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The Effects of Polyamines on the Melting of Polyriboinosinic Acid Plus Polyribocytidylic Acid Complex in Solution

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The thermal transition profile (melting profile) of the double-helical complex of polyriboinosinic acid plus polyribocytidylic acid, poly(I+C), into random coils in solution has been examined by the observation of the ultraviolet absorbance at 248 mμ of the solution. The effects of Na^+, Mg^{2+}, NH_4^+, $CH_3NH_3^+$, $NH_3^+(CH_2)_nNH_3^+$ (where $n=2$, 3, 4, 5, 6, and 8), spermidine, spermine, actinomycin D, and poly-L-lysine upon the melting profile have been observed. Among the diamines examined, that with $n=3$ has been found to have the maximum effect. Poly-L-lysine has been found to form a complex with poly(I+C) in the solution with a stoichiometric mole ratio of $NH_2 : P=0.5 : 1$.

The interactions of polyamines with nucleic acids seem to play important roles in some stages of many biological processes. Spermine $NH_3^+(CH_2)_3NH_2^+(CH_2)_4NH_2^+(CH_2)_3NH_3^+$, for example, which is widely distributed in biological materials,(1) is known to affect the deoxyribonucleic acid (DNA) multiplication in some bacterial systems. In the chromosomes of higher organisms, DNA is found as a complex with histon, which is a lysine- and arginine-rich basic protein. In view of such biological importance, we have attempted to obtain fundamental knowledge about the interactions of polyamines with nucleic acids.

Polyriboinosinic acid (poly I) and polyribocytidylic acid (poly C) are synthetic polymers which have the same ribosephosphate backbone as natural ribonucleic acid (RNA).(2) A 1 : 1 mixture of poly I and poly C is known to form a double-stranded helical complex, poly(I+C), in solution.(3) Its structure(4) is considered to be similar to that of natural double-stranded DNA.(5) Some general discussions of the "melting" of such a double-helical nucleic acid were given in a previous paper.(6) Along this line we have been observing the effects of polyamines on the "melting" profile of poly(I+C) in solution. Quite recently, however, a similar work with poly(I+C), which was done independently of our work, has been reported by Mehrotra and Mahler,(7) as have been a few similar examinations made previously with DNA.(8-10) Some of our results, which differ from those reported in other papers, will be presented in this paper and Discussed.

Experimental

The polyriboinosinic acid (poly I) and polyribocytidylic acid (poly C) used in this work were prepared by the use of the polynucleotide phosphorylase obtained from *Azotobacter vinelandii*. Both polynucleotides showed a sedimentation coefficient of 4s—8s in ultracentrifugal examination. The polynucleotide concentration in each standard solution was determined by measuring the phosphorus content.(11,12) Ethylenediamine dihydrochloride $NH_3^+(CH_2)_2NH_3^+\cdot 2Cl^-$, 1, 4-diaminobutane

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2) M. Grunberg-Manago, P. J. Ortiz and S. Ochoa, *Science*, **122**, 907 (1955).

3) D. R. Davies and A. Rich, *J. Am. Chem. Soc.*, **80**, 1003 (1958).

4) D. R. Davies, *Nature*, **186**, 1030 (1960).

5) R. Langridge, W. E. Seeds, H. R. Wilson, C. W. Hooper, M. H. F. Wilkins and L. D. Hamilton, *J. Biophys. Biochem Cytol.*, **3**, 767 (1957).

6) M. Tsuboi, This Bulletin, **37**, 1514 (1964).

7) B. D. Mehrotra and H. R. Mahler, *Biochim. Biophys. Acta*, **91**, 78 (1964).

8) H. R. Mahler, B. D. Mehrotra and C. W. Sharp, *Biochem. Biophys. Res. Comm.*, **4**, 79 (1961).

9) M. Mandel, *J. Mol. Biol.*, **5**, 435 (1962).

10) H. Tabor, *Biochemistry*, **1**, 496 (1962).

11) P. S. Chen, T. Y. Toubarer and H. Warner, *Anal. Chem.*, **28**, 1756 (1956).

