*	123–125	+23.2	25.66	6.03	25.81	6.41
9	D-Lysine*	123–125	–24.0	25.66	6.03	25.85	5.93
10	D,L-Leucine	194–195	—	33.07	6.94	33.10	6.79
11	D,L-Phenylalanine	203–204	—	39.40	5.84	39.25	5.94
12	D,L-Methionine	219	—	28.62	6.09	28.55	6.11
13	D-Lysyl-D-leucine	106–107	–4.72	34.21	6.46	34.48	6.74
14	D-Lysyl-D-phenylalanine	120–121	–15.7	39.58	5.54	39.80	5.75
15	D-Lysylglycine	82–83	–13.1	26.32	5.25	26.19	4.98

*Analyses for hemihydrate.

Table 2. *Effects of various concentrations of salts (I) on the T_m of the helix-coil transition of poly rI·poly rC in 25mM-sodium phosphate buffer, pH 6.15 (25mM with respect to Na^+)*

The T_m of the poly rI·poly rC helix in the absence of salts (I) is found to be $48.8 \pm 0.4^\circ\text{C}$. At any one concentration of salts (I) T_m curves were run concurrently for each enantiomeric pair. Under these conditions $\Delta T_m = T_{m(L)} - T_{m(D)}$ is found to be reproducible to $\pm 0.3^\circ\text{C}$. The T_m measurements were carried out with the polynucleotides at a concentration of 60–70 μmol of P/l.

Compound	Derivative of (I)	T_m ($^\circ\text{C}$)				No. of positive charges/molecule	
		0.5mM	1.0mM	3.0mM	5.0mM	50 $^\circ\text{C}$	30 $^\circ\text{C}$
1	Glycine	—	62.8	68.0	69.8	2.00	2.00
2	L-Alanine	58.4	61.6	66.5	69.2	2.00	2.00
3	D-Alanine	56.5	59.8	64.0	66.8	2.00	2.00
2 and 3	DL-Alanine	—	60.0	65.6	67.4	2.00	2.00
4	L- α -Aminobutyric acid	57.2	59.8	65.6	67.5	1.88	2.00
5	D- α -Aminobutyric acid	55.8	57.4	62.8	64.6	1.85	2.00
4 and 5	DL- α -Aminobutyric acid	—	60.0	63.8	—	—	—
6	L-Proline	59.6	62.6	67.6	69.2	2.00	2.00
7	D-Proline	54.6	55.6	61.6	63.0	2.00	2.00
6 and 7	DL-Proline	—	61.8	66.0	—	—	—
8	L-Lysine	77.2	79.2	82.5	82.2	2.90	3.00
9	D-Lysine	75.8	77.7	80.4	80.4	2.98	3.00
8 and 9	DL-Lysine	—	78.6	80.7	—	—	—
10	DL-Leucine	—	55.0	59.0	60.3	1.75	2.00
11	DL-Phenylalanine	—	51.7	54.4	55.4	1.68	1.90
12	DL-Methionine	—	66.8	72.2	74.5	1.68	1.80

Table 3. *Effects of various concentrations of salts (I) on the T_m of the helix-coil transition of poly rA·poly rU in 25mM-sodium phosphate buffer, pH 6.25 (25mM with respect to Na^+)*

The numbering system for the compounds is the same as that used in Table 1. The T_m of the poly rA·poly rU helix in the absence of salts (I) is found to be $43.5 \pm 0.4^\circ\text{C}$. T_m curves for each enantiomeric pair were run concurrently. The T_m measurements were carried out with the polynucleotides at a concentration of 60–70 μmol of P/l.

Compound	Derivative of (I)	T_m ($^\circ\text{C}$)						No. of positive charges/molecule	
		0.1mM	0.3mM	0.5mM	1.0mM	3.0mM	5.0mM	50 $^\circ\text{C}$	30 $^\circ\text{C}$
2	L-Alanine	44.4	47.9	50.7	54.1	62.0	65.2	2.00	2.00
3	D-Alanine	44.1	47.9	50.8	54.1	60.8	65.2	2.00	2.00
4	L- α -Aminobutyric acid	—	—	—	—	62.4	64.0	1.88	2.00
5	D- α -Aminobutyric acid	—	—	—	—	62.0	64.5	1.85	2.00
6	L-Proline	—	—	—	—	62.8	64.5	2.00	2.00
7	D-Proline	—	—	—	—	61.8	63.7	2.00	2.00

Table 4. *Effects of salts (I) on the T_m of the helix-coil transition of calf thymus DNA in 25mM-sodium phosphate buffer, pH 6.25 (25mM with respect to Na^+)*

The numbering system for the compounds is the same as that used in Table 1. The T_m of calf thymus DNA in the absence of salts (I) is found to be $73.4 \pm 0.3^\circ\text{C}$. T_m curves for each enantiomeric pair were run concurrently. The T_m measurements were carried out with the DNA at a concentration of 60–70 μmol of P/l.