12) B. N. Ames and D. T. Dubin, *J. Biol. Chem.*, **235**, 769 (1960).

dihydrochloride $NH_3^+(CH_2)_4NH_3^+\cdot 2Cl^-$, 1, 5-diaminopentane dihydrochloride $NH_3^+(CH_2)_5NH_3^+\cdot 2Cl^-$, 1, 6-diaminohexane dihydrochloride $NH_3^+(CH_2)_6NH_3^+\cdot 2Cl^-$ and 1, 8-diaminooctane dihydrochloride $NH_3^+(CH_2)_8NH_3^+\cdot 2Cl^-$ were obtained from the Tokyo Kasei Co. 1, 3-Diaminopropane dihydrochloride was obtained by introducing hydrogen chloride into 1, 3-diaminopropane (purchased from the Tokyo Kasei Co.) dissolved in ethanol. Spermidine trihydrochloride $NH_3^+(CH_2)_3NH_2^+(CH_2)_4NH_3^+\cdot 3Cl^-$ was obtained from the Nutritional Biochemicals Corporation, and spermine tetrahydrochloride $NH_3^+(CH_2)_3NH_2^+(CH_2)_4NH_2^+(CH_2)_3NH_3^+\cdot 4Cl^-$ from the Sigma Chemical Co. Poly-L-lysine hydrobromide with average molecular weight of 60000 was purchased from Pilot Chemicals, Inc. The poly-L-lysine concentration in our standard solution was determined by hydrolyzing it with 6 N hydrochloric acid and by then measuring the lysine content by the use of 2, 4-dinitrofluorobenzene.[13]

The ultraviolet absorption measurements were made by the use of an Ito Spectrophotometer, model QU-3. The temperature of the sample chamber was controlled by circulating ethylene glycol from a Haake Thermostat, model F. The sample chamber was thermally insulated from the other parts of the spectrophotometer by using two insulating plates and by having cooling water flow between the two plates. The temperature of the sample solution was measured with a cupper-Constantan thermocouple.

Results and Discussion

The Melting of Poly(I+C) Observed by Tracing the Absorbance at 248 mμ.—First, a few preliminary examinations were made of the ultraviolet absorption spectra of our samples of polyribonucleotides and their mixtures. It was found that our poly I and poly C in a citrate buffer (pH 7.0) give exactly the same spectra as those reported by Davies and Rich[3] in the 220—300 mμ region. The spectrum of a 1 : 1 mixture of these polymers indicates that a strong interaction takes place in 0.01 M sodium chloride plus 0.01 M sodium citrate buffer (pH 7.0), while no interaction takes place in a 0.001 M sodium-citrate buffer (pH 7.0) without sodium chloride. In the 0.05 M sodium chloride plus 0.01 M sodium-citrate buffer (pH 7.0), the absorbance was measured at 233 mμ for several mixtures containing varying proportions of poly I and poly C, while the total nucleotide concentration was kept constant. The resulting mixing curve is similar to what Davies and Rich[3] observed at 235 mμ in a cacodylate buffer (pH 6.7). It is composed of two straight lines which intersect sharply at a 1 : 1 mole ratio, demonstrating the formation of a 1 : 1 complex, poly(I+C). This complex is considered to have a double-helical conformation.[3,4] When solution of this complex is heated, there is a general increase in absorbance in the 230—270 mμ region. This increase is considered to correspond to the "melting" of the double-helical poly(I+C) into single-stranded random coils, poly I and poly C. The increase in absorbance is greatest at 248 mμ. Therefore, the melting process of poly(I+C) was followed by observing the absorbance at 248 mμ throughout the rest of the present work. The melting temperature, T_m, is defined as the midpoint of the increase in absorbance just mentioned.

The Effect of Na⁺.—The melting process of poly(I+C) is influenced by the Na^+ concentration in the solvent in two ways. When the Na^+ concentration is lowered, the melting temperature, T_m, of poly(I+C) is lowered, and at the same time the melting process loses its reversibility. As may be seen in Fig. 1, the T_m of poly(I+C) is 65°C in the medium with 0.1 M Na^+ while it is 49.5°C with 0.01 M Na^+. In the former solution the re-formation of the complex takes place rapidly (within 9 min.), while in the latter it takes a very long time (about 24 hr.). This lower reaction rate may be attributed to a high activation energy. Without shielding by a cation atmosphere, two negatively-charged polymers (poly I and poly C) cannot easily approach to each other. In Fig. 2, T_m is plotted against the logarithm of the Na^+ concentration [s]. As was expected in a previous paper,[6] the curve gives an S shape. On the basis of what was described in Section 3 in a previous paper,[6] $K'=1/[s]$ at the inflection point. Here, K' is the equilibrium constant at T_m of each of the reactions:

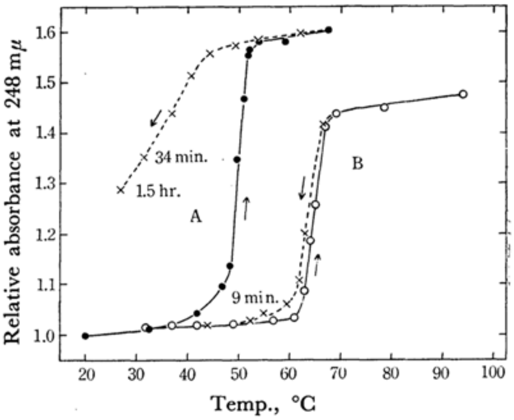

Fig. 1. The variation of the absorbance of poly-(I+C) solutions at 248 mμ with temperature. Full line: heating curve, broken line: cooling curve.

A Poly I 2.5×10^{-5} M plus poly C 2.5×10^{-5} M in 0.01 M NaCl plus 0.01 M Li-citrate buffer, pH 7.0.

B poly I 2.5×10^{-5} M plus poly C 2.5×10^{-5} M in 0.1 M NaCl plus 0.01 M Li-citrate buffer, pH 7.0.

Each of the figures given on the cooling curves indicates the time taken by cooling the solution from the highest temerature given to the temperature in question.

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[13] D. T. Dubin, *J. Biol. Chem.*, **235**, 783 (1960).

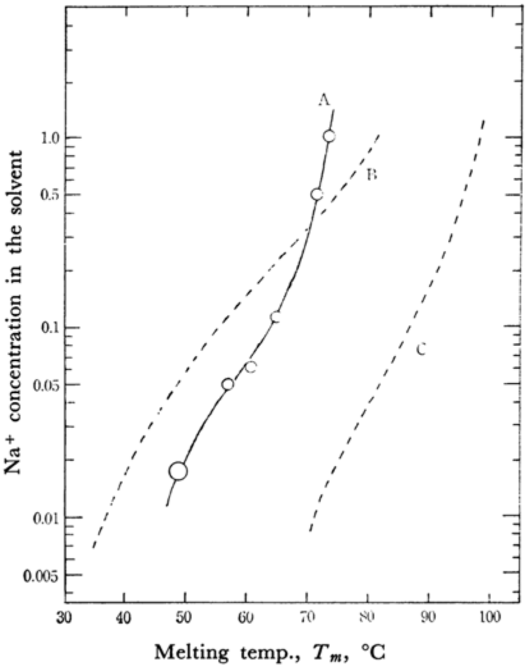

Fig. 2. The dependence of T_m on Na^+ concentration.
A Equimolar mixture of poly I and poly C.
B Equimolar mixture of poly A and poly U (Details will be given in our next paper.)
C DNA (the dependence of T_m on K^+ concentration, J. Marmur and P. Doty, *J. Mol. Biol.*, **5**, 109 (1961).)

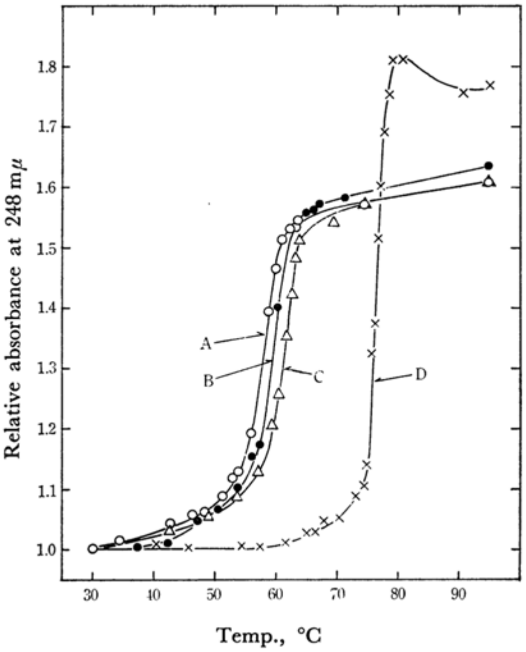

Fig. 3. The variation of the absorbance of poly(I+C) solutions at 248 mμ with temperature.
A Poly I 2.5×10^{-5} M plus poly C 2.5×10^{-5} M in 0.05 M NaCl plus 0.001 Na-citrate buffer, pH 7.0
B Solution A plus 0.02 M $CH_3NH_3^+Cl^-$
C Solution A plus 0.02 M NH_4Cl
D Solution A plus 0.01 M $MgCl_2$