Compound	Derivative of (I)	Concn. (mM)	T_m ($^\circ\text{C}$)	No. of positive charges/molecule	
				50 $^\circ\text{C}$	30 $^\circ\text{C}$
6	L-Proline	5.0	87.0	2.00	2.00
7	D-Proline	5.0	86.2	2.00	2.00
8	L-Lysine	3.0	90.8	2.98	3.00
9	D-Lysine	3.0	90.0	2.98	3.00

Table 5. *Effects of 2 mM-lysyl dipeptides (II) on the T_m of the helix-coil transition of poly rI·poly rC, poly rA·poly rU and calf thymus DNA in 25 mM-sodium phosphate buffer, pH 6.25 (25 mM with respect to Na^+)*

T_m values of the poly rI·poly rC, poly rA·poly rU and calf thymus DNA helices in the absence of salts (II) are found to be 50.3 ± 0.4 , 44.6 ± 0.2 and $73.4 \pm 0.3^\circ\text{C}$, respectively. It should be noted that the T_m of poly rI·poly rC and poly rA·poly rU reported in Tables 2 and 3 respectively refer to different lot numbers and sources. T_m curves were run concurrently for each enantiomeric pair. The T_m measurements were carried out with the polynucleotides at a concentration of 60–70 μmol of P/l.

Compound	T_m ($^\circ\text{C}$)			No. of positive charges/molecule	
	Poly rI·poly rC	Poly rA·poly rU	Calf thymus DNA	50 $^\circ\text{C}$	30 $^\circ\text{C}$
L-Lysyl-L-leucine	56.4	48.1	76.8	1.95	1.98
D-Lysyl-D-leucine (13)	54.6	46.7	76.1	1.95	1.98
L-Lysyl-L-phenylalanine	53.2	46.3	75.2	1.84	1.91
D-Lysyl-D-phenylalanine (14)	51.8	45.5	74.8	1.84	1.91
L-Lysylglycine	53.4	46.6	75.0	1.87	1.94

Table 6. ΔT_m values for poly rI·poly rC, poly rA·poly rU and calf thymus DNA in the presence of the L- and D-enantiomers of salts (I) and (II)

Derivative of (I) or (II)	T_m ($^\circ\text{C}$)								
	poly rI·poly rC					poly rA·poly rU			Calf thymus DNA
	0.5 mM	1.0 mM	2.0 mM	3.0 mM	5.0 mM	2.0 mM	3.0 mM	5.0 mM	2.0 mM
Alanine	1.9	1.8		2.5	2.4				
α -Aminobutyric acid	1.4	2.4		2.8	2.9				
Lysine	1.4	1.5		2.1	1.8				
Proline	5.0	7.0		6.0	6.2		1.7	1.9	
Lysyl-leucine			1.8			1.4			0.7
Lysylphenylalanine			1.4			0.8			0.4

T_m studies

The effects of salts (I) and (II) on the thermal denaturation of poly rI·poly rC, poly rA·poly rU and calf thymus DNA are summarized in Tables 2–5. A number of observations can be made. (1) The degree of stabilization of poly rI·poly rC, poly rA·poly rU and calf thymus DNA is dependent on the nature of the amino acid. (2) The L-enantiomers of salts (I) and (II) stabilize the poly rI·poly rC helix more than do the D-enantiomers. The same is true for calf thymus DNA in the presence of salts (I) and (II). The L-enantiomers of salts (II) stabilize the poly rA·poly rU helix more than do the D-enantiomers. However, only the L-proline derivative (compound 6) of salts (I) shows a significantly greater stabilization than the D-proline derivative (compound 7). There is no difference in the stabilization of poly rA·poly rU in the presence of the alanine and α -aminobutyric acid enantiomeric-pair compounds 2, 3, 4 and 5. (3) There is very little difference in $\Delta T_m = T_{m(L)} - T_{m(D)}$ going from alanine to α -aminobutyric acid (R going from

methyl to ethyl) for the poly rI·poly rC helix. However, a much greater T_m is observed for the proline derivatives. ΔT_m in the presence of salts (II) increases in the following order: calf thymus DNA < poly rA·poly rU < poly rI·poly rC (see Table 6). (4) As the R group of salts (I) increases in size and becomes more hydrophobic, the relative amount of stabilization of poly rI·poly rC decreases. The same is observed for poly rA·poly rU in the presence of salts (I), but the decrease in stabilization is smaller. As the R group of salts (I) becomes more polar, the relative degree of stabilization of poly rI·poly rC increases. (5) The relative amount of stabilization of poly rI·poly rC, poly rA·poly rU and calf thymus DNA is greater for salts (I) than for salts (II). (6) Changing R from a non-polar to a polar group does not alter the relative order of stabilization by the L- and D-enantiomers.

There are several ways these results may be interpreted. Diammonium salts have been shown to interact with adjacent phosphate groups on the polynucleotide chain (Gabbay, 1966, 1967, 1968; Gabbay & Shimshak, 1968). Gabbay *et al.* (1968)

have also shown that the D-enantiomers of salts (I) inhibit the ribonuclease-catalysed hydrolysis of poly rA to a greater extent than do the L-enantiomers. The diammonium salts (I) and (II) would be expected to stabilize the double helix by interacting with adjacent phosphate groups and diminishing interchain repulsion. Increasing the hydrophobic nature of group R would tend to decrease the dielectric constant of the environment surrounding the phosphate groups, thus increasing the free energy of the double helix. The free energy of the random coils would also be increased, but, since there is the additional interchain repulsion in the double helix, the free energy of the latter is expected to increase more than the free energy of the random coils in a medium of low dielectric constant. Likewise, when group R becomes more polar, the dielectric constant of the medium surrounding the phosphate groups would increase, leading to a relative stabilization of the double helix with respect to the random coils.

The exact nature of the difference between the interactions of the L- and D-enantiomers is much more difficult to visualize. If the interaction of the diammonium salts with both the double helix and the random coils at the T_m is considered, there are three possible situations that can exist.

Situation I. The dissociation constant, K_H , of the diammonium salt-helix complex is of the same order of magnitude as the dissociation constant, K_C , of the diammonium salt-random coil complex, i.e. $K_H \sim K_C$:

If $K_H \sim K_C$, in order for the L-enantiomer to stabilize more than the D-enantiomer then: (1) $K_{H(L)} < K_{H(D)}$ and $K_{C(L)} \sim K_{C(D)}$; (2) $K_{H(L)} \sim K_{H(D)}$ and $K_{C(L)} > K_{C(D)}$; or (3) $K_{H(L)} < K_{H(D)}$ and $K_{C(L)} > K_{C(D)}$.

Situation II. The dissociation constant for the helix-diammonium salt complex is less than the dissociation constant of the random coil-diammonium salt complex ($K_H < K_C$):

If $K_H < K_C$, in order for the L-enantiomer to stabilize more than the D-enantiomer then $K_{H(L)} < K_{H(D)}$.

Situation III. The dissociation constant of the helix-diammonium salt complex is greater than the dissociation constant of the random coil-diammonium salt complex ($K_H > K_C$):

If $K_H > K_C$, in order for the L-enantiomer to stabilize more than the D-enantiomer then $K_{C(L)} > K_{C(D)}$.

There is some indication that, for single-stranded poly rA, the D-enantiomer interacts more strongly than does the L-enantiomer. As cited above, Gabbay *et al.* (1968) have found that the D-enantiomers of salts (I) inhibit the ribonuclease-catalysed hydrolysis of poly rA more than do the L-enantiomers. This finding, however, does not give any indication of the interactions of the enantiomeric pairs with the double-stranded poly rA·poly rU. There is also the possibility that the difference in inhibition is due, not to a binding phenomenon alone, but also to a steric factor involving the displacement of optically active diammonium salts by an optically active catalyst, i.e. ribonuclease.

Dissociation constants

In the preceding section the stabilization of double-stranded polynucleotides in the presence of salts (I) and (II) was discussed. In the present section an attempt is made to determine which of the three proposed situations best explains the difference in stabilization between the L- and D-enantiomeric pairs. In order to determine the dissociation constants it is necessary to know the concentration of free and bound salts (I). Preliminary experiments showed that the ninhydrin assay for the determination of the concentrations of α -amino acid amides (I) (for details see the Experimental section) does not distinguish between free and bound salts. It was therefore necessary to find a method that would determine one or the other. It was considered that the standard procedures for determining dissociation constants, e.g. equilibrium dialysis, were too time-consuming for the number of determinations necessary, and that the errors involved were too large. The method chosen was a modification of the procedure used by Fasella, Hammes & Schimmel (1965) to determine the binding of cytidine 3'-phosphate to ribonuclease. The modified method involves a competition between Dowex 50W (X2) cation-exchange resin and the polynucleotides for salts (I). Knowledge of the dissociation constant for salts (I) and

Table 7. *Diammonium salt concentration in supernatant as a function of time*

The initial diammonium salt concentration was 20 mM. For details of procedure, see the Experimental section.