$$\mathrm{Poly(I + C) + Na^+ = poly(I + C)Na^+}$$
$$\mathrm{poly(I + C)Na^+ + Na^+ = poly(I + C)Na^+_2}$$

....

and

$$\mathrm{poly(I+C)Na^+_{N-1}+Na^+=poly(I+C)Na^+_N}$$

From an inspection of Fig. 2, $K'=15$—$20\ \mathrm{M}^{-1}$. An inspection of Fig. 2 also shows that the T_m change in the variation of $[s]$ is smaller than that for poly(A+U) and that for DNA. Very probably this means that the α value is smaller for poly(I+C) than for poly(A+U) or for DNA.⁶⁾ Here α is the increase in the enthalpy of the melting of poly(I+C) caused by the adsorption of one cation.⁶⁾

The Effects of a Few Other Cations.—As may be seen in Fig. 3, the effect of the ammonium ion on the T_m of poly(I+C) is almost the same as that of Na^+. The effect of the methylammonium ion is somewhat smaller, while the effect of Mg^{2+} is markedly greater than the effect of the ammonium ion.

The Effects of Diamines.—When a NH_3^+-$(CH_2)_nNH_3^+$-type diamine is added to the poly(I+C) solution, an appreciable elevation of T_m is again observed (Fig. 4). The amount of elevation ΔT_m, is found to depend upon the value of n, the number of the methylene groups placed between the two amino groups of the diamine. When the concentration of the diamines is kept constant (0.01 M), the greatest ΔT_m value of poly(I+C) is found at $n=3$. $NH_3^+(CH_2)_3NH_3^+$ has an effect on the T_m of poly(I+C) greater not only than $NH_3^+(CH_2)_2NH_3^+$ but also than Mg^{2+}. This fact suggests that the $n=3$ cation functions not only as a shielder of the PO_2^- charges of the poly I and poly C chains, but also as a clamp which tends to keep the two polynucleotide chains together and gives an additional stability to the intertwined double-helical structure. If so, the $NH_3^+\cdots NH_3^+$ distance in this cation should reflect the distance from an oxygen atom of a PO_2^- group belonging to a poly I chain to that belonging to the poly C chain which is intertwined with the poly I chain.

The Effects of Spermidine and Spermine.—The effect of spermidine, $NH_3^+(CH_2)_3NH_2^+$-$(CH_2)_4NH_3^+$ (see Fig. 5, B), is much greater than that of the 1, 3-diaminopropane described above (Fig. 4, C). Thus, the addition of the former in only half the amount (0.005 M) of the latter causes a greater change in the T_m value (from 57°C to 84°C, i. e., $\Delta T_m=27$°C). The effect of spermine, $NH_3^+(CH_2)_3NH_2^+(CH_2)_4NH_2^+(CH_2)_3NH_3^+$, is even more dramatic (Fig. 5, C). Only 0.0005 M

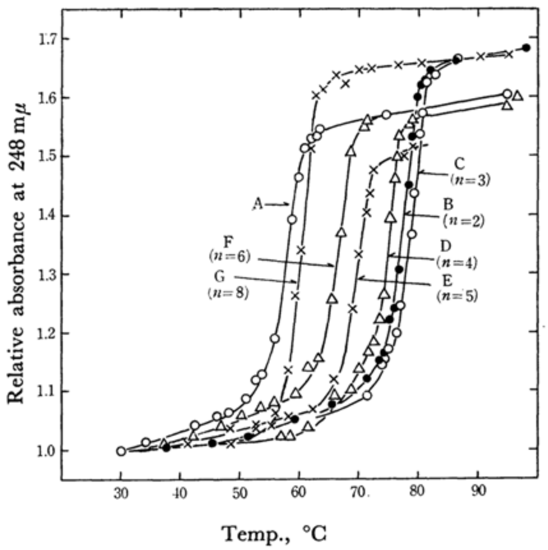

Fig. 4. The variation of the absorbance of poly(I+C) plus diamine ($NH_3^+(CH_2)_nNH_3^+$) solutions at 248 mμ with temperature.