Incubation time (min)	E_{570} of 0.10 ml sample	Total concn. in supernatant (mM)
15	0.698	5.25
30	0.491	3.74
60	0.461	3.52
120	0.478	3.64

Table 8. *Various models for the interactions of the diammonium ions with polyions*

Model 1	$^+\text{NR}_3\text{SO}_3^-$	Eqns. (1), (2)	$n = x = 1$
Model 2	$\begin{pmatrix} ^+\text{NR}_3\text{SO}_3^- \\ ^+\text{NR}_3\text{SO}_3^- \end{pmatrix}$	Eqn. (1)	$n = x = 2$
Model 3	$\begin{pmatrix} ^+\text{NR}_3\text{SO}_3^- \\ ^+\text{NR}_3\text{SO}_3^- \\ ^+\text{NR}_3\text{SO}_3^- \\ ^+\text{NR}_3\text{SO}_3^- \end{pmatrix}$	Eqn. (1)	$n = 4, 6, \dots$ $x = 2$
Model 4	$\begin{pmatrix} ^+\text{NR}_3\text{SO}_3^- \\ ^+\text{NR}_3\text{SO}_3^- \end{pmatrix}$	Eqn. (2)	$n = x = 2$
Model 5	$\begin{pmatrix} ^+\text{NR}_3\text{SO}_3^- \\ ^+\text{NR}_3\text{SO}_3^- \\ ^+\text{NR}_3\text{SO}_3^- \\ ^+\text{NR}_3\text{SO}_3^- \end{pmatrix}$	Eqn. (2)	$n = 4, 6, \dots$ $x = 2$

the resin would enable the free salt (I) concentration in the presence of polynucleotide to be determined. The Law of Mass Action was used to determine the dissociation constant for the salts (I)-resin complex. A simpler treatment using a partition coefficient, $K_D = \text{salts (I)}_{\text{solution}} / \text{salts (I)}_{\text{resin}}$, was attempted first, but the errors were too great. The large deviations in the partition coefficient are probably due to the variation in the Na^+ concentration released by the resin on binding various concentrations of salts (I). An attempt was made to increase the total Na^+ concentration, but Na^+ concentrations high enough to be considered constant did not permit enough salt (I) to bind to the resin. Therefore a more rigorous treatment, using the Law of Mass Action, was employed.

Description of the system. The amount of resin and Na^+ added was held constant, and the total diammonium salt concentration was varied. Before any concentration studies were made, the length of time for the system to come to equilibrium was determined, and it was found that the diammonium

salt concentration in the supernatant remained constant after 30 min (see Table 7).

The models considered are shown in Table 8, and the dissociation constants corresponding to each one were determined. Model 1 refers to a simple electrostatic interaction between single charges. Models 2 and 3 refer to the interaction of the diammonium salts as monomers or x -mers with single sulphonate groups. In models 4 and 5 the possibility of a certain amount of ordering in the free polymer is considered.

Calculation of the dissociation constants for the interaction of salts (I) and Dowex 50W (X2). The equations describing the above model systems can be reduced to two general equations if the following assumptions are made. (1) The binding of one unit of salts (I) to the resin, either as the monomer or x -mer, is independent of the binding of any other unit to the resin. This assumption should be valid at ratios of salts (I) to resin less than 1. The highest ratio of salts (I) to resin used was 0.5 for the alanine and α -aminobutyric acid derivatives (I).

(2) The binding of each unit is independent of the position on the polymer, i.e. all sulphonate groups or units of sulphonate groups are equivalent. Assumption (1) describes the interaction of salts (I) with single sulphonate groups, and assumption (2) describes the interaction of salts (I) with units of sulphonate groups. Varying n generates the specific equation describing each model.

$$K_{R(I)} = \frac{[\text{SO}_3^-\text{Na}^+]^n [(\text{NR}_3)_x]^{n/x}}{[(\text{SO}_3^-\text{NR}_3)_n][\text{Na}^+]^n}$$

$$K_{R(II)} = \frac{[(\text{SO}_3^-\text{Na}^+)_n][(\text{NR}_3)_x]^{n/x}}{[(\text{SO}_3^-\text{NR}_3)_n][\text{Na}^+]^n}$$

where n is the number of positive charges interacting with n negative charges: $n = 1, 2, 4, 6, \dots$ for diammonium salts and $n = 1, 3, 6, 9, \dots$ for triammonium salts; x is the number of positively charged nitrogens per molecule of the amides (I).

Since the amount of resin is held constant, a new $K_{R(I)}$ can be defined:

$$K'_{R(I)} = K_{R(I)}(\text{ml of resin})^{n-1} = \frac{(RNa)^n(d)^{n/x}}{(Rd)(Na^+)^n} \quad (3)$$

where d = mequiv. of NR_3 uncomplexed; RNa = mequiv. of SO_3^-Na^+ uncomplexed; Rd = mequiv. of SO_3^-NR_3 ; Na^+ = mequiv. of free Na^+ . It should be noted that both $K'_{R(I)}$ and $K_{R(II)}$, for $n = x = 1$, reduce to the equation describing model 1. The experimental quantity d was determined from the extinction values obtained from the ninhydrin assays, and converted into mequiv. calculated from standard curves.

Results of the salts (I)-Dowex 50 (X2) dissociation studies. Each concentration at 50°C was run in duplicate and the dissociation constants for each value of n were calculated by means of an IBM 720

computer. The dissociation constants at 30°C were run in triplicate at one concentration. To determine which model best describes the interactions, the assumption was made that the value of n that best describes the equilibrium is that value which gives the smallest error within experimental error over the concentration range used. Model 2, with $n = x = 2$, gives the best results, i.e. the least error, with the diammonium salts. Table 9 gives the dissociation constants, $K'_{R(I)}$, at 30 and 50°C. It is noted that the dissociation constants for the dipositive enantiomeric pairs are approximately the same, i.e. the differences are within the experimental error.

Calculation of the dissociation constants for the interaction of salts (I) with polynucleotides

The same type of models were chosen as in the resin-salts (I) equilibria. Making the same assumptions, the following equations can be written:

$$K_{N(I)} = \frac{[(\text{PO}_4^-\text{Na}^+)_n][(\text{NR}_3)_x]^{n/x}}{[(\text{PO}_4^-\text{NR}_3)_n][\text{Na}^+]^n} \quad (4)$$

$$K_{N(II)} = \frac{[(\text{PO}_4^-\text{Na}^+)]^n [(\text{NR}_3)_x]^{n/x}}{[(\text{PO}_4^-\text{NR}_3)_n][\text{Na}^+]^n} \quad (5)$$

where n is the number of positive charges interacting with n negative charges on the polynucleotide; $n = 1, 2, 4, 6, \dots$ for diammonium salts and $n = 1, 3, 6, 9, \dots$ for triammonium salts; x is the number of positively charged nitrogens per molecule of salt (I).

$K_{N(I)}$ describes the interaction of salts (I) with units of phosphate groups. This implies a certain amount of ordering in the uncomplexed polynucleotides. $K_{N(II)}$ describes the interaction of salts (I) with single phosphate groups.

The dissociation constants, $K_{N(I)}$ and $K_{N(II)}$, may be rewritten in the more convenient forms:

$$K_{N(I)} = \frac{(NNa)(d)^{n/x}}{(Nd)(Na^+)^n} \quad (6)$$

Table 9. Dissociation constants ($K'_{R(I)}$, $n = 2$ and $n = 3$) for salts (I) and Dowex 50W (X2) at 30 and 50°C

Derivative of (I)	n	$K'_{R(I)}$	
		30°C	50°C
L-Alanine	2	0.0289 ± 0.0032	0.0293 ± 0.0025
D-Alanine	2	0.0251 ± 0.0019	0.0316 ± 0.0035
L- α -Aminobutyric acid	2	0.0165 ± 0.0009	0.0233 ± 0.0023
D- α -Aminobutyric acid	2	0.0174 ± 0.0009	0.0271 ± 0.0025
L-Lysine	3	—	0.00139 ± 0.00019
D-Lysine	3	—	0.000707 ± 0.000055

and

$$K_{N(II)} = \frac{(NNa)^n(d)^{n/x}}{(Nd)(Na^+)^n} \quad (7)$$

where NNa is the mequiv. of $PO_4^-Na^+$ uncomplexed; Nd is the mequiv. of $PO_4^-NR_3$; d is the mequiv. of NR_3 uncomplexed. Since K_R and K_N are the dissociation constants for competing equilibria, d can be calculated from the resin-salt (I) dissociation constant:

$$d = \frac{K'_{R(I)}(Rd)(Na^+)^n}{(RNa)^n(\text{ml of soln.})} \quad (8)$$

The experimentally determined quantity, D , is no longer free salt (I), but free salt (I) plus salt (I) bound to polynucleotide ($D = Nd + d$).