A Poly I 2.5×10^{-5} M plus poly C 2.5×10^{-5} M in 0.05 M NaCl plus 0.001 M Na-citrate buffer, pH 7.0
B Solution A plus 0.01 M $NH_3^+CH_2CH_2NH_3^+$
C Solution A plus 0.01 M $NH_3^+(CH_2)_3NH_3^+$
D Solution A plus 0.01 M $NH_3^+(CH_2)_4NH_3^+$
E Solution A plus 0.01 M $NH_3^+(CH_2)_5NH_3^+$
F Solution A plus 0.01 M $NH_3^+(CH_2)_6NH_3^+$
G Solution A plus 0.01 M $NH_3^+(CH_2)_8NH_3^+$

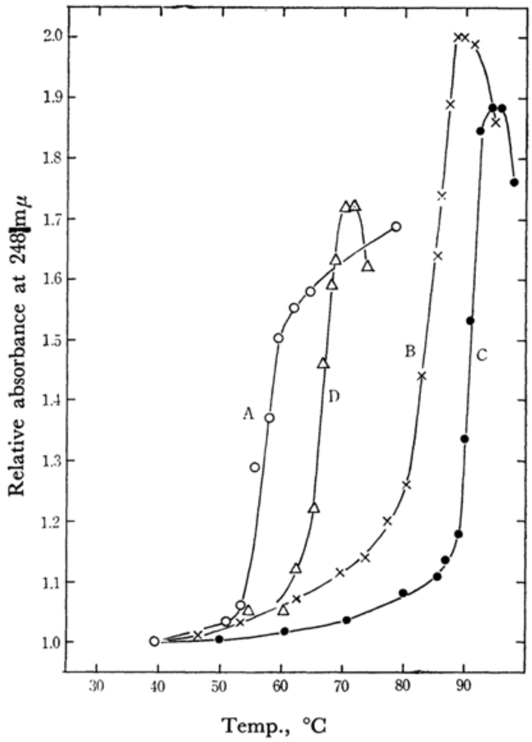

Fig. 5. The variation of the absorbance of poly(I+C) plus polyamines at 248 mμ with temperature.

A Poly I 2.5×10^{-5} M plus poly C 2.5×10^{-5} M in 0.05 M NaCl plus 0.001 M Na-citrate buffer, pH 7.0
B Solution A puls 0.005 M spermidine trihydrochloride
C Solution A plus 0.0005 M spermine tetrachloride
D A solution obtained by a dialysis of the solution C against 0.05 M NaCl plus 0.001 M Na-citrate buffer, pH 7.0, for 40 hr.

of it causes an elevation of T_m from 57°C to 91°C. This suggests that the equilibrium constant, K', of the binding reaction of spermine to poly(I+C) is to the order of 10^4 or more. Mandel[9] examined the interaction of spermine with DNA, and found that the extent of the increase in T_m is proportional to the adenine-thymine content of the DNA sample. On the basis of what he found, one might suggest that there is no effect of spermine upon a double-helical polynucleotide structure without an adenine-thymine pair. What we have observed here, however, shows that this is not necessarily the case. There is certainly a marked effect of spermine upon poly(I+C), a double-helical polynucleotide without an adenine-thymine or an adenine-uracil pair. As may be seen in Fig. 5, D, the dialysis of the poly(I+C) plus spermine solution against the buffer for 40 hr. results in a great lowering of T_m, from 91°C to 66°C. Its T_m, however, is still appreciably higher than that of poly(I+C) alone in the same buffer. This fact indicates that the poly(I+C)-spermine binding is a very strong one; however, there seems to be no chemical reaction between them.

The Effect of Actinomycin D.—In a previous work,[14] 31.8 μg./ml. of actinomycin D was found to cause an elevation of the T_m of DNA from 72°C to 87°C in a 0.01 M tris buffer. The same amount of actinomycin D, however, has now been found to have almost no effect on the melting profile of poly(I+C) in 0.05 M sodium chloride plus a 0.01 M sodium citrate buffer.