Results of the salts (I)-polynucleotide dissociation studies

Concentration studies were run in duplicate first to determine the type of binding, and dissociation constants for each value of n were calculated by using an IBM 720 computer. The assumption was again made that the value of n that best describes the equilibrium is that value which gives the smallest error within the experimental error over the concentration range used. This corresponds to the model describing the interaction between salt (I) and units of two phosphate groups $K_{N(I)}$ ($n = 2$). Table 10 gives the dissociation constants for the

diammonium salts and the single- and double-stranded polynucleotides at 50°C and 30°C.

The following observations can be made (see Table 10).

(1) Poly rA·poly rU.

(a) $K_H \sim K_C$ at 30 and 50°C.

(b) The dissociation constants for the alanine and α -aminobutyric acid derivatives show no appreciable differences between enantiomers.

(c) The differences in K_H for the alanine and α -aminobutyric acid derivatives are small.

(2) Poly rI·poly rC.

(a) $K_H < K_C$ at 50°C, and $K_H \sim K_C$ at 30°C.

(b) $K_{H(L)} < K_{H(D)}$ at 30 and 50°C.

(c) K_H (L-alanine) $< K_H$ (L- α -aminobutyric acid) and K_H (D-alanine) $< K_H$ (D- α -aminobutyric acid) at 30 and 50°C.

(3) There is a greater temperature-dependence for the binding of salts (I) to the single-stranded poly rI and poly rC than to the single-stranded poly rA and poly rU. The L- and D- α -aminobutyric acid derivatives also show a greater temperature-dependence than do the L- and D-alanine derivatives for poly rI and poly rC.

(4) The binding of salts (I) to the double-stranded poly rI·poly rC and poly rA·poly rU helices shows very little temperature-dependence.

On going back to the original problem of what causes the differences in T_m between the L- and D-enantiomers, it is difficult to come to a clear-cut conclusion. It has been shown by the dissociation constant studies that situation III [dissociation of the salt (I)-double-stranded polynucleotide com-

Table 10. *Dissociation constants for the salt (I)-polynucleotide complex at 30 and 50°C in 50 mM-sodium phosphate buffer, pH 6.0 (50 mM with respect to Na⁺)*

Polynucleotide	$K_{N(I)} (n = 2)$			
	L-Alanine		D-Alanine	
	50°C	30°C	50°C	30°C
Poly rA	1.1 $\pm$ 0.1	1.2 $\pm$ 0.4	1.4 $\pm$ 0.4	1.3 $\pm$ 0.5
Poly rU	1.3 $\pm$ 0.3	0.76 $\pm$ 0.10	2.0 $\pm$ 0.7	1.4 $\pm$ 0.5
Poly rA·poly rU	1.4 $\pm$ 0.6	1.3 $\pm$ 0.2	1.4 $\pm$ 0.3	1.1 $\pm$ 0.3
Poly rI	1.7 $\pm$ 0.1	1.0 $\pm$ 0.3	1.7 $\pm$ 0.5	1.0 $\pm$ 0.1
Poly rC	2.2 $\pm$ 0.3	1.7 $\pm$ 0.4	4.1 $\pm$ 0.1	1.9 $\pm$ 0.7
Poly rI·poly rC	0.51 $\pm$ 0.02	0.38 $\pm$ 0.08	1.2 $\pm$ 0.1	0.84 $\pm$ 0.11
	L- α -Aminobutyric acid		D- α -Aminobutyric acid	
	50°C	30°C	50°C	30°C
Poly rA	1.9	1.7 $\pm$ 0.1	2.3 $\pm$ 0.1	1.6 $\pm$ 0.2
Poly rU	1.5 $\pm$ 0.4	0.79 $\pm$ 0.30	1.5 $\pm$ 0.1	1.2 $\pm$ 0.4
Poly rA·poly rU	1.1 $\pm$ 0.3	1.1 $\pm$ 0.1	1.5 $\pm$ 0.1	1.1 $\pm$ 0.2
Poly rI	2.8 $\pm$ 0.7	0.72 $\pm$ 0.08	3.0 $\pm$ 1.2	1.3 $\pm$ 0.3
Poly rC	6.8 $\pm$ 0.9	1.5 $\pm$ 0.3	7.0 $\pm$ 0.1	1.0 $\pm$ 0.2
Poly rI·poly rC	1.3 $\pm$ 0.1	0.97 $\pm$ 0.30	1.7 $\pm$ 0.3	1.7 $\pm$ 0.3

plex is greater than the dissociation constant of the salt (I)-single-stranded polynucleotide complex] does not explain the results, since in all cases $K_H \leq K_C$.

The behaviour of the double-stranded poly rI·poly rC helix at the T_m is not the same as that of the double-stranded poly rA·poly rU helix at the T_m . This difference appears to be due to the interactions of salts (I) with the single-stranded polynucleotides rather than the double-stranded polynucleotides. For poly rA·poly rU $K_H \sim K_C$ near the T_m , and any results must be explained by looking at both the single and double strands. For poly rI·poly rC $K_H < K_C$ near the T_m , and the temperature-dependence for the single strands is greater than for the double strands; i.e. K_C increases faster than K_H with a change in temperature. The behaviour of poly rI·poly rC at the T_m can thus be explained by considering only the interactions with the double-stranded polynucleotide.

The dissociation-constant results are in line with the T_m studies. The poly rA·poly rU helix did not show any difference in T_m in the presence of the D- and L-enantiomers of salts (I), except for the proline enantiomeric pairs, nor was there any appreciable difference in the interactions of the D- and L-alanine and D- and L- α -aminobutyric acid derivatives with the single-stranded poly rA and poly rU or the double-stranded poly rA·poly rU. The poly rI·poly rC helix did show a difference in the T_m in the presence of the D- and L-enantiomers of salts (I), and this difference is reflected in the dissociation constants. For the double-stranded poly rI·poly rC $K_{H(L)} < K_{H(D)}$ for both pairs of enantiomers near the T_m .

For the interactions of salts (I) with the polynucleotides, the results are summarized below.

(1) For the poly rA·poly rU system at the T_m the binding of the diammonium salts to both the helix and the random coil should be considered (situation I).

(2) For the poly rI·poly rC system at the T_m it is possible to consider only the binding of the diammonium salts to the helix (situation II).

(3) The interaction of polynucleotides with the amino acid amide derivatives appears to be dependent on the base as well as the amino acid.

EXPERIMENTAL

Synthesis

The general reactions for the synthesis of salts (I) and (II) are shown in Scheme 1 and discussed above. Analyses were performed by George Robertson, Florham Park, N.J., U.S.A. Routine i.r. spectra were taken on a Perkin-Elmer model 137 Infracord i.r. spectrophotometer. The p.m.r. spectra were taken on a Varian model A-60 spectrometer. Optical rotations were measured with a Perkin-

Elmer model 141 polarimeter. Melting points were measured on a Mel-temp apparatus and are uncorrected. Benzyloxycarbonyl chloride, benzyloxycarbonylglycine, L- and D- α -aminobutyric acid and glycine ethyl ester hydrochloride were purchased from Aldrich Chemicals Inc., Milwaukee, Wis., U.S.A. The benzyloxycarbonyl derivatives of L-, D- and DL-alanine, L-lysine, L- and D-proline, L- and DL-methionine, L- and DL-leucine and L- and DL-phenylalanine were purchased from Mann Research Laboratories Inc., New York, N.Y., U.S.A. D-Leucine, D-phenylalanine and L-valine are also purchased from Mann Research Laboratories Inc. D-Lysine was purchased from International Chemical and Nuclear Co., Irvine, Calif., U.S.A. Isobutyl chloroformate was purchased from Eastman Chemicals, Rochester, N.Y., U.S.A., and stored over CaCO_3 at 0°C . *unsym.*-NN-Dimethylethylenediamine was purchased from Aldrich Chemicals Inc. and purified by distillation (b.p. 101°C) from KOH pellets.

T_m determination

Poly rA (lot no. 55451), poly rU (lot nos. 63671 and 76106), poly rC (lot nos. 63217 and 46114) and poly rI (lot no. 64437) were purchased from Calbiochem, Los Angeles, Calif., U.S.A. Calf thymus DNA (DNA 642) was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Stock solutions of the homopolymers were prepared by the procedure described by Gabbay (1967). T_m determinations were run as described by Gabbay & Kleinman (1967).