The Effect of Poly-L-lysine.—When poly-L-lysine is added to $0.8_3\times10^{-5}$ M to a solution of 2.5×10^{-5} M poly(I+C) ($NH_2:P=0.16_6:1$), two-step melting is observed instead of one-step melting (Fig. 6, A). Roughly two-thirds of the total increase in absorbance at 248 mμ takes place at 57°C, and the rest at 89°C. When the concentration of poly-L-lysine is increased to $1.6_6\times10^{-5}$ M (now $NH_2:P=0.33:1$), only 1/3 of the total increase in absorbance takes place at the first-step melting; the rest takes place at the second step. However, the melting temperatures remain at 57°C and 89°C (Fig. 6, B). The melting temperature of the first step (57°C) is the same as

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[14] M. Tsuboi, S. Higuchi, Y. Kyogoku, K. Matsuo and A. Wada, *Chem. Pharm. Bull.*, **12**, 501 (1964).

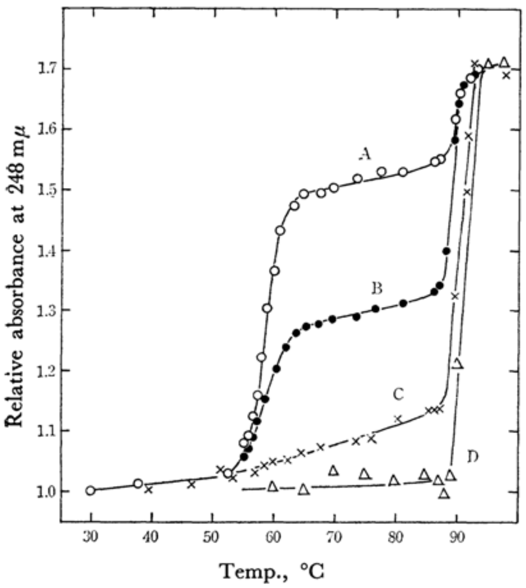

Fig. 6. The variation of the absorbance of poly-(I+C) plus poly-L-lysine solutions with temperature. Solvent: 0.05 M NaCl in 0.001 M Na-citrate buffer, pH 7.0.

- A Poly I 2.5×10^{-5} M plus poly C 2.5×10^{-5} M plus poly-L-lysine $0.8_3\times10^{-5}$ M (NH_2 : P= 0.16_6 : 1).
- B Poly I 2.5×10^{-5} M plus poly C 2.5×10^{-5} M plus poly-L-lysine $1.6_6\times10^{-5}$ M (NH_2 : P= 0.33 : 1).
- C Poly I 2.5×10^{-5} M plus poly C 2.5×10^{-5} M plus poly-L-lysine 2.5×10^{-5} M (NH_2 : P= 0.5 : 1).
- D Poly I 2.5×10^{-5} M plus poly C 2.5×10^{-5} M plus poly-L-lysine $3.7_5\times10^{-5}$ M (NH_2 : P= 0.75 : 1).

the T_m value of poly(I+C) alone in the solvent in question (0.05 M sodium chloride plus 0.001 M sodium citrate buffer, pH 7.0). When the concentration of poly-L-lysine is increased to 2.5×10^{-5} M (now NH_2 : P=0.5 : 1), the first-step melting at 57°C almost disappears, and only the melting at 89°C takes place (Fig. 6, C).