Determination of dissociation constants

Preparation of resin. To approx. 25g of wet resin (Dowex 50W; X2; H^+ form) was added 1 litre of 0.1M-NaOH. The resin was stirred overnight at room temperature. The resin was allowed to settle and the supernatant was removed with the aspirator. Approx. 1 litre of glass-distilled water was added and the resin was stirred for 15 min. The resin was allowed to settle and the supernatant was removed. The washing was repeated until the pH of the supernatant remained constant. The resin was filtered and air-dried for at least 2 days.

Determination of Q (mequiv. of SO_3^-Na^+ /g of dry resin). To approx. 1g of wet resin (Dowex 50W; X2; H^+ form) was added 100.0ml of 0.100M-NaOH. The resin was equilibrated with stirring for 2 days. The resin was quantitatively filtered and then washed to remove excess of NaOH, and the filtrate and washings were titrated with 0.100M-HCl to a phenolphthalein end point. The Na^+ form of the resin was air-dried and weighed to $\pm 0.2\text{mg}$. $Q = 3.80 \pm 0.06$ mequiv. of SO_3^-Na^+ /g of dry resin.

Determination of the dissociation constants of polynucleotide-diammonium salt complexes. (a) Equilibrium between resin and diammonium salts. In a typical run, Dowex 50W (X2; Na^+ form) (30.0mg) was put into a water-jacketed glass vessel, which was connected to a Haake constant-temperature water circulator. Diammonium salt (0.10–1.00ml, 5–50mM), 0.50ml of 0.20M-sodium phosphate buffer, pH 6.10 (0.20M with respect to Na^+), and glass-distilled water to give a total volume of 2.00ml were added. The solution was stirred for 1h at

constant temperature. The resin was allowed to settle, and the supernatant was carefully transferred to a small test tube with a Pasteur pipette. The amount of diammonium salt in solution was determined by a ninhydrin assay.

(b) Equilibrium between polynucleotide and diammonium salt. In a typical run, 30.0mg of Dowex 50W (X2; Na⁺ form) was put into the apparatus. Diammonium salt (0.10–1.00ml, 2.5–25mM), polynucleotide (1.00ml, 6.00mM in 0.10M-sodium phosphate buffer, pH6.15) and glass-distilled water to 2.00ml were added. Poly rA (lot no. 11849), poly rU (lot no. 41855), poly rI (lot no. 39726) and poly rC (lot no. 212726) were purchased from Miles Laboratories Inc., Elkhart, Ind., U.S.A. The stock solutions were checked for purity by column chromatography. The solution was stirred for 1h at constant temperature. The supernatant was withdrawn and the amount of diammonium salt was determined by a ninhydrin assay.

(c) Ninhydrin assay. The following reagents were used. (1) Ninhydrin solution: ninhydrin (Fisher Scientific Co., New York, N.Y., U.S.A.) (1.5g) was dissolved in 20ml of ethanol; N₂ was bubbled through, the bottle was sealed with a serum cap and the solution was stored in a desiccator over CaCl₂. (2) KCN–pyridine reagent: 2ml of 0.01M-KCN was diluted to 100.0ml with spectroscopic-grade pyridine; N₂ was bubbled through and the solution was stored in a desiccator over CaCl₂. (3) 80% phenol: reagent-grade solid phenol (Allied Chemical Co., Marcus Hook, Pa., U.S.A.) (80.0g) was dissolved in 20ml of ethanol with gentle heating; N₂ was bubbled through and the solution was stored in a desiccator over CaCl₂. (4) 60% ethanol: ethanol (225ml) was mixed with 150ml of glass-distilled water.

The diammonium salt solution (0.10–0.50ml) was pipetted into a 10cm test tube. Glass-distilled water was added to bring the volume to 0.50ml. KCN–pyridine reagent (2.0ml) and 80% phenol (2.0ml) were pipetted in with a pipetting bulb. The test tube was capped with

a serum cap and a 2.5cm hypodermic needle was inserted into the serum cap. The solution was heated in a hot-water bath at 98°C for 100s. Ninhydrin (0.2ml) was added through the hypodermic needle by using a 0.25ml tuberculin syringe fitted with a Luer Lok. The solution was mixed by shaking quickly and the reaction was allowed to proceed for 300s. The reaction was quenched in an ice–water bath and the solution was diluted to 10ml with 60% ethanol. The E_{570} value was read against 60% ethanol on a Beckman DU-2 spectrophotometer equipped with a Gilford automatic cuvette-positioner. For a reagent blank 0.50ml of glass-distilled water, treated as above, was used. Unknown concentrations were determined by plotting known concentrations versus the E_{570} values of the ninhydrin solutions after reaction.

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