These experimental results indicate that there is a complex of poly-L-lysine and poly(I+C) with a definite mole ratio and with a T_m of 89°C in the solution. It has recently been shown[15] that, in poly-L-lysine plus DNA system, all the poly-L-lysine molecules added are bound by the DNA molecules and no poly-L-lysine molecules remain free, if the amount added is less than the amount sufficient to saturate the DNA molecules with poly-L-lysine. This is probably the case also for the poly-L-lysine plus poly(I+C) system. If so, the composition of the poly-L-lysine+poly-(I+C) complex in question can easily be determined. The NH_2: P ratio in the complex should be 0.5 : 1, since the melting of poly(I+C) free from poly-L-lysine (at 57°C) disappears at this mole ratio. This knowledge of the composition of the complex enables us to conjecture to some extent about its structure. It may be concluded, for example, that a molecular model similar to that of deoxyribonucleoprotamine, as proposed by Wilkins et al.[16,17] is not acceptable for the poly-L-lysine+poly(I+C) complex. In this model, an almost fully-extended polypeptide chain winds helically around the doubly-stranded polynucleotide chains. The pitch of the polypeptide helix is the same as that of polynucleotide helices. This model requires one amino acid residue per nucleotide residue, i. e., NH_2 : P=1 : 1, while in the poly-L-lysine+poly(I+C) complex in question, NH_2: P=0.5 : 1. In a fully-extended polypeptide chain, one amino acid residue corresponds to a translation of 3.4 Å along the chain axis. In the A-form of poly(I+C), the translation along the helix axis per nucleotide pair is 3.0 Å.[4] Therefore, such a chain axis of poly-L-lysine should be inclined about

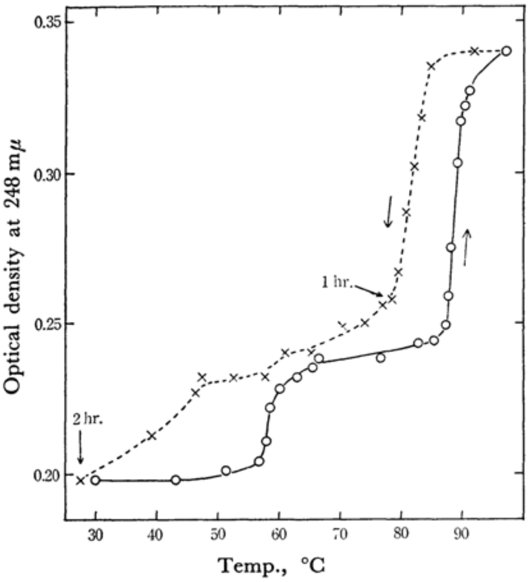

Fig. 7. The variation of the absorbance of a poly(I+C) plus poly-L-lysine solution on heating (full line) and on cooling (broken line). Poly I 2.5×10^{-5} M plus poly C 2.5×10^{-5} M plus poly-L-lysine $1.6_6\times10^{-5}$ M (NH_2: P=0.33: 1) in 0.05 M NaCl plus 0.001 M Na-citrate buffer, pH 7.0. Each of the figures given on the cooling curve indicates the time taken by cooling the solution from 97°C to the temperature in question.

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[15] M. Tsuboi, K. Matsuo and P. O. P. Ts'o, *J. Mol. Biol.*, in press.

[16] M. Feughelman, R. Langidger, W. E. Seeds, A. R. Stokes, H. R. Wilson, C. W. Hooper, M. H. F. Wilkins, R. K. Barclay and L. D. Hamilton, *Nature*, **175**, 834 (1955).

[17] M. H. F. Wilkins, *Cold Spring Har. Quant. Biol.*, **21**, 75 (1956).

arc cos (3.0/3.4) = 28° to the helix axis of the poly(I+C) in the complex in question. Thus a possible model of the complex may be suggested in which the fully-extended poly-L-lysine chain winds around the double helices of poly(I+C) core along a helix with a long pitch.

The poly-L-lysine concentration was further increased from that corresponding to $NH_2 : P = 0.5 : 1$. As may be seen in Figs. 6, C and D, the poly-L-lysine plus poly(I+C) solution with $NH_2 : P = 0.75 : 1$ gives a heating curve similar to that of the solution with $NH_2 : P = 0.5 : 1$. There is, however, a slight difference between them. In the latter there is a gradual increase in absorbance from 50°C to 88°C before a steep increase in absorbance takes place, whereas in the former no such gradual increase is observed; the curve is practically horizontal up to 89°. This fact may be taken as indicating that the excess poly-L-lysine molecules in the solution with $NH_2 : P = 0.75 : 1$ serve to keep the complex rigid throughout the emperature range from 50°C to 89°C, and that without such excess poly-L-lysine molecules the poly-L-lysine+poly(I+C) complex is somewhat flexible.

Figure 7 shows the results of an examination of the reversibility of the two-step meltings now in question. As may be seen here, an almost complete reformation of the poly-L-lysine+poly(I+C) complex takes place in the solvent in question when the solution is kept at about 80°C for an hour after the melting.

